Regenerative treatment options for intervertebral disc degeneration *in vitro*

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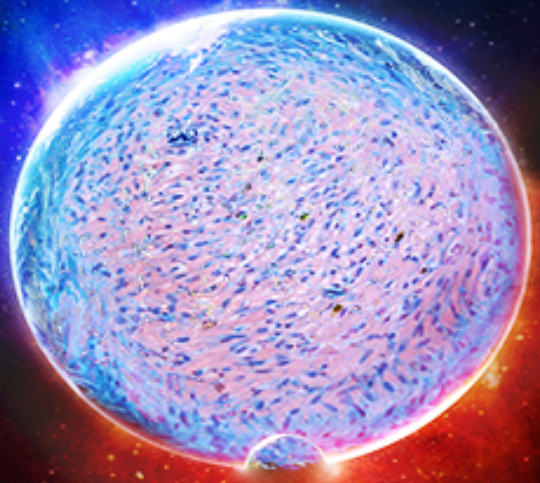

Honours Programme

01-09-2014 - 01-09-2015

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Summary

Introduction. Intervertebral disc degeneration (IVDD) is a major medical issue and is related with low back pain in both humans and dogs. During IVDD, notochordal cells (NCs) are being replaced by chondrocyte-like cells (CLCs) in the nucleus pulposus (NP). Current treatments for IVDD do not cure the degenerated intervertebral disc (IVD) and therefore, (growth factor-based) regenerative therapies are proposed. Transforming growth factor beta 1 (TGF-ß$_1$), bone morphogenetic protein 2 (BMP-2) and notochordal cell (NC)-derived factors (present in NC-conditioned medium (NCCM)) stimulate extracellular matrix (ECM) deposition *in vitro*. Their regenerative potency was never explored in comparative studies. Furthermore, in order to develop new treatment strategies, the NC-secreted factor(s) need to be determined.

Therefore, the **main aims** of this project were
(1) to compare the species-specific NCCM potency on human CLCs,
(2) to determine the response of canine CLCs to soluble (peptides and proteins) and pelletable (protein aggregates and exosomes) NCCM fractions,
(3) and lastly, to compare the regenerative and cell adhesion capacity of TGF-ß$_1$ and BMP-2 on canine CLCs.

Material and Methods. Species-specific NCCM was produced from porcine, canine and human NC-rich NP tissue. CLCs from degenerated human IVDs were cultured in micro-aggregates in the species-specific NCCM for 28 days. For evaluation of the biologic effect of NCCM, NCCM + was generated from healthy NCs and NCCM – was generated from dedifferentiated NCs. NCCM + was separated into a soluble (NCCM S) and pelletable (NCCM P) fraction by ultracentrifugation. Canine CLCs isolated from degenerated discs were cultured in alginate in control culture medium, TGF-ß$_1$-supplemented medium, NCCM –, NCCM +, NCCM P (1X and 10X concentrated) or NCCM S for 28 days. The regenerative effect of TGF-ß$_1$ and BMP-2 was tested on CLCs from degenerated human, canine CD and NCD IVDs in negative control, TGF-ß$_1$ (10 ng/mL)- or BMP-2 (250 ng/mL)-supplemented culture medium for 28 days. Furthermore, to determine whether pretreatment with growth factors may facilitate the NCCM effects on CLCs, CLCs were pretreated for 5 days with BMP-2- or TGF-ß$_1$-supplemented culture medium and thereafter received chondrogenic control-, TGF-ß$_1$-, BMP-2-supplemented medium or canine NCCM until day 28. Read out parameters were apoptosis (RT-qPCR), cell proliferation (DNA content, RT-qPCR) and ECM production (RT-qPCR, glycosaminoglycan (GAG) content and release, Safranin O/Fast Green and Alcian Blue staining, IHC).

Results. Human CLC proliferation and ECM deposition was significantly increased by human, canine and porcine NCCM. Human NCCM was less potent than porcine and canine NCCM in inducing GAG deposition, but more potent in inducing collagen type II deposition. NCCM + was more potent in increasing the GAG and DNA content of the canine CLCs than NCCM –. The soluble and pelletable NCCM fractions were equally potent in increasing the GAG and DNA content, but not as potent as NCCM +. TGF-ß$_1$ and BMP-2 treatment stimulated human and canine CLC cell adhesion and GAG deposition, whereas only TGF-ß$_1$ induced fibrotic (re)differentiation. Even in CLCs pretreated with growth factors, NCCM was less potent in enhancing canine CLC proliferation and ECM deposition than TGF-ß$_1$ and BMP-2.

Conclusion. All regenerative mediators (NCCM, NCCM soluble and pelletable fraction, TGF-ß$_1$ and BMP-2) showed regenerative effects on degenerated human CLCs. Canine and porcine NCCM were more potent than human NCCM and their NP tissue is more easily obtained, indicating that pigs and dogs can serve as suitable NC donors in future studies. Moreover, both pelletable and soluble NCCM fractions exerted considerable and similar regenerative effects on canine CLCs indicating that both fractions should be further studied in order to identify the bioactive NC-secreted factors. BMP-2 exerted a significant regenerative effect on human and canine CLCs and did not induce fibrotic (re)differentiation as observed with TGF-ß$_1$ treatment, making it a more preferable regenerative medicine candidate.

General introduction

The healthy intervertebral disc

The healthy intervertebral disc (IVD) consists of an inner nucleus pulposus (NP), surrounded by a layered annulus fibrosus (AF) which is in turn bordered by the cartilaginous endplates (EP) (Figure 1)[1]. The gelatinous-like NP tissue, a remnant of the notochord, contains a high amount of water. The extracellular matrix (ECM) of the NP mainly consists of proteoglycans and collagen type II fibers. The water in the NP tissue is attracted by negatively charged glycoaminoglycans (GAGs), which are proteoglycan side chains. The most important GAG is aggrecan. Numerous notochordal cells (NCs) can be found in the young, healthy NP. NCs are found in clusters and have large characteristic vacuolae which presume to have a function in lowering osmotic stress by releasing their contents[2]. The exact function of NCs is not fully explained yet, but it is debated that the NCs can give rise to chondrocyte-like cells (CLCs) (Figure 2)[2-4]. These CLCs gradually replace the NCs during maturation and degeneration of the IVD.

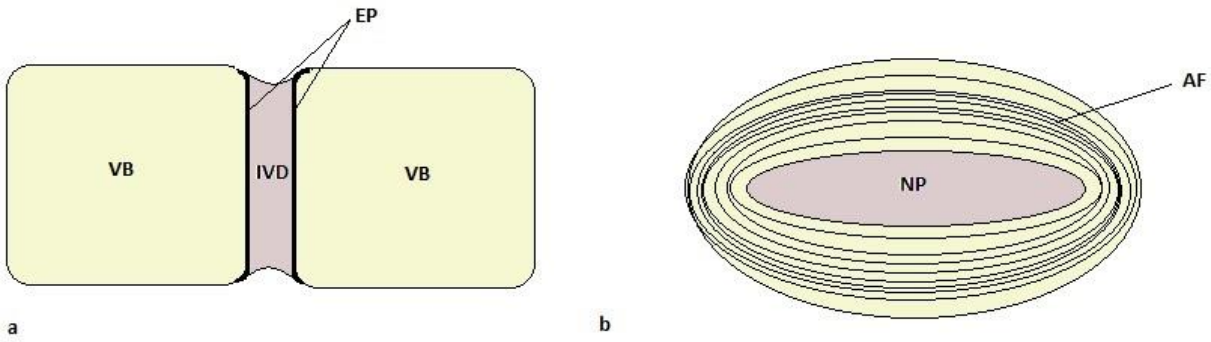


Figure 1. Simplified illustration showing structures at the intervertebral disc level. a) The intervertebral disc (IVD) and endplates (EP) with the adjacent vertebral bodies (VB). b) Macroscopic view of the intervertebral disc in transverse plane, with the nucleus pulposus (NP) surrounded by the annulus fibrosus (AF).

The transition from NP to AF is marked by a change in composition, from gelatinous to fibrous/cartilaginous. The AF is concentrically layered around the NP tissue and consists of regularly patterned collagen type I fibers in the outer rings and collagen type I and II fibers and a higher concentration of proteoglycans in the less distinctive inner rings.

After disc compression, the hydrostatic balance inside the NP changes. Pressure changes are different in each part of the NP, depending on the movement and compression. The most inner part of the AF also contributes to the maintenance of the hydrostatic pressure by consisting of more collagen type II fibers than the outer parts of the AF[5].

EPs, in turn, form a physiological border between the IVD and the adjacent vertebral body. These cartilaginous EPs are semipermeable and allow small molecules to reach the NP and AF cells. This is essential, because the NP and the inner layers of the AF lack blood and nerve supply[6].

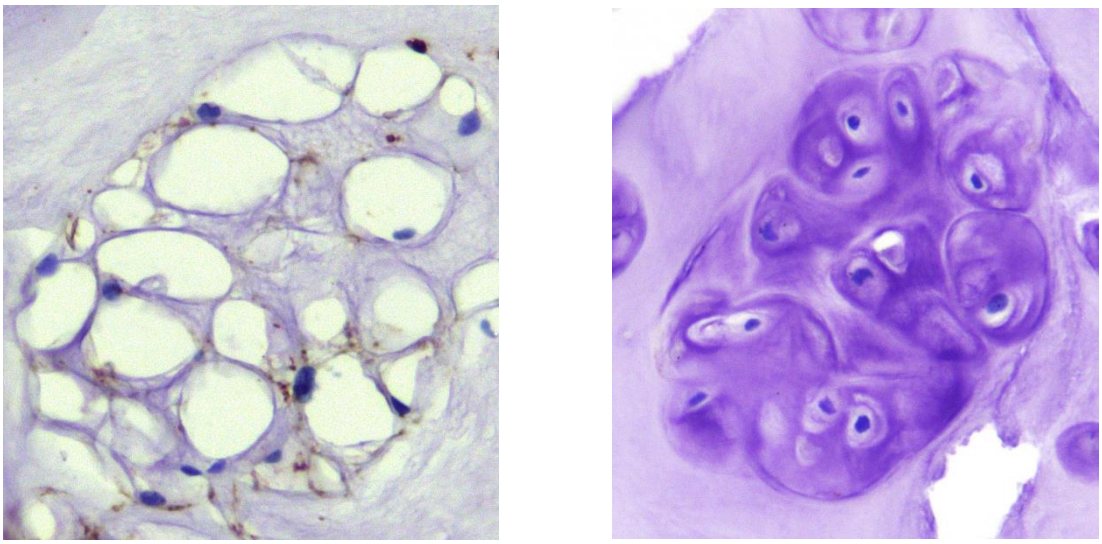


Figure 2. Left: Notochordal cells (NCs) with characteristic large vacuolae. Right: Smaller chondrocyte-like cells (CLCs) which have lost the large vacuolae. 20x magnification, canine nucleus pulposus (NP) tissue (non-chondrodystrophic and chondrodystrophic breed, respectively).

Intervertebral disc degeneration

A medical disorder which can occur in both humans and dogs, is intervertebral disc degeneration (IVDD). This disease is characterized by a shift in cell type from NCs to smaller, non-vacuolated CLCs and degradation of ECM by matrix metalloproteinases (MMPs), matrix remodeling proteins. The collagen type II content decreases, while the collagen type I content increases, and the IVD gains a more fibrotic phenotype. Degradation of proteoglycans in combination with matrix dehydration leads to the incapability to cope with forces exerted on the vertebral column and thus the IVD. During IVDD, the GAG and water content decreases in the NP, changing the biomechanical environment of the IVD cells. Because of the changed IVD matrix, diffusion of nutrients becomes impaired, which further deteriorates the health of the IVD cells and healthy matrix synthesis. Since the avascular IVD exhibits inadequate matrix repair, a vicious circle develops. The extraordinary loading of forces on the AF and the structural changes will eventually lead to cracks and tears, which predispose for IVD herniation[5,6].

Dog breeds with IVD disease can be classified into two groups (based on predisposition, location of the disease and the age of concurrence): non-chondrodystrophic (NCD) and chondrodystrophic (CD) breeds. NCD breeds (i.e. Dobermann dogs and German Sherperds[7]) show pathological changes at caudal cervical or lumbosacral levels, while cervical or thoracolumbar levels are more commonly affected in CD dogs. The mean age of occurrence is 6-8 and 3-7 years in NCD and CD breeds, respectively. On the whole, CD breeds (i.e. Beagles, French Bulldogs and Dachshunds) can be distinguished based on appearance. They have disproportionally short limbs due to disturbed endochondral ossification. CD and NCD dog breeds have been shown to be suitable animal models for human IVDD, due to similarities of the IVD in e.g. pathology, histology, GAG content and biochemical properties during degeneration as described by Bergknut *et al*.[8].

Several therapies are being proposed for treatment of IVDD, because it is causing major health and economic issues. Low back pain prevalence in human patients is as high as up to 85% and has a high global ranking in causing working disability[9]. Up to 40% of low back pain can be related to IVDD[5]. This

was responsible for €3.5 billion in costs in 2007 in the Netherlands alone[10,11]. Current treatment for IVDD (humans and dogs) include surgery, physiotherapy and medicinal therapy. All current treatment options are based on nerve (cord) decompression or pain relief, but do not cure the IVD[4].

A switch in therapy

To break the vicious circle of IVDD, other promising therapies should be considered. Following this, it is proposed that the use of regenerative therapies like cell-, growth factor- and/or gene-based therapy, could be the future. Regenerative therapies aim at restoring healthy tissue and improving tissue functionality, for instance by stimulating ECM production[12]. Cell-based therapies (e.g. CLCs, mesenchymal stem cells (MSCs) and NCs) and growth factor-based therapies (e.g. transforming growth factor beta and bone morphogenetic protein) are proposed to be promising regenerative therapy options[4,12,13].

Why are NCs chosen as a starting point for the development of a regenerative therapy?

The round, vacuolated NCs are found in clusters in the young, healthy IVD and are proposed embryonic notochord remnants. NCs are being replaced by CLCs during IVDD or aging. Disappearance of NCs by aging takes place at a species-specific time point. The separation of NCs from CLCs is possible by fluorescence-activated cell sorting analysis (FACS), because NCs are smaller than CLCs[14,15].

NCs have several proposed functions: progenitor, regulatory and protection. It is suggested that they are indirectly able to induce ECM deposition by stimulating CLCs or by producing matrix themselves as they turn into CLCs[16,17]. As presumed progenitors, they can replete the CLC pool[2,4]. Also, they have shown to induce MSC differentiation to a more NP-like phenotype[18]. The presumed protective capacity is shown on bovine CLCs by suppressing the caspase pathways which are linked to apoptosis[19]. Moreover, soluble factors secreted by NCs are necessary for preserving a healthy IVD[20]. Altogether, it seems reasonable to suspect a regenerative effect of NCs.

Notochordal cell-conditioned medium (NCCM), which contains NC secreted factors, can be produced *in vitro*[21,22] and further studied in order to identify the bioactive substances mediating the regenerative effects.

Growth factor-based therapies

a. Transforming growth factor beta

Transforming growth factor beta (TGF-ß) has shown to interfere in cell proliferation and differentiation, but also to induce ECM production[23-26]. Following this, TGF-ß has been used for the chondrogenic differentiation of MSCs and CLCs and has been acting as a positive control in cell culture experiments where other growth factors/regenerative therapies are tested[13,27].

The effects of TGF-ß are initiated by binding to the TGF-ß type II receptor (TßR-II), which possesses serine/threonine kinase activity (Figure 3). Binding of TGF-ß to the TßR-II triggers a phosphorylation (activation) in the GS domain of TGF-ß receptor I (TßR-I). Activated TßR-I in turn activates receptor-activated Smads (R-Smads) by phosphorylation of their C-terminus[23,24]. The effects of TGF-ß signaling are mediated via two different TßR-Is: activin-like kinase (ALK) 1 and ALK 5. ALK 5 activation leads to activated Smad 2 and 3 signalling, which form hetero-oligomers with common-mediator (Co-Smad) Smad 4. The R-smad - Smad 4 complex is transferred to the cell nucleus where it initiates

transcriptional processes[28]. Activation via ALK 1, in turn, triggers the activation of Smad 1/5/8. Signaling via the ALK 1 pathway results in an upregulation of matrix metalloproteinase 13 (MMP 13) and collagen type X, genes associated with hypertrophic differentiation[28-30], whereas ALK 5 activation leads to an increased production of GAGs and collagen type I and II[25]. In addition to R- and Co-Smads, inhibitory Smads (I-Smads) connect to type I receptors and prevent R-Smads from binding[23,31]. Common I-Smads include Smad 6 and 7[24], but Smad 1 and 5 also inhibit Smad 3-initiated pathways[32]. The mentioned pathways and mediators intertwine to regulate TGF-ß signaling. In addition, TGF-ß signaling can also be mediated via Smad-independent pathways, e.g. the MAPK pathways[23].

In MSCs, Smad 2/3 signaling has been shown to stimulate transcription via SRY-type high mobility group box 9 (Sox 9)[33]. Sox 9 is an important transcription factor in chondrogenic differentiation, both in early and successive stages of cartilage development and maintenance[34]. It is responsible for the expression of multiple genes connected to chondrogenesis, like aggrecan and collagen type II[35-38].

b. Bone morphogenetic proteins

Bone morphogenetic proteins (BMPs) are classed as members of the TGF-ß superfamily and are produced by e.g. osteoblasts and chondrocytes. Initially, it was found that BMPs have osteogenic potential. However, more functions are described nowadays including angiogenesis and chondrogenesis[39,40].

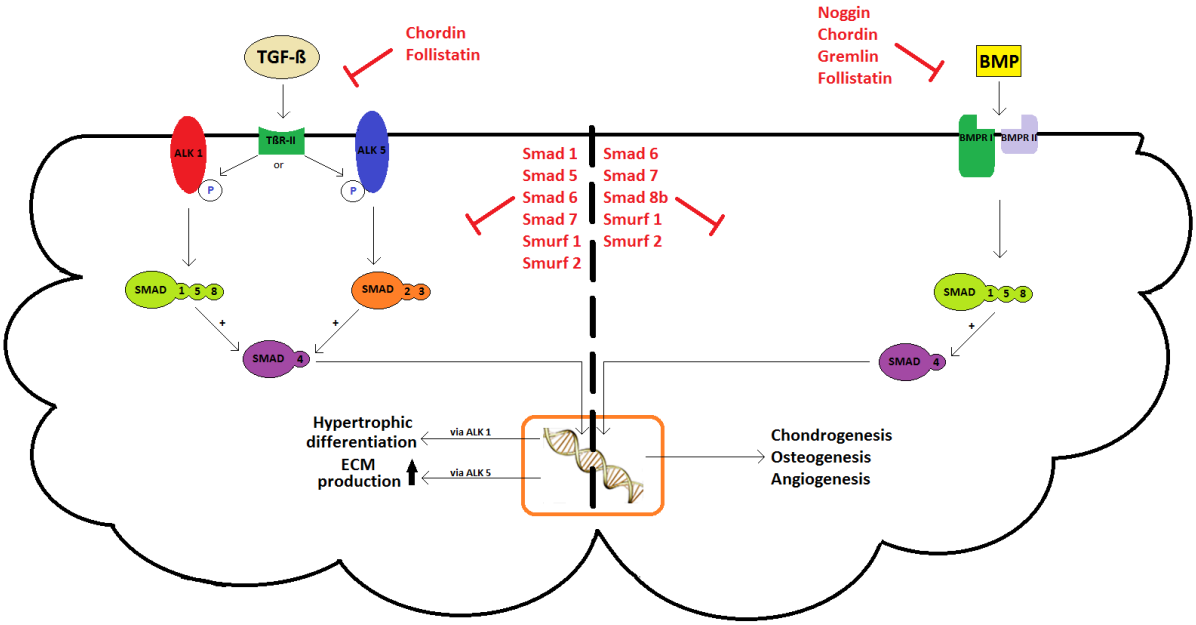


Figure 3. Simplified schematic representation of the TGF-ß and BMP signaling pathways. Left: TGF-ß binding activates ALK 1 or ALK 5 receptor via phosphorylation (P) of SMAD 1/5/8 or 2/3, respectively. A complex with SMAD 4 is formed and shuttled to the cell nucleus leading to hypertrophic differentiation (via ALK 1) or extracellular matrix (ECM) production (ALK 5). Right: BMP binding activates SMAD 1/5/8 which binds to SMAD 4 before this complex is transferred to the cell nucleus resulting in e.g. chondrogenesis, osteogenesis or angiogenesis. Antagonists, both extra- and intracellular, are depicted in red[39,41-43].

Activation of the BMP signaling pathway is initiated by BMP ligands binding either to BMP receptor I (BMPR I, also named ALK 1-7), which attracts BMP receptor II (BMPR II) or to pre-formed receptor complexes[39,42]. After BMP binding, Smad 1/5/8 are activated, they bind to Smad 4 and this complex is transferred to the nucleus where it can influence transcriptional processes by direct DNA binding or association with binding proteins[44]. Antagonists of the BMP signaling include amongst others I-Smads 6 and 7, Smurf 1 and 2 (Figure 3). Multiple transcriptional and epigenetic regulators also intervene in both BMP and TGF-ß signaling pathways[41].

Aim and scope of the HP project

NCCM, produced from NC-rich NP tissue, has the ability to stimulate ECM production in human CLCs *in vitro*. NCCM of multiple species has been studied, but has never been compared regarding the (regenerative) potency. Therefore, the first aim of this HP project was to compare the species-specific NCCM regenerative potency on human CLCs and to see whether NC-secreted bioactive fractions can be suitable regenerative therapy candidates for IVDD (Chapter 1). The second aim was to study the differential responses of canine CD CLCs towards individual NCCM fractions (Chapter 2) in order to determine which fraction of the NCCM is biologically active. This is the first step taken in the process of defining which bioactive substances elicit the NCCM effect and use those in the development of new treatment strategies.

TGF-$ß_1$ and BMP-2 have also shown to stimulate ECM production in multiple species CLCs and considered as possible regenerative targets. However, never before were they tested on canine CLCs and their effects compared. Therefore, the third aim of this study was to investigate and compare the effects of TGF-$ß_1$ and BMP-2 on human, canine CD and NCD CLCs to determine their regenerative potential as an IVDD treatment.

Furthermore, pretreatment of canine CLCs with growth factors is proposed to enhance cell-cell adhesion and stimulate cell responsiveness. Enhanced cell adhesion capacity would be necessary, because NCCM-treated canine CD CLC micro-aggregates disintegrated after 28 days of culture (unpublished data). Therefore, the aim of the fourth study was to compare the effects of two growth factors (TGF-$ß_1$ and BMP-2) pretreatment on canine CD CLC micro-aggregates and to observe if this pretreatment can maintain cell-cell adhesion after treatment with NCCM.

Chapter 1: The species-specific regenerative effect of notochordal cell-conditioned medium on degenerated human nucleus pulposus cells

Abstract

Introduction. Intervertebral disc degeneration (IVDD) is a major medical issue and is related with low back pain in both humans and dogs. Notochordal cell-conditioned medium (NCCM), produced from notochordal cell (NC)-rich nucleus pulposus (NP) tissue, has the ability to stimulate extracellular matrix (ECM) production in human chondrocyte-like cells (CLCs) *in vitro*. NCCM of multiple species has been studied, but has never been compared regarding their (regenerative) potency. The aim of this study was to compare the species-specific NCCM regenerative potency on human CLCs and to see whether NC-secreted bioactive factors can be suitable regenerative therapy candidates for IVDD. **Material and Methods.** NCCM was produced by culturing porcine, canine and human NC-rich NP tissue for four days. CLCs from degenerated human IVDs (Thompson score 3) were cultured in micro-aggregates in species-specific NCCM or positive/negative control culture media for 28 days. Read out parameters were apoptosis (RT-qPCR), cell proliferation (DNA content, RT-qPCR) and matrix production (RT-qPCR, glycosaminoglycan (GAG) content, Safranin O/Fast Green staining and immunohistochemistry). **Results.** Matrix production and the DNA content of CLC micro-aggregates cultured in NCCM derived from all species was significantly increased compared with the negative controls. Human NCCM was less potent than porcine and canine NCCM in inducing GAG production by human CLCs. Only human NCCM, however, induced collagen type II deposition. **Conclusion.** NCCM derived from all species stimulated ECM production of human CLCs, indicating a cross-species effect. Canine and porcine NCCM were more potent than human NCCM in stimulating GAG production. This indicates that pigs and dogs can serve as a suitable NC donors in future studies that aim at identifying the bioactive NC-secreted fractions for the treatment of IVDD.

Introduction

Intervertebral disc degeneration (IVDD) is related to low back pain in both humans and dogs[6,45]. Current treatments, like surgery and medicinal therapies, aim at the relief of pain and neurological deficits, but do not cure the degenerated intervertebral disc (IVD)[5,46]. It is proposed that regenerative therapies like cell-, growth factor- and gene-based therapies could be the future. Therefore, they are being studied for that purpose[4]. Notochordal cell-conditioned medium (NCCM), which contains notochordal cell (NC)-secreted fractions, is a promising potential regenerative medicine candidate for IVDD. Canine and porcine NCCM already showed their value by stimulating extracellular matrix (ECM) production in human or canine chondrocyte-like cells (CLCs) *in vitro*[47]. The regenerative effect of NCCM derived from different species has, however, never been compared. In order to study which species is most favorable to collect NC-rich nucleus pulposus (NP) tissue, produce NCCM, and eventually isolate bioactive factors from, NP tissue from three different species (porcine, canine and human) was used in the present study. The main aim of this study was to compare the species-specific regenerative effect of NCCM on degenerated human CLCs and hence to evaluate whether strategies based on human, porcine or canine NCs have the best potential for translation in both human and dogs.

Materials and methods

Degenerated IVD tissue from human donors (Thompson grade 3) was used for CLC isolation and healthy human, porcine and canine NP tissue (Thompson grade 1) was used to produce NCCM. The

human tissue used for CLC isolation was kindly provided by the Department of Orthopaedics, University Medical Centre Utrecht, the Netherlands. Human IVDs were obtained during a standard post mortem diagnostic procedure in which part of the spine (L2-L5) was collected within 48 hours after death, as approved by the scientific committee of the Pathology department of the University Medical Centre Utrecht. Anonymous use of redundant tissue for research purposes is a standard treatment agreement with patients in the University Medical Centre Utrecht (Local Medical Ethical Committee (METC) number 12-364). The material was used in line with the code 'Proper Secondary Use of Human Tissue' as installed by the Federation of Biomedical Scientific Societies.

Whole porcine spines were obtained from the slaughterhouse in accordance with local regulations. Whole healthy canine spines were collected after euthanizing non-chondrodystrophic (NCD) dogs in unrelated research studies, as approved by the Animal Ethics Committee. An overview of used donors is given in Table 1.

Table 1. Overview of the used human, porcine and canine donors.

Human CLCs	**Donor number**	**Age** (years)	**Gender**
CLC donor pool[1]			
	S14-80	63	Female
	S13-128	49	Male
	S13-142	47	Female

Human NCCM[2]	**Donor number**	**Age**
NCCM HX[3]		
	S14-88	1 day
	S14-114	6 days
NCCM NX[4]		
	S13-206	2 days
	S13-228	5 days

Canine NCCM[2]	**Donor number**	**Age** (months)	**Gender**	**Breed**
NCCM HX				
	104755	18	Female	Mixed
	898503	23	Female	Mixed
	913375	20	Female	Mixed
	920738	18	Female	Mixed

Porcine NCCM[2]	**Donor number**	**Age** (months)
NCCM HX		
	P2	3
	P3	3
	P4	3
	P5	3

[1]Donor chondrocyte-like cells (CLCs) were pooled before use, derived from Thompson grade 3 intervertebral discs (IVDs).

[2]Produced from nucleus pulposus (NP) tissue derived from Thompson grade 1 IVDs.

[3]Notochordal cell-conditioned medium (NCCM) generated under hypoxic conditions (5% O_2).

[4]NCCM generated under normoxic conditions (21% O_2).

Collection of CLCs

Human CLCs were already available (kindly provided by the Department of Orthopaedics, University Medical Centre Utrecht, the Netherlands). Canine NP tissue was collected by careful separation from the AF and kept in hgDMEM + Glutamax (Invitrogen, 31966) + 2% P/S (PPA Laboratories, P11-010). The collected tissue was washed with hgDMEM + Glutamax + 1% P/S. The tissue was subsequently digested with 0.15% pronase and 0.15% collagenase II at 37 °C (45 minutes and overnight, respectively). The next day, the digested tissue was filtered over a 70 µm cell strainer and washed with hgDMEM + Glutamax + 10% Fetal bovine serum (FBS) (High performance, Gibco, 16000-044) + 1% P/S. Finally, the cells were counted, re-suspended in hgDMEM + Glutamax + 10% FBS and 10% DMSO (Merck, 1.02950.0500), divided in cryovials (Corning, 430488) and stored at -196 °C until use.

Production of canine, porcine and human NCCM

The NPs were cultured under hypoxic conditions (37 °C, 5% O_2, 5% CO_2) (S14-88, S14-114 and all canine and porcine NP tissue) and normoxic conditions (21% O_2) (S13-206 and S13-228) in hgDMEM + 1% P/S (1 gram tissue per 30 mL medium). After 4 days, the culture medium with NP tissue was filtered through a 40 µm filter. Subsequently, the culture medium was centrifuged twice for 10 minutes (200 and 500 g) and the supernatant was collected after removal of cell debris (pellet). The supernatant was then filtered again over a 3 KDa filter tube (Amicon Ultra-15 Centrifugal filter) under simultaneous centrifugation for 45 minutes (4000 g, 4 °C). The isolated NC-secreted factors were resuspended in fresh hgDMEM + 1% P/S and this NCCM was stored at -70 °C until use.

Table 2. Contents of expansion and chondrogenic medium.

Expansion medium	**Concentration**
DMEM, high glucose, GlutaMAX (TM), pyruvate (Invitrogen, 31966)	
FBS (High performance, Gibco, 16000-044)	10%
Penicillin/Streptomycin (PPA Laboratories, P11-010)	1%
Ascorbic acid 2-phosphate (Sigma, A8960)	0.1 mM
Dexamethasone (Sigma, D1756)	0.001 µM
bFGF (AbD Serotec, PHP105)	1 ng/mL
Chondrogenic medium	
DMEM, high glucose, GlutaMAX (TM), pyruvate (Invitrogen, 31966)	
ITS+ premix (Corning, 354352)	1%
L-Proline (Sigma,P5607)	0.04 mg/mL
Penicillin/Streptomycin (PPA Laboratories, P11-010)	1%
Ascorbic acid 2-phosphate (Sigma, A8960)	0.1 mM
Human Serum Albumin (Sanquin, Albuman)	1.25 mg/mL

Culture

Human CLCs were expanded in expansion medium (Table 2) in T175 culture flasks (Greiner bio-one, Cellstar, 660175) until they reached passage 2. At 90% confluence, the cells were counted, pooled and plated with a density of 35,000 cells and 50 µL medium/well in a Ultra Low Cluster, Round Bottom, Ultra Low Attachment, 96 Well plate (Corning Costar, 7007). Micro-aggregates were formed after centrifugation at 500 rpm for 5 minutes and cultured in 21 % O_2, 37 °C for 28 days.

The micro-aggregates were treated with chondrogenic medium (Table 2) (control), chondrogenic medium with supplementation of transforming growth factor beta (TGF-ß$_1$) (10 ng/mL, R&D Systems, 240-B-010), and three different sources of NCCM (porcine, dog and human) for 28 days.

Read-out parameters

RT-qPCR was performed to compare gene expression profiles of the different conditions. Safranin O/Fast Green staining and a Di-Methyl Methylene Blue (DMMB) assay were performed to determine GAG deposition. Cell proliferation was assessed by measuring the DNA content of the micro-aggregates. Collagen type I, II and X deposition was determined using immunohistochemistry.

Gene expression profiling

At day 7, micro-aggregates were harvested for RNA isolation, 6 micro-aggregates for control and TGF-ß$_1$ and 4 micro-aggregates for the species-specific NCCM-treated CLCs. These micro-aggregates were repeatedly frozen in liquid nitrogen and crushed using pestles (Argos, 9951-901). The RNA was isolated using the RNeasy Micro Kit (Qiagen, 74004) following manufacturers' instruction with an extra DNase step to ensure DNA removal. The RNA quality was assessed by Agilent 2100 Bioanalyzer and RNA Nanochip kit (5067-1511, Agilent Technologies, Amstelveen, the Netherlands). cDNA was formed using the iScript^{TM} cDNA Synthesis Kit (Bio-Rad Laboratories, 170-8891), following manufacturer' instructions. Gene expression levels were assessed with SYBR Green RT-qPCR (Biorad 170-8880, Herculas, CA) in the *CFX384* Touch Real-Time PCR Detection System (Bio-Rad). 40 cycles were assessed. RT-qPCR was performed for genes associated with matrix production (Collagen type I (*COL1A1*), II (*COL2A1*) and X (*COL10A1*), Aggrecan (*ACAN*)), matrix remodeling (A Disintegrin And Metalloproteinase with Thrombospondin Motifs (*ADAMTS5*), Metalloproteinase 13 (*MMP13*), Tissue inhibitor of Metalloproteinase-1 (*TIMP1*)), cell proliferation (Cyclin D1 (*CCND1*)), apoptosis (B-cell lymphoma 2 (*BCL2*), BCL2-associated X protein (*BAX*), Caspase 3 (*CASP3*)). Sex determining region Y-box 9 (*SOX9*) gene expression was evaluated for the differentiation process towards a chondrocytic phenotype. Genes of interest and reference genes are depicted in Table 3. The Normfirst method was used for visualization of the n-fold gene expression. ΔCt values were calculated (corrected for reference genes, $Ct_{average\ reference\ genese} - Ct_{gene\ of\ interest}$) and averaged. The $E^{\Delta Ct}$ was calculated using the negative control micro-aggregates as calibrator.

Table 3. Information of the human primers used in the current study.

Primer name	Sequence[1]	Number of steps	Amplicon size	Temperature annealing (°C)
TBP[2]	5' TGCACAGGAGCCAAGAGTGAA 3' 5' CACATCACAGCTCCCCACCA 3'	2	132	63.5
YWHAZ[2]	5' ACTTTTGGTACATTGTGGCTTCAA 3' 5' CCGCCAGGACAAACCAGTAT 3'	2	94	64
HPRT[2]	5' TATTGTAATGACCAGTCAACAG 3' 5' GGTCCTTTTCACCAGCAAG 3'	2	192	60
SDHA[2]	5' TGGGAACAAGAGGGCATCTG 3' 5' CCACCACTGCATCAAATTCATG 3'	3	86	58
ACAN	5' CAACTACCCGGCCATCC 3' 5' GATGGCTCTGTAATGGAACAC 3'	2	160	63.5
COL2A1	5' AGGGCCAGGATGTCCGGCA 3' 5' GGGTCCCAGGTTCTCCATCT 3'	2	195	63.5
CCND1	5' AGCTCCTGTGCTGCGAAGTGGAAAC 3' 5' AGTGTTCAATGAAATCGTGCGGGGT 3'	2	480	65
ID1	5' CTCTACGACATGAACGGCTGT 3' 5' TGCTCACCTTGCGGTTCTG 3'	2	76	65
PAI1	5' GCTGGTGAATGCCCTCTAC 3' 5' GGCAGCCTGGTCATGTTG 3'	2	318	65
ALK5	5' GCCGTTTGACTGAAGGCTG 3' 5' GGGCATCCCAAGCCTCATC 3'	2	146	61
COL1A1	5' TCCAACGAGATCGAGATCC 3' 5' AAGCCGAATTCCTGGTCT 3'	2	191	61
MMP13	5' TCCCAGGAATTGGTGATAAAGTAGA 3' 5' CTGGCATGACGCGAACAATA 3'	2	123	64
BCL2	5' ATCGCCCTGTGGATGACTGAG 3' 5' CAGCCAGGAGAAATCAAACAGAGG 3'	2	125	64
BAX	5' GGACGAACTGGACAGTAACATGG 3' 5' GCAAAGTAGAAAAGGGCGACAAC 3'	2	150	60
CASP3	5' CAGTGGAGGCCGACTTCTTG 3' 5' TGGCACAAAGCGACTGGAT 3'	2	102	58
ADAMTS5	5' GCCAGCGGATGTGCAAGC 3' 5' ACACTTCCCCCGGACGCAGA 3'	3	130	62.5
ALK1	5' GCAACCTGCAGTGTTGCATC 3' 5' CGGATCTGCTCGTCCAGCAC 3'	3	139	62.5
SOX9	5' CCCAACGCCATCTTCAAGG 3' 5' CTGCTCAGCTCGCCGATGT 3'	3	242	65.5
AXIN2	5' GGTAGGCATTTTCCTCCATCAC 3' 5' AGCCAAAGCGATCTACAAAAGG 3'	3	104	57
TIMP1	5' CTTCTGGCATCCTGTTGTTG 3' 5' GGTATAAGGTGGTCTGGTTG 3'	2	153	64
COL10A1	5' CACTACCCAACACCAAGACA 3' 5'CTGGTTTCCCTACAGCTGAT 3'	2	225	61

[1]Forward and reverse primer, respectively.

[2]Reference genes.

GAG deposition

At day 14 and 28, micro-aggregates (n=8 for control and TGF-ß$_1$, n=4 for species-specific NCCM) were harvested for the quantitative measurement of GAG production in duplo. Micro-aggregates were washed with Hanks Balanced Salt Solution (HBSS, Gibco) and freeze dried. Each micro-aggregate was digested overnight at 60 °C with 75 µL Papain digestion solution to release GAGs (1.57 mg cysteine HCL (Sigma, C7880) and 250 µg papain (Sigma, P3125) per mL Papain buffer (100 mL Papain buffer: 3.13 g H_2NaPO_4*2 H_2O (Boom, 21254) + 0.326 g EDTA (Merck, 1.00944.1000), adjust to pH 6))[48]. The digested micro-aggregates were diluted (1:10) in PBS-EDTA and a standard line was made in duplo by diluting Chondroitin sulphate C 0.5 mg/mL (Sigma, C4384) with PBS-EDTA. 100 µL was pipetted into a 96 well plate (Greiner, 655191). Next, 200 µL DMMB staining solution (Sigma-Aldrich, 341088) was added to the wells and the extinction was measured directly at 540 and 595 nm using a microplate reader (Model 3550, Bio-Rad). The GAG content was calculated using the polynomic standard line. GAG release into the culture medium was not evaluated due to the high concentration of GAGs in the NCCM itself, which would have given a biased comparison.

DNA content

DNA content was measured using the Qubit dsDNA High Sensitivity Assay Kit (Qiagen, Valencia, CA) according to the manufacturers' instructions.

Histology

At day 28, micro-aggregates were harvested after fixation with 200 µL NBF + 10% eosin overnight. After embedding the micro-aggregates in alginate, they were put in 70% ethanol until dehydration. Afterwards, the micro-aggregates were embedded in paraffin, cut in 5 µm thick sections using a microtome (Leica) and mounted on microscope slides (Klinipath B.V., KP-3056).

For Safranin O/Fast Green staining, the sections were deparaffinized and dehydrated in xylene twice, 96% ethanol, 80% ethanol, 70% ethanol, 60% ethanol and PBS, each for 5 minutes. Then the sections underwent a citrate (citrate buffer, pH 6, 10 mM citric acid (Merck, 1.00244.0500) + 0.05% Tween 20 (Boom, 76021765)) treatment for 15 minutes (RT) and were washed (demi-water, 5 minutes). Subsequently, the sections were dipped in Mayers hematoxylin (J.T.Baker® Chemicals, 3870) for 5 seconds and washed again with demi-water for 5 minutes. The sections were stained with 0.4% aqueous Fast Green (Sigma-Aldrich, F7252) for 4 minutes before dual treatment with fresh 1% acetic acid for 3 minutes. Next, the sections were stained with 0.125% aqueous Safranin O (Sigma-Aldrich, 58884) for 7 minutes. The stained sections were subjected to 96% ethanol twice for 3 minutes, 100% ethanol for 2 minutes and xylene twice for 3 minutes. The sections were mounted using Vectamount (Vector Laboratories, H5000). Images were acquired using an Olympus BX60 microscope with colorview III digital camera and cell imaging software (Olympus, Zoeterwoude, the Netherlands).

For the collagen type II staining, the sections were first deparaffinized and dehydrated as described previously. Next, they were blocked with 0.3% H_2O_2 (Boom B.V., 51008600.9025) for 10 minutes and washed twice with 0.1% PBST for 5 minutes each. Antigen retrieval was performed with 1 mg/mL pronase (Roche Diagnostics, 11459643001) and 10 mg/mL hyaluronidase (Sigma-Aldrich, H3506) both for 30 minutes at 37 °C. Next, blocking was performed with 5% PBS/BSA for 30 minutes at 37 °C. The sections were then incubated with collagen type II mouse monoclonal antibody (DSHB, II-II6B3)

(1/1000 in PBS 5%) overnight at 4 °C. As a negative control, Mouse IgG_1 (3877, Santa Cruz Biotechnology) was used; no false positive staining was observed in these sections. The next day, the sections were washed twice for 5 minutes with 0.1% PBST and incubated at room temperature for 1 hour with the secondary antibody (EnVision+ System-HRP Goat anti-Mouse, DAKO, K4001). The sections were washed again and incubated with DAB peroxidase substrate solution (DAKO, K3468) for 1 minute. Next, the sections were rinsed in Milli-Q and demi-water and counterstained with Hematoxylin QS solution (Vector Laboratories, H3404) for 1 minute. The sections were rinsed for 15 minutes in tap water and afterwards dehydrated through 70% and 80% ethanol (both 3 minutes), 95% ethanol (twice, both 5 minutes), 100% ethanol (5 minutes) and xylene (twice, both 5 minutes). The sections were mounted and images were acquired as described previously.

The sections for collagen type I staining were processed as described previously for collagen type II staining, but with the collagen type I (ab6308, Abcam) mouse primary monoclonal antibody (1/1000 in 5% BSA/PBS 5%).

A staining for collagen type X was performed as described previously for the collagen type II staining with the following adjustments. Antigen retrieval was performed with 0.5% pepsin (DAKO, S3002) for 20 minutes at 37 °C and hyaluronidase (Sigma-Aldrich, H3506). Dual endogenous enzyme peroxidase block (DAKO, S2003) and goat serum were used (incubated at RT for 5 and 30 minutes, respectively) for blocking. The primary collagen type X antibody (Quartett, 2031501005) or negative control antibody (Mouse IgG_1 (3877, Santa Cruz Biotechnology)) were used, both 1:50 diluted with PBS.

Statistical analysis

Statistics were performed using IBM SPSS Statistics 22. First, normality was checked by the Shapiro Wilks test. Subsequently, a Kruskal Wallis and a Mann Whitney U test were performed on all non-normally distributed data. All normally distributed data was tested by a One-Way Anova. Afterwards, post hoc corrections for multiple comparisons were performed by Benjamini & Hochberg False Discovery Rate. A p-value < 0.05 was considered significant for all tests.

Results

Notochordal cell-conditioned medium produced in hypoxic or normoxic conditions

No significant differences were found in DNA, GAG and GAG/DNA content of human CLCs cultured in human NCCM produced under normoxic (NX) or hypoxic (HX) conditions (Figure 1). Thus, the oxygen status during NCCM generation does not result in a differential biologic activity on human CLCs. Though the natural habitat of the NC cells is hypoxic, they can secrete their active factors in NCCM produced under normoxic conditions[14,21]. Therefore, the results of these two NCCM conditions were pooled in the rest of the study.

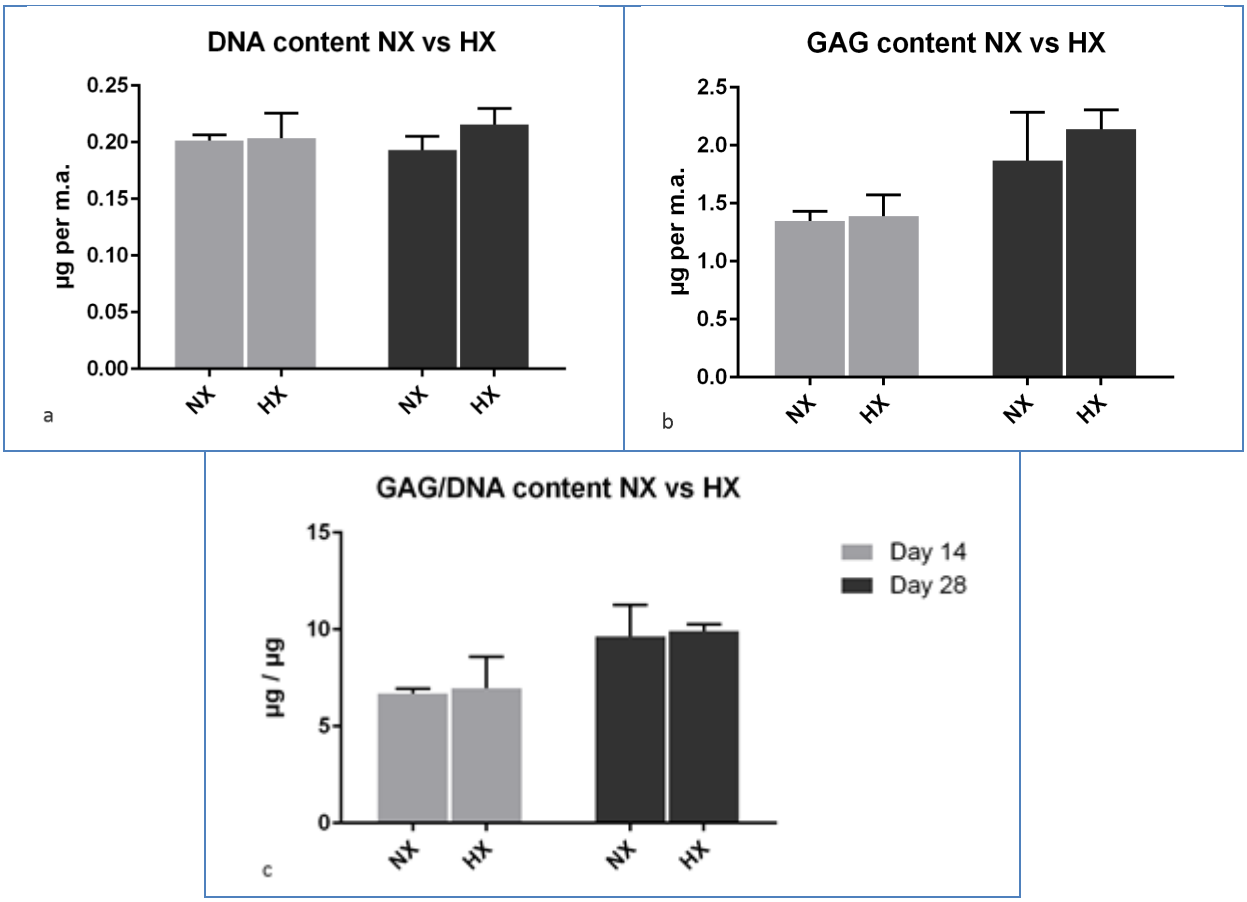


Figure 1. Mean DNA (a), GAG (b) and GAG/DNA (c) content ±S.D. of human CLC micro-aggregates at day 14 and 28 cultured in human NCCM produced under normoxic (NX, 21% O_2) or hypoxic (HX, 5 O_2) conditions.

Gene expression

Matrix production and chondrogenic differentiation

COL1A1, *COL2A1* and *ACAN* gene expression was significantly increased in the TGF-ß$_1$-treated micro-aggregates compared with controls (Figure 2a, b and c). *COL1A1* expression was significantly increased in porcine and canine NCCM-treated micro-aggregates versus human NCCM-treated micro-aggregates. NCCM-treated micro-aggregates showed a tendency towards upregulation of *COL2A1* gene expression, but this was not significantly different from the negative control group. *ACAN* gene expression was significantly downregulated in the porcine and human NCCM-treated micro-aggregates compared with the negative control micro-aggregates (Figure 2a). *COL10A1* gene expression was only detectable in the TGF-ß$_1$-treated micro-aggregates (data not shown). *SOX9* gene expression was evaluated for the differentiation process towards a chondrocytic phenotype and was significantly downregulated in all conditions but canine NCCM. Gene expression of *SOX9* in canine NCCM-treated micro-aggregates was significantly less downregulated than in the micro-aggregates treated with porcine and human NCCM (Figure 2d).

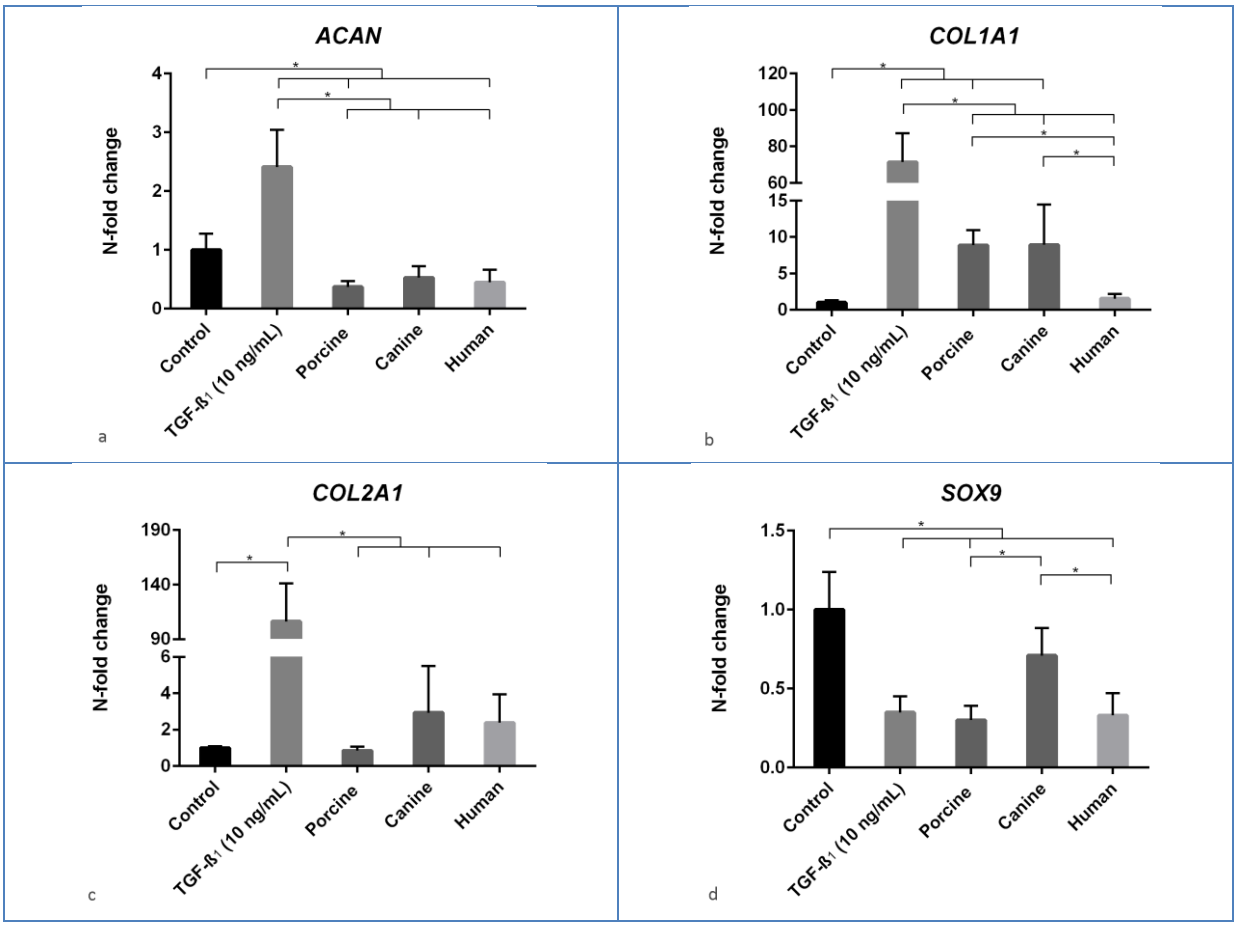


Figure 2. Relative gene expression levels ±S.D. of *ACAN* (a), *COL1A1* (b), *COL2A1* (c), *COL10A1* (d), and *SOX9* (e). Significance level of $p<0.05$. For each target gene, expression levels in the control culture group were set at 1.

Matrix remodeling

ADAMTS5 gene expression, responsible for cleavage of aggrecan, was significantly upregulated in TGF-ß$_1$-treated micro-aggregates compared with human NCCM-treated micro-aggregates (Figure 3a). *MMP13* gene expression, responsible for collagen degradation, was significantly upregulated in all the conditions but canine NCCM (Figure 3b). *TIMP1* gene expression (inhibitor of MMPs) was significantly downregulated in TGF-ß$_1$-treated micro-aggregates, but significantly upregulated in porcine NCCM-treated micro-aggregates (Figure 3c). *TIMP1* expression was significantly more upregulated in porcine NCCM- versus human NCCM-treated micro-aggregates.

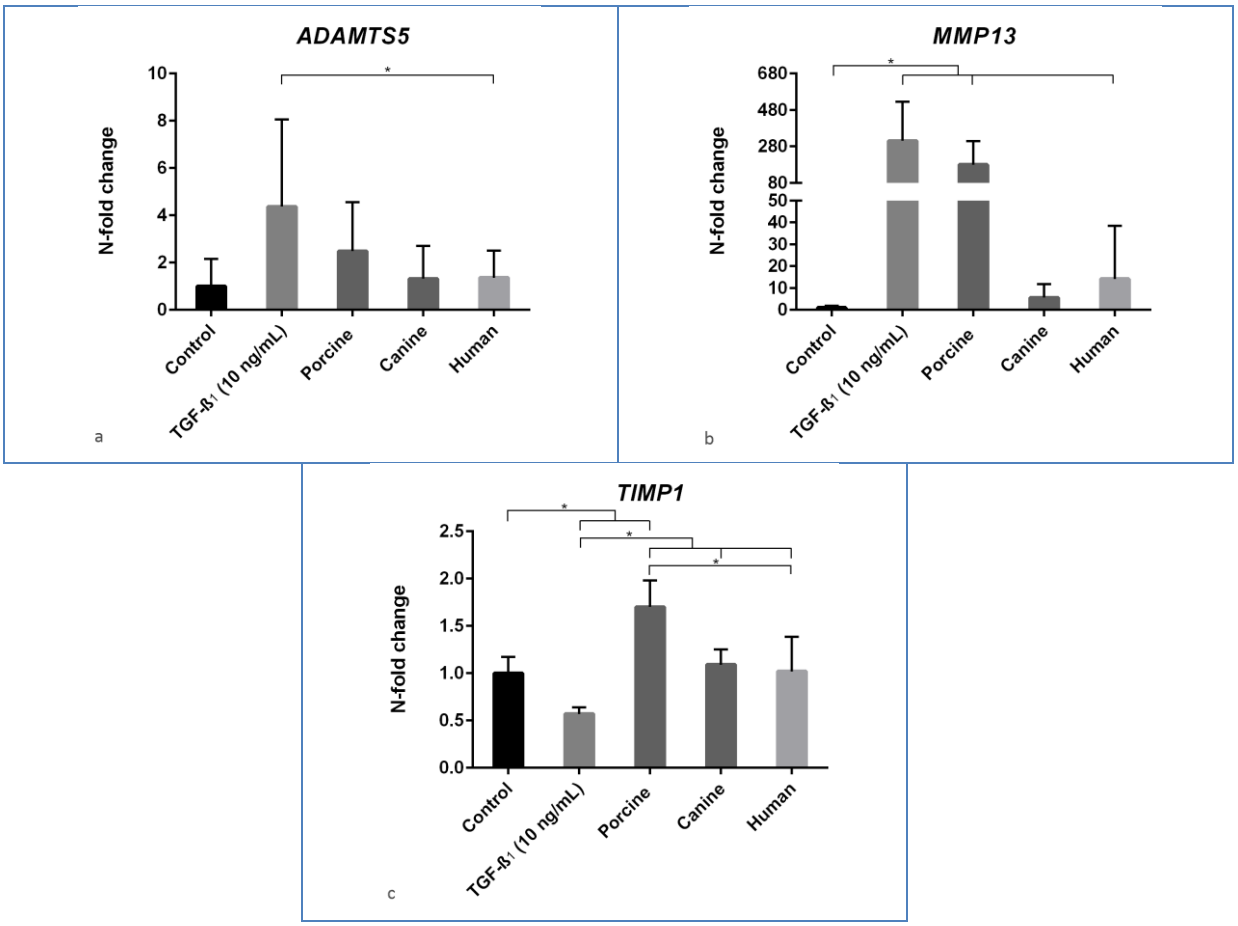


Figure 3. Relative gene expression levels ±S.D. of *ADAMTS5* (a), *MMP13* (b), and *TIMP1* (c). Significance level of $p<0.05$. For each target gene, expression levels in the control culture group were set at 1.

Cell proliferation and apoptosis

The expression of *CASP3* and *BAX*, both apoptosis-related genes, was not significantly affected by the different culture treatments (Figure 4a and c). *BCL2* expression, an anti-apoptotic gene, was significantly downregulated compared with controls in all culture conditions but human NCCM (Figure 4b). Expression of *CCND1,* a cell proliferation marker, was significantly upregulated in control and canine NCCM-treated versus human NCCM-treated micro-aggregates (Figure 4d).

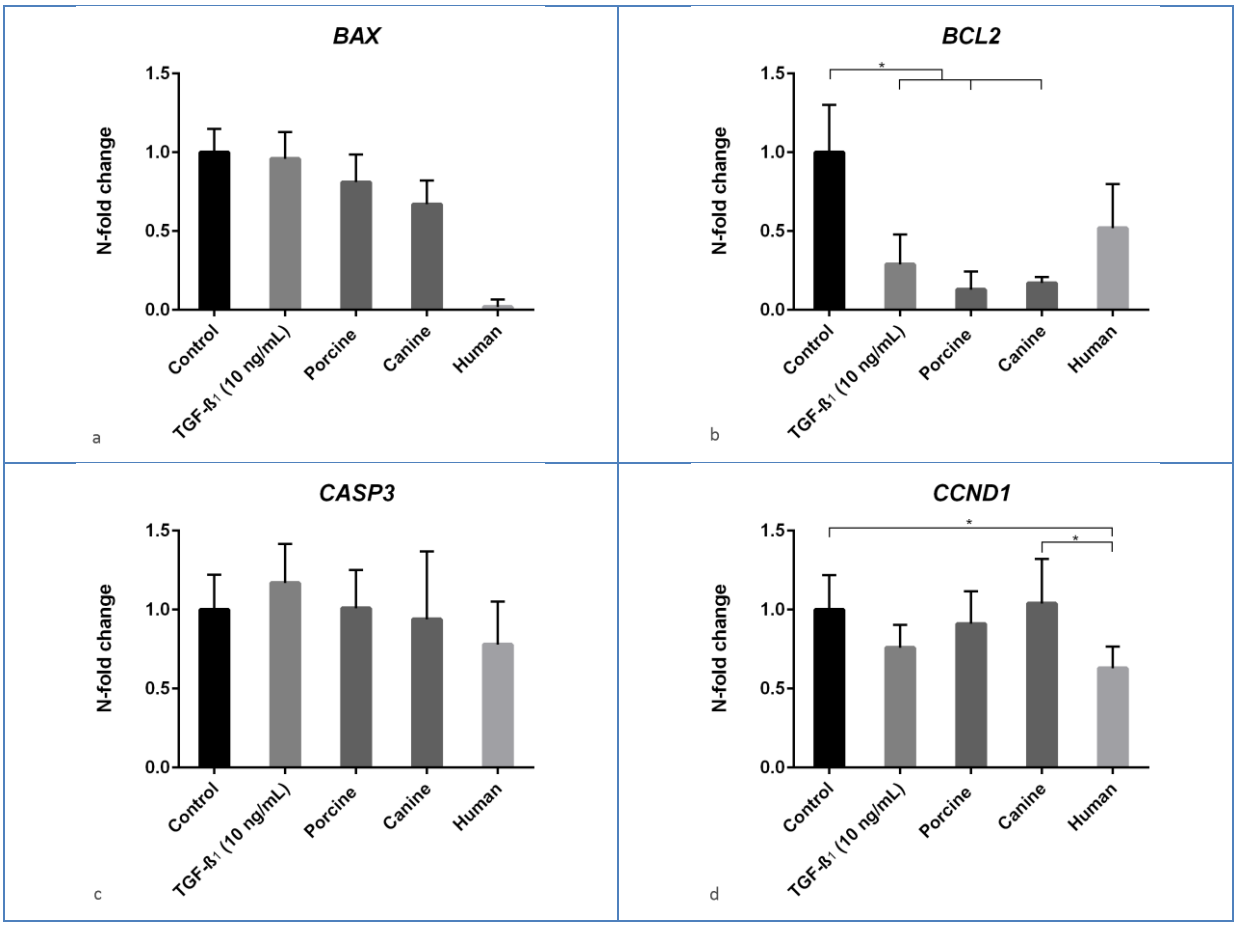


Figure 4. Relative gene expression levels ±S.D. of *BAX* (a), *BCL2* (b), *CASP3* (c), and *CCND1* (d). Significance level of $p<0.05$. For each target gene, expression levels in the control culture group were set at 1.

DNA, GAG and DNA/GAG content

NCCM derived from all species significantly increased the DNA, GAG and GAG/DNA content compared with controls, but not as potent as TGF-$ß_1$ on both time points (Figure 5a, b and c). No significant difference was found between the DNA and GAG/DNA content of porcine NCCM- and canine NCCM-treated micro-aggregates, on both time points. Porcine and canine NCCM were significantly more potent than human NCCM in inducing GAG deposition.

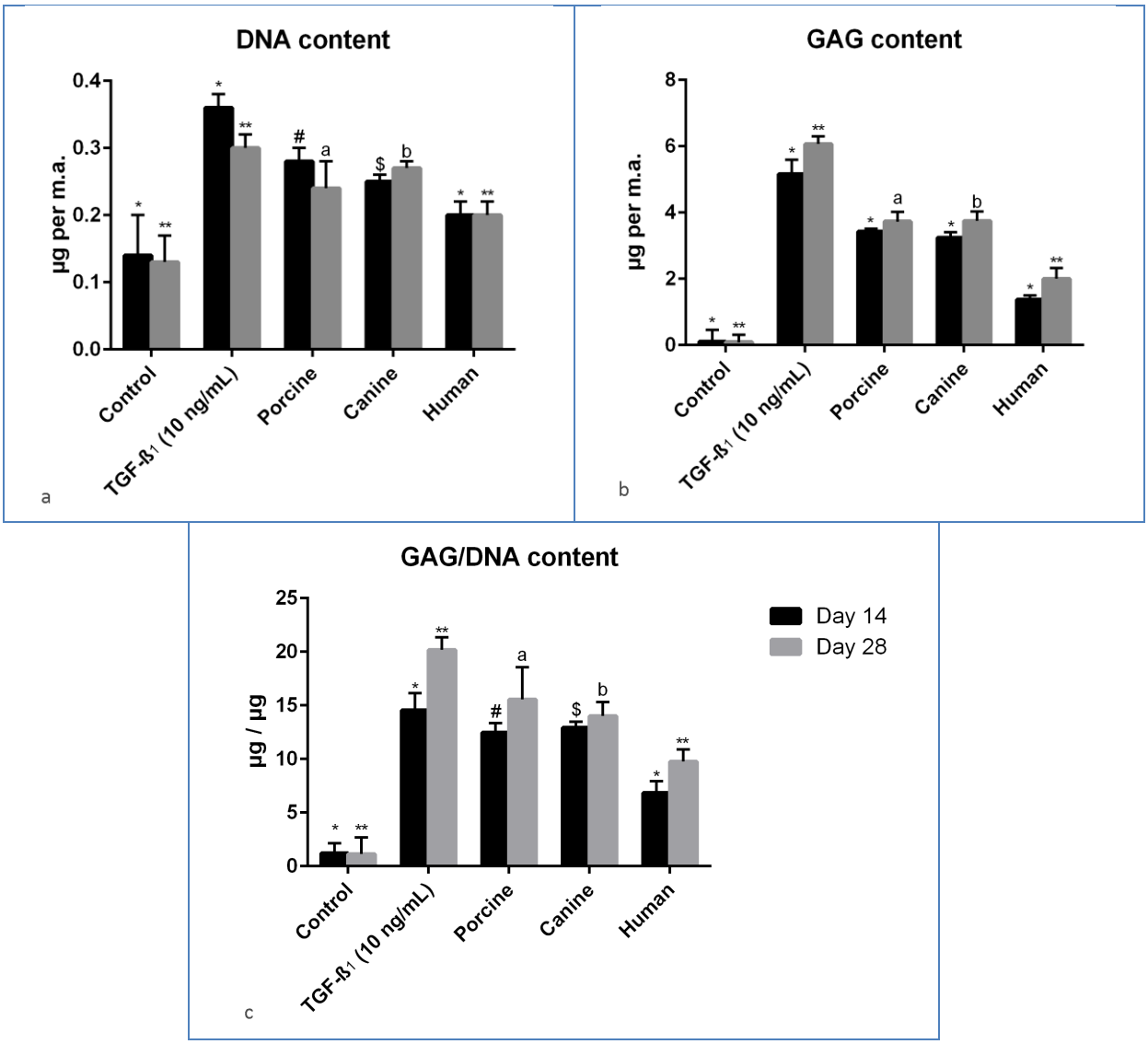


Figure 5. Mean DNA (a), GAG (b) and GAG/DNA (c) content ±S.D. of human chondrocyte-like cell (CLC) micro-aggregates at day 14 and 28. *, **: significantly different from all other conditions at that time point. #, a: significantly different from all other conditions but canine notochordal cell-conditioned medium (NCCM) at that time point. $, b: significantly different from all other conditions but porcine NCCM at that time point. Significance level of $p<0.05$.

Safranin O/Fast Green staining and immunohistochemistry of collagen type I, II, and X

The Safranin O/Fast Green staining indicated that there was little GAG deposition in the control micro-aggregates at day 28 (Figure 6). The TGF-$ß_1$-treated micro-aggregates showed an intense Safranin O (GAG) staining. The micro-aggregates treated with porcine NCCM in turn showed a more intense staining compared with the canine and human NCCM-treated micro-aggregates. The TGF-$ß_1$-treated micro-aggregates showed a fibrotic outer rim and the matrix deposition appeared to be denser than in the micro-aggregates from all other conditions. Micro-aggregates cultured in TGF-$ß_1$, porcine and canine NCCM medium showed collagen type I deposition, with the highest intensity in the TGF-$ß_1$-treated micro-aggregates (Figure 6). Collagen type II deposition was present in all micro-aggregates cultured in human NCCM (Figure 6). Most intense staining was seen in the outer layers of the micro-aggregate. Collagen type X was not detected in the micro-aggregates regardless the tested culture condition (Figure 6).

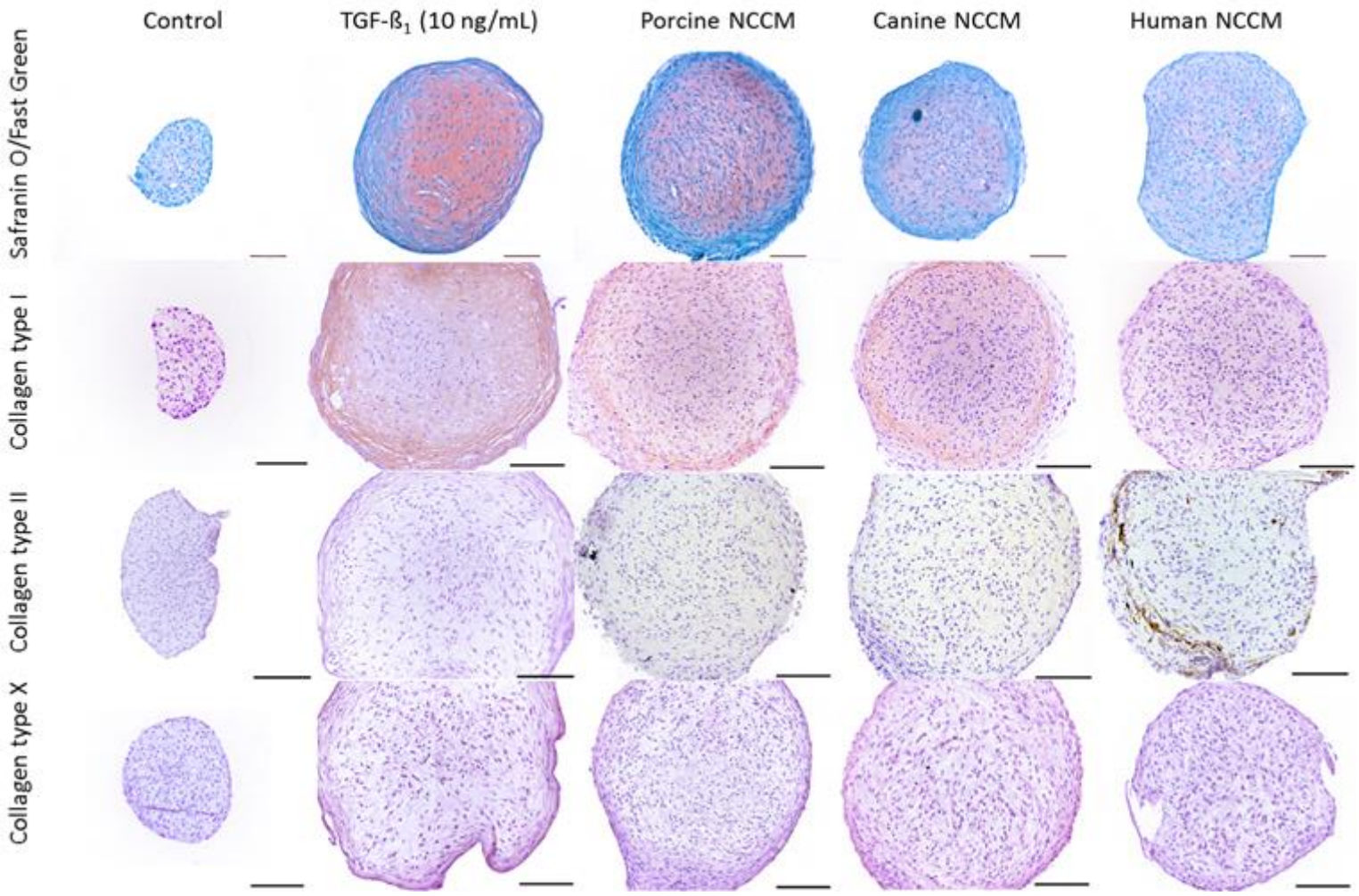


Figure 6. Overview of the Safranin O/Fast Green, collagen type I, II and X immunohistochemistry at day 28. The displayed pictures are representatives for all donors. Scale bar represents 100 µm.

Discussion

Previous studies on canine and porcine NCCM have already shown positive effects on CLCs in terms of cell proliferation and matrix production[22]. However, species-specific NCCM has not yet been compared regarding its regenerative potency. In addition, the impact of secreted factors from human NCs has never been determined (most probably because of low availability of human NC-rich NP tissue). However, it is important to know if the effect of homologous human NC-secreted factors on degenerated human CLCs is comparable or even superior to the secreted factors by canine and porcine NCs. Therefore, the main aim of this study was to compare the species-specific regenerative effect of NCCM on degenerated human CLCs and hence to evaluate whether strategies based on human, porcine or canine NCs have the best potential for translation in human and dogs.

Cross-species effect of NCCM

The present study shows that NCCM derived from all tested species increased both the DNA and GAG content of CLC micro-aggregates compared with controls, indicating a cross-species effect. However, *CCND1* (cell proliferation marker) gene expression was not upregulated at day 7. Upon growth factor stimulation, *CCND1* is upregulated by Ras during the G2 phase of cell mitosis, and is downregulated in the S phase[49]. This may indicate that most of the CLCs were in S phase of the cell cycle when RT-qPCR was performed or that cell proliferation had already peaked before or would have peaked after day 7 of culture (the moment of RT-qPCR). Another explanation may be that cell proliferation was not stimulated at all, but that only cell apoptosis was prevented by the species-specific NCCM compared with controls. However, RT-qPCR did not indicate that anti-apoptotic effects were induced by NCCM. Future studies should measure Cyclin D1 in combination with Ras and cell senescence marker protein levels to clarify this.

While NCCM derived from all species significantly increased GAG deposition in human CLC micro-aggregates, the human NCCM was not as potent in inducing GAG deposition as porcine and canine NCCM. This could indicate (a) that different factors – with a higher potential to stimulate GAG deposition – are present in porcine and canine NCCM than in human NCCM, (b) that the bioactive NCCM factors are more abundantly present in porcine and canine NCCM than in human NCCM or (c) that factors in porcine and canine NCCM have a more active conformational structure than the factors in human NCCM. Although GAG deposition was stimulated, *COL2A1* and *ACAN* gene expression was not significantly upregulated in the NCCM-treated micro-aggregates. De Vries *et al.* showed an increase in *ACAN* and *COL2A1* gene expression in a 28 day culture of CD CLCs using NCD NCCM[50], Abbott *et al.* showed an increase in *COL2A1* gene expression of human CLCs using porcine NCCM[47] and Potier *et al.* showed a downregulation of both genes in a 28 day culture of bovine CLCs[22]. However, these studies use different cell types from different donor species, different donor ages and NCCM was not generated in the same manner, which all limit the comparison of inter-study results. Interestingly, collagen type II protein was only present in the CLC micro-aggregates treated with human NCCM. The latter supports our hypothesis that there are different factors present in human NCCM than in canine and/or porcine NCCM that effectuate a differential biologic response.

TGF-ß$_1$ supplementation induced chondrogenic differentiation of human chondrocyte-like cells

TGF-ß$_1$ addition significantly increased the GAG, DNA and GAG/DNA content of the micro-aggregates compared with the other culture conditions. In line with these findings, *COL1A1*, *COL2A1*, *COL10A1* and *ACAN* gene expression was upregulated, while gene expression of ECM remodeling genes

(*ADAMTS5*, *MMP13* and *TIMP1*) indicated highly active tissue. This combination indicates the remodeling of collagen type II and aggrecan in the first 7 days of the culture[5]. This might also explain why positive collagen type II staining on TGF-$ß_1$-treated micro-aggregates was not encountered.

Wang *et al.* already showed an increase in *ADAMTS5* gene expression after 48 hours of TGF-$ß_1$ treatment in human CLCs. This, in combination with our findings suggests a long-term effect of TGF-$ß_1$ on human CLCs, detectable on RT-qPCR on day 7[26]. Other studies report results in line with our finding about the remodeling genes *MMP13* and *TIMP1*, i.e. upregulation of *MMP13* and downregulation of *TIMP1* gene expression in TGF-ß-treated CLCs. However, other studies show a downregulation of *ADAMTS5*[18,47,51,52]. Explanation could be differences in age and IVD grade of the CLC donors used.

The use of TGF-$ß_1$ as a regenerative mediator to stimulate a healthy NP phenotype has several drawbacks. An upregulation of *COL1A1 and COL10A1* expression in the TGF-$ß_1$-treated micro-aggregates indicates fibrotic (re)differentiation (as found in the annulus fibrosus (AF)), which is not desirable in the loadbearing IVD[6]. These findings are in line with the fibrotic rim surrounding these micro-aggregates [52]. Moreover, there was an increase in collagen type I gene and protein expression in canine and porcine NCCM-treated compared with human NCCM-treated micro-aggregates. This indicates that, besides TGF-$ß_1$, also canine and porcine NCCM can induce mild fibrosis in human CLCs. Although the positive effects of TGF-$ß_1$ on degenerated human CLCs in terms of matrix production and proliferation look promising for use as regenerative mediator, the drawbacks (i.e. fibrotic (re)differentiation) may limit the functionality for the use in the IVD *in vivo*. Future research should investigate whether the identified NC-secreted factors may, when applied in the proper concentration, induce ECM production in a similar manner without this undesirable side effect.

Study limitations

The present study has several limitations, including donor age variability. Human and also porcine donors were considerably younger than the canine NC donors. Unfortunately, inter- and intra-species differences in life span and age of NC loss makes the use of 'comparable' NC donors almost impossible. Additionally, RT-qPCR was only performed at day 7 due to volume limitation of the human NCCM (about 1 mL of NCCM was available per human donor after two years of NCCM collection). Peaks in target gene expression might have been missed by measuring only at day 7. Future research should (when possible) include multiple time points for gene expression analysis. The human NCCM volume limitation also restricted the statistical power of the present study (only n=4). Furthermore, also due to the human NCCM volume limitations, CLCs derived from degenerated (Thompson grade 3) IVDs of three human donors were pooled. The effects of species-specific NCCM was therefore not tested on CLCs derived from human IVDs with other degeneration grades or on other species' CLCs. Another limitation is that in the present study only *ACAN* gene expression was determined, but other important GAGs (decorin, lumican or versican) of the NP ECM should be measured in future studies[22]. Moreover, passage 2 cells were used, but dedifferentiation during the expansion phase is a common drawback[53,54]. Also, cells were cultured *in vitro* under normoxic conditions and a differential response may be expected when supplementing NCCM to an explant system or *in vivo* to the hypoxic NP. In addition, CLC isolation was performed with great care by macroscopic separation of the NP from the AF and EPs, but it cannot be excluded that cells from the AF/EP were isolated, collected and cultured as well[53,54]. Lastly, the focus of the present study was

aimed at regeneration, however disease modifying action of NCs would also be an interesting field of research. Anti-angiogenic, neurogenic or inflammatory actions would be potential mechanisms to study in future research[55].

Clinical relevance and future research

In conclusion, NCCM derived from all species stimulated GAG deposition and proliferation of human CLCs. Human NCCM was less potent in stimulating GAG deposition (but more potent in inducing collagen type II production) compared with porcine and canine NCCM. The observed cross-species effect indicates that the factors present in NCCM work via non-specific pathways, which (a) support study findings of resemblances in IVD de- and regeneration among species[8] or (b) indicates that there are other factors present in NCCM produced from different species[14,18,22]. Since both canine and porcine NC-secreted factors exerted potent regenerative effects on human CLCs and the fact that canine and porcine NCs are more easily available than human NCs, porcine and canine donors are suitable to identify the bioactive factors resulting into IVD regeneration. Human NCCM was not as potent in inducing GAG deposition, but was the only NCCM that promoted collagen type II deposition. Therefore, an optimal balance between species-specific bioactive factors may optimize healthy NP matrix production. Future research should aim at identifying the bioactive factors present within the species-specific NCCM. When the bioactive NC-secreted factors have been identified, synthetic recombinant products can be developed and tested. Eventually, they can potentially be used clinically for the treatment of low back pain in both dogs and man.

Chapter 2: Investigation of the biologic effects of the soluble and pelletable fraction of the notochordal cell-conditioned medium

Abstract

Introduction. Regenerative treatment options for intervertebral disc degeneration (IVDD) aim at restoring a healthy nucleus pulposus (NP) phenotype. Notochordal cell-conditioned medium (NCCM) has shown to stimulate extracellular matrix (ECM) production in bovine and canine chondrocyte-like cells (CLCs) and in human mesenchymal stromal cells (MSCs). However, the bioactive NCCM factors have not been elucidated yet. The aim of this study was to compare the effect of the soluble and pelletable NCCM fraction on canine chondrodystrophic (CD) CLCs and hence to see which NCCM fraction should be further investigated in order to develop a new regenerative treatment strategy. **Material and Methods**. NCCM was generated by culturing healthy notochordal cell (NC)-rich NP tissue of 7 NCD canine donors for 4 days (NCCM +) in plain hgDMEM culture medium. NCs were thereafter dedifferentiated by culturing this healthy NP tissue in FBS-containing hgDMEM culture medium for 21 days. Thereafter, conditioned medium was generated by culturing these dedifferentiated NCs for 4 days in plain hgDMEM (NCCM –). NCCM + was separated into a soluble (NCCM S) and pelletable (NCCM P) fraction by ultracentrifugation. CLCs from degenerated CD canine IVDs were cultured in alginate beads in control culture medium, transforming growth factor beta 1 (TGF-$ß_1$)-supplemented medium, NCCM –, NCCM +, NCCM P (in 2 concentrations: P 1X and P 10X) and NCCM S for 28 days. Read out parameters were cell proliferation (DNA content) and ECM production (glycosaminoglycan (GAG) content, Alcian Blue staining). **Results.** NCCM + was more potent in increasing the GAG and DNA content of the CLC-containing alginate beads than NCCM –. The soluble and pelletable NCCM fraction were equally potent in increasing the GAG and DNA content, but not as potent as NCCM +. **Conclusion.** NCCM created from healthy NCs had a better regenerative potential than NCCM created from dedifferentiated NCs. Both the pelletable and soluble NCCM fraction exerted considerable and similar regenerative effects on canine CD CLCs. Therefore, both fractions should be further investigated for bioactive NC-secreted factor isolation/identification.

Introduction

Advancements in treatment options for intervertebral disc degeneration (IVDD) aim at restoring degenerated nucleus pulposus (NP) tissue to a healthy phenotype. Regenerative therapies (e.g. cell-, growth factor- and/or gene-based therapy) show promising results in terms of extracellular matrix (ECM) production (e.g. proteoglycans and collagen type II)[12,13,22,45,56,57]. In this respect, notochordal cell-conditioned medium (NCCM) produced from notochordal cell (NC)-rich NP tissue has been studied for the use as a regenerative therapy and has shown to stimulate ECM production (proteoglycan and collagen) in bovine and canine chondrocyte-like cells (CLCs) and in human mesenchymal stromal cells (MSCs). A 3D alginate bead culture system has already been established and has shown to be a stable culture method in previous NCCM studies[14,18,19,22,50].

NCCM contains multiple bioactive factors. In order to elucidate which factors induce the regenerative effects, NCCM can be divided in a soluble (peptides and proteins) and pelletable (microvesicles and large protein aggregates) fraction by ultracentrifugation. These vesicles and cellular exosomes can be isolated from several cell types and are considered to mediate cellular communication. Proposed mechanisms include exosomal membrane protein interaction, exosomal membrane protein

interaction with cleavage of soluble ligands and exosomes-target cell fusion, but also tissue stromal supporter (derived from MSCs)[58,59]. The effect of these individual NCCM fractions has not been studied yet. The potent regenerative NCCM fraction(s) has/have to be studied further to identify the regenerative bioactive NC-secreted factor(s). Functional studies and identification of the NC-secreted bioactive factors will give insight into the working mechanism of NCCM. Recombinant production and supplementation of the NC-secreted bioactive factor(s), presumably in combination with other growth factors as a combinatory treatment, may be a step forward in creating an optimal regenerative therapy for IVDD *in vivo*. Therefore, the aim of this study was to compare the effect of the soluble and pelletable NCCM fraction on canine chondrodystrophic (CD) CLCs and hence to see which NCCM fraction should be further investigated for NC-secreted bioactive factor (with best IVDD regenerative treatment potential) identification.

Materials and methods

Whole healthy canine spines were collected after euthanizing CD dogs for unrelated studies, as approved by the Animal Ethics Committee. Tissue from 4 canine CD donors was used for CLC isolation (Table 1).

Table 1. Overview of the used canine chondrodystrophic (CD) donors.

Canine	**Donor number**	**Age** (years)	**Gender**	**Breed**
CLC CD donor pool[1]				
	402932	10.1	Male	Beagle
	7140314	3.3	Male	Beagle
	7207859	4.8	Male	Beagle
	7199261	5.2	Male	Beagle

[1]Donor cells were pooled before use.

Production of NCCM and NCCM fractions

NCCM + was produced as described previously (Chapter 1). Briefly, healthy canine NP tissue (Thompson grade 1) (Table 3) was cultured under hypoxic conditions (37 °C, 5% O_2, 5% CO_2) in hgDMEM + 1% P/S (1 gram tissue per 30 mL medium). After 4 days, the tissue and culture medium were filtered through a 40 µm filter. Subsequently, the culture medium containing the NC-secreted factors was centrifuged twice for 10 minutes (200 and 500 g) and the supernatant was collected after removal of cell debris (pellet). This supernatant was then filtered over a 3 KDa filter tube (Amicon Ultra-15 Centrifugal filter) under simultaneous centrifugation for 45 minutes (4000 g, 4 °C) and the isolated NC-secreted factors were resuspended in fresh hgDMEM + 1% P/S (NCCM+). After NCCM + generation, the NC-rich NP tissue was cultured for 14 days in hgDMEM (High performance, Gibco, 16000-044) + 10% fetal bovine serum to create dedifferentiated NCs, before the 4 day hypoxic culture (37 °C, 5% O_2, 5% CO_2) for NCCM – generation started. NCCM+ was divided into a soluble (NCCM S) and pelletable fraction (NCCM P) by (ultra)centrifugation according to van der Vlist *et al*., 2012 protocol[60]. Briefly, after ultracentrifugation for 30 minutes at 10,000 g and 65 minutes at 100,000 g (both at 4°C), the supernatant (NCCM S) was separated from the pellet (NCCM P). Then, the pellet of 1 mL NCCM was re-suspended in 20 µL PBS + 0.2 % BSA. NCCM P 1x and NCCM S were applied to the micro-aggregates in the same concentration as present in NCCM+, whereas NCCM P was also added at a 10x higher concentration (NCCM P 10x). The P 10X control condition consisted of

only a 0.2% BSA solution, similar to the BSA concentration of the NCCM P 10X condition. NCCM - / +/ S / P was stored at -70°C until use (6 months)[60].

Culture

The effect of the soluble and pelletable NCCM fraction was tested previously by our research group on canine CLC micro-aggregates, but these NCCM-treated canine CLC micro-aggregates lost cell coherence with disintegrated micro-aggregates as a result. This effect was never observed in human NCCM-treated CLC micro-aggregates nor in canine CLC control and TGF-$ß_1$-treated micro-aggregates. Thus, there are fractions present in (canine) NCCM that negatively regulate canine CLC cell adhesion, but not human CLC cell adhesion. For this reason, the alginate bead culture system was employed and has been successfully used for *in vitro* NCCM studies.

NP tissue from canine CD donors was collected as described previously (Chapter 1). Cells were expanded in expansion medium in T175 culture flasks (Greiner bio-one, Cellstar, 660175) until passage 2 was reached. Next, the cells were counted, pooled and a suspension of 6,000,000 cells/mL chondrogenic medium was made (Chapter 1). This solution was diluted 1:1 with 2.4% alginate (Sigma, A2033-100G). Alginate beads were formed using a 23G needle and polymerized for 15 minutes in sterile calcium chloride/HEPES (15 g/L calcium chloride (Boom, 50380) + 2.383 g/L HEPES (Sigma, H4034)). Beads were rinsed twice with Hanks Balanced Salt Solution (HBSS, Gibco) and 1 bead/well was plated in a Ultra Low Cluster, Round Bottom, Ultra Low Attachment, 96 Well plate (Corning Costar, 7007) (Table 2). Medium was changed twice a week and beads were treated once a week with calcium chloride/HEPES to prevent disintegration. Beads were cultured at 21% O_2, 5% CO_2, and 37°C for 28 days.

Table 2. Overview of the used conditions.

Condition	Concentration
Control[1] (chondrogenic medium)	
TGF-$ß_1$[2]	10 ng/mL (R&D Systems, 240-B-010)
NCCM[3] –	
NCCM +	
NCCM P[4] 1X	6.67 µL/mL
NCCM P 10X	66.67 µL/mL
P 10X control	66.67 µL/mL
NCCM S[5]	6.67 µL/mL

[1]Chondrogenic medium, negative control (Chapter 1)
[2]Positive control
[3]Notochordal cell conditioned medium (NCCM)
[4]Pelletable fractions isolated from NCCM
[5]Soluble fractions isolated from NCCM

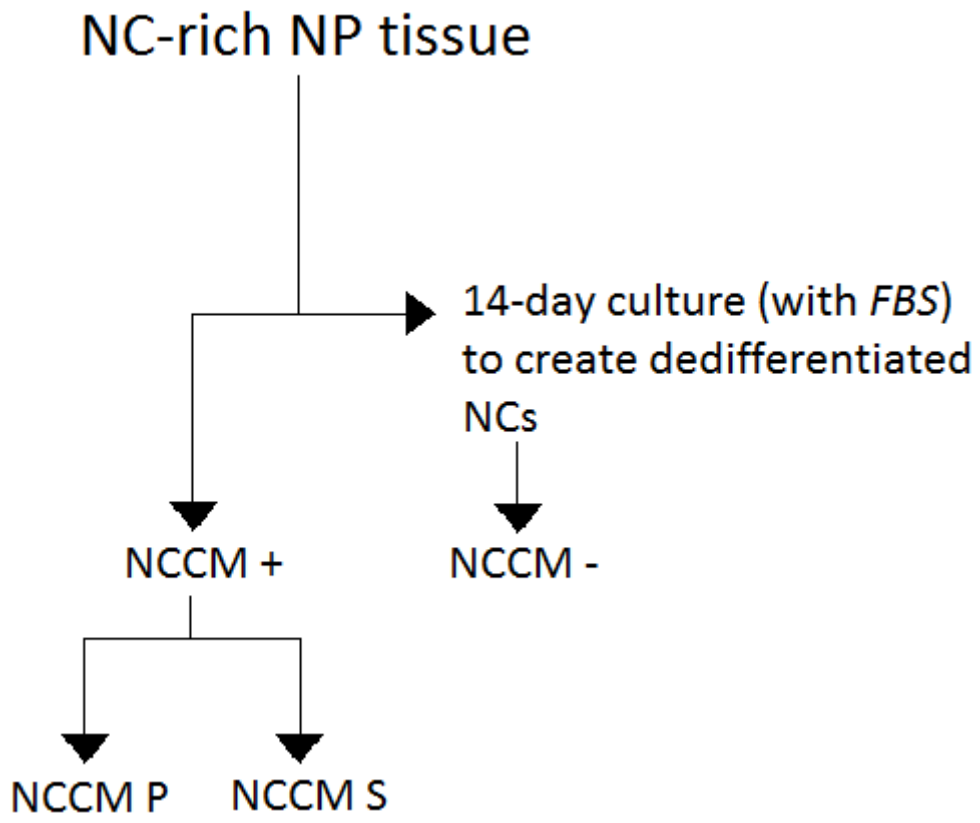


Figure 1. Production of notochordal cell-conditioned medium (NCCM) and NCCM fractions from notochordal cell (NC)-rich nucleus pulposus (NP) tissue. NCCM +: NCCM produced from healthy NCs. NCCM –: NCCM produced from dedifferentiated NCs. NCCM P: Pelletable fraction. NCCM S: Soluble fraction.

Table 3. Overview of the used notochordal cell-conditioned medium (NCCM) donors.

Donor number	**Age** (years)	**Gender**	**Breed**
843181	4.3	Female	Mixed breed
865575	3.8	Female	Mixed breed
853968	4.0	Female	Mixed breed
903728	2.9	Female	Mixed breed
898473	3.2	Female	Mixed breed
911364	2.7	Female	Mixed breed
892190	3.2	Female	Mixed breed

Read-out parameters

Gene expression profiling

At day 7, alginate beads were harvested for RNA isolation (n=7 for each condition) and processed as described previously (Chapter 1) with a 5 minute sodium citrate buffer (16.2 g/L tri-sodium citrate-2-hydrate (Merck, 6448) + 8.8 g/L sodium chloride (Merck, 6404) + 6.0 g/L HEPES (Sigma, H4034)) incubation step (to dissolve alginate) before the addition of RLT buffer + ß-mercaptoethanol (βME). cDNA production and RT-qPCR was performed and analyzed as described previously with the same canine primers and reference genes (Chapter 1).

GAG deposition

At day 28, micro-aggregates (n= 7 per condition; NCCM +, NCCM –, NCCM P 1X, NCCM P 10X and NCCM S in duplo) were harvested for the quantitative measurement of glycosaminoglycan (GAG) production. An additional washing step was included to remove GAGs originating from the NCCM before harvesting. pH of the Di-Methyl Methylene Blue (DMMB) staining solution was adjusted to 1.5 in order to have no interference from the alginate[61]. GAG release into the culture medium was not evaluated due to the high concentration of GAGs in the NCCM itself, which would have given a biased comparison.

DNA content

DNA content was measured by the use of the Qubit dsDNA High Sensitivity Assay Kit (Qiagen, Valencia, CA) according to the manufacturers' protocol.

Histology

At day 28, alginate beads (n=7 per condition) were harvested after treatment with 4% formaldehyde/$CaCl_2$ 200 µL NBF overnight. Thereafter, the beads were put in 70% ethanol and dehydrated. Afterwards, the pellets were embedded in paraffin, sliced at 5 µm thickness by a microtome (Leica) and mounted (Klinipath B.V., KP-3056).

An Alcian Blue staining, in which GAGs are stained dark blue, was performed. For this purpose, sections were deparaffinized and dehydrated as described previously. Next, the sections were stained with Hematoxylin QS solution (Vector Laboratories, H3404) for 10 seconds, and rinsed in tap water (10 minutes). Subsequently, the sections were stained with Alcian Blue staining solution at pH 2.5 (1 g Alcian Blue (05500, Fluka) + 3 mL glacial acetic acid (100063, Merck) + 97 mL demi water) for 10 minutes and rinsed for 2 minutes in tap water. The stained sections were subjected to 95% ethanol twice for 3 minutes, 100% ethanol for 2 minutes and xylene twice for 3 minutes. The sections were mounted using Vectamount (Vector Laboratories, H5000). Images were acquired using an Olympus BX60 microscope with colorview III digital camera and cell imaging software (Olympus, Zoeterwoude, the Netherlands).

Collagen type I and II immunohistochemistry (IHC) were performed as described previously (Chapter 1). Briefly, sections were deparaffinized (xylene), dehydrated (ethanol) and blocked with 0.3% H_2O_2 for 10 minutes and washed with 0.1% PBST. Antigen retrieval was performed with 1 mg/mL pronase (Roche Diagnostics 11459643001) and 10 mg/mL hyaluronidase (Sigma-Aldrich, H3506) both for 30 minutes at 37 °C. Blocking was performed with 5% PBS/BSA for 30 minutes at 37 °C. The sections were then incubated with collagen type II mouse monoclonal antibody (DSHB, II-II6B3) (1:2000 in PBS 5%) or with the collagen type I (ab6308, Abcam) mouse primary monoclonal antibody (1:1500 in 5% BSA/PBS 5%) overnight at 4 °C. As a negative control, Mouse IgG_1 (3877, Santa Cruz Biotechnology) was used; no false positive staining was observed in these sections. The next day, the sections were washed with 0.1% PBST and incubated with the secondary antibody (EnVision+ System-HRP Goat anti-Mouse, DAKO, K4001). DAB peroxidase substrate (DAKO, K3468) was used and the sections were counterstained with Hematoxylin QS solution (both 1 minute). All sections were dehydrated through ethanol and xylene. Next, sections were mounted and images were acquired using an Olympus BX60

microscope with colorview III digital camera and cell imaging software (Olympus, Zoeterwoude, the Netherlands).

Statistical analysis

Statistics were performed using IBM SPSS Statistics 22. First, normality was checked by the Shapiro Wilks test. Subsequently, a Kruskal Wallis and a Mann Whitney U test were performed on all non-normally distributed data. All normally distributed data was tested by a One-Way Anova. Afterwards, post hoc corrections for multiple comparisons were performed by Benjamini & Hochberg False Discovery Rate. A *p*-value < 0.05 was considered significant for all tests.

Results

DNA, GAG and GAG/DNA content CD beads

The DNA content of the CLC-containing alginate beads significantly decreased in all cultured conditions compared with day 0 (Figure 2a). Only in NCCM P 10X, the DNA content was significantly higher compared with the negative control. However, the DNA content in the NCCM P 10X condition was not significantly different from the P 10x control. NCCM P 1X and NCCM S had a significantly lower DNA content compared with TGF-β_1.

NCCM - – treated alginate beads deposited significantly less GAGs than all other conditions. Micro-aggregates treated with NCCM + produced and incorporated significantly more GAGs than any other condition at day 28 (Figure 2b). Furthermore, treatment with TGF-β_1, NCCM P 1X, NCCM P 10X and NCCM S resulted in significantly more GAG deposition than the negative control alginate beads. These four conditions were equally potent in inducing GAG deposition. NCCM P 10X significantly increased GAG deposition in the CLCs compared with P 10X control.

All conditions significantly increased the GAG/DNA content of the alginate beads compared with day 0, however only NCCM –, +, P 1X and S significantly increased the GAG/DNA content compared with the negative control (Figure 2c). Additionally, NCCM –, P 1X and S were equally potent and increased the GAG/DNA content significantly more than TGF-β_1. NCCM P 10X significantly increased the GAG/DNA content of the alginate beads compared with P 10X control.

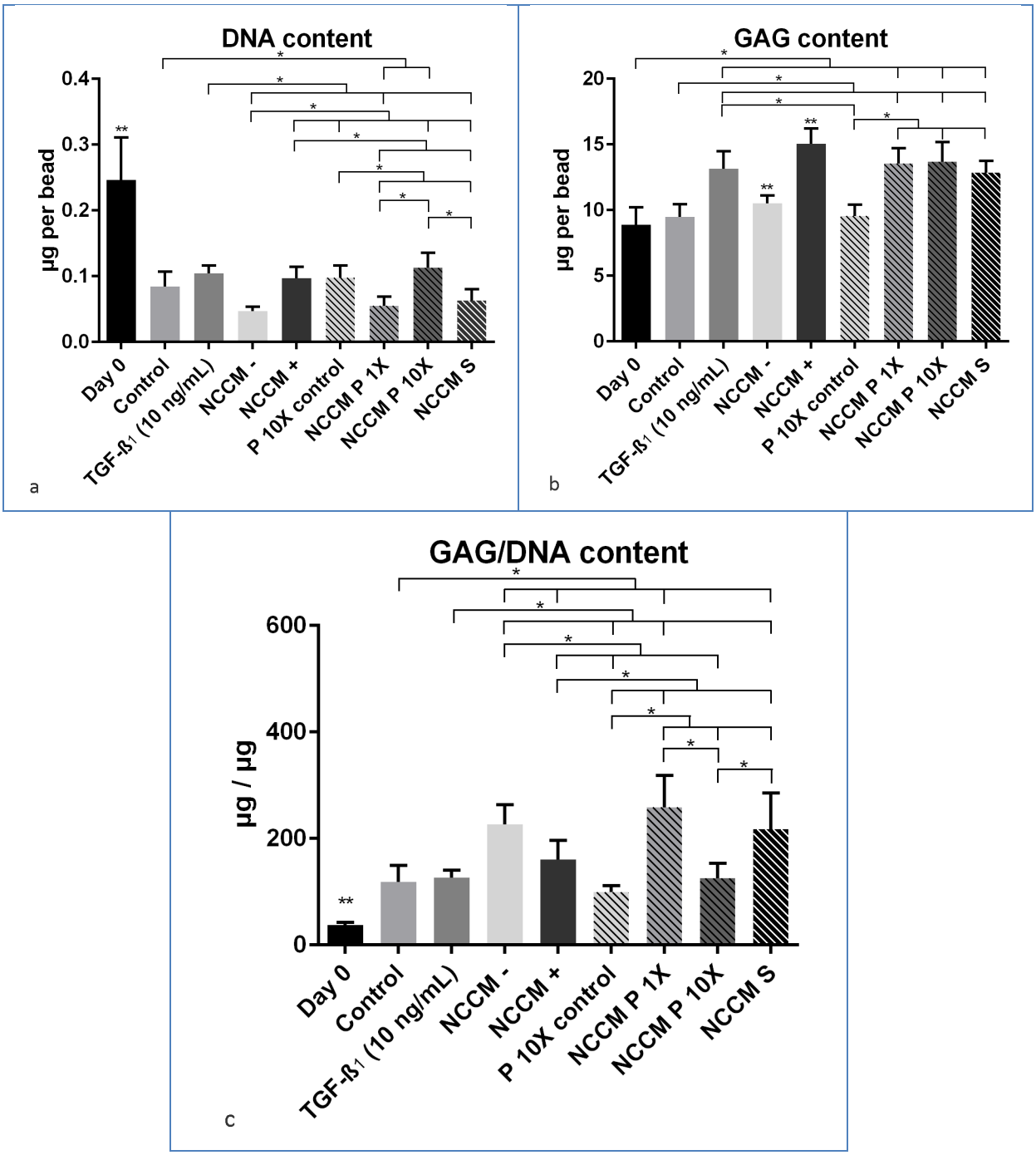


Figure 2. Mean DNA (a), GAG (b) and GAG/DNA (c) content ±S.D. of canine CD alginate beads at day 28. * = $p<0.05$. **: significantly different from all other conditions. Significance level of $p<0.05$.

Alcian Blue staining

Alginate beads treated with negative control medium, NCCM P 10X and NCCM S hardly showed any positive staining (Figure 3). The most intense staining was observed around the CLCs of alginate beads treated with NCCM –, NCCM +, NCCM P 1X, NCCM P 10X control and TGF-$ß_1$.

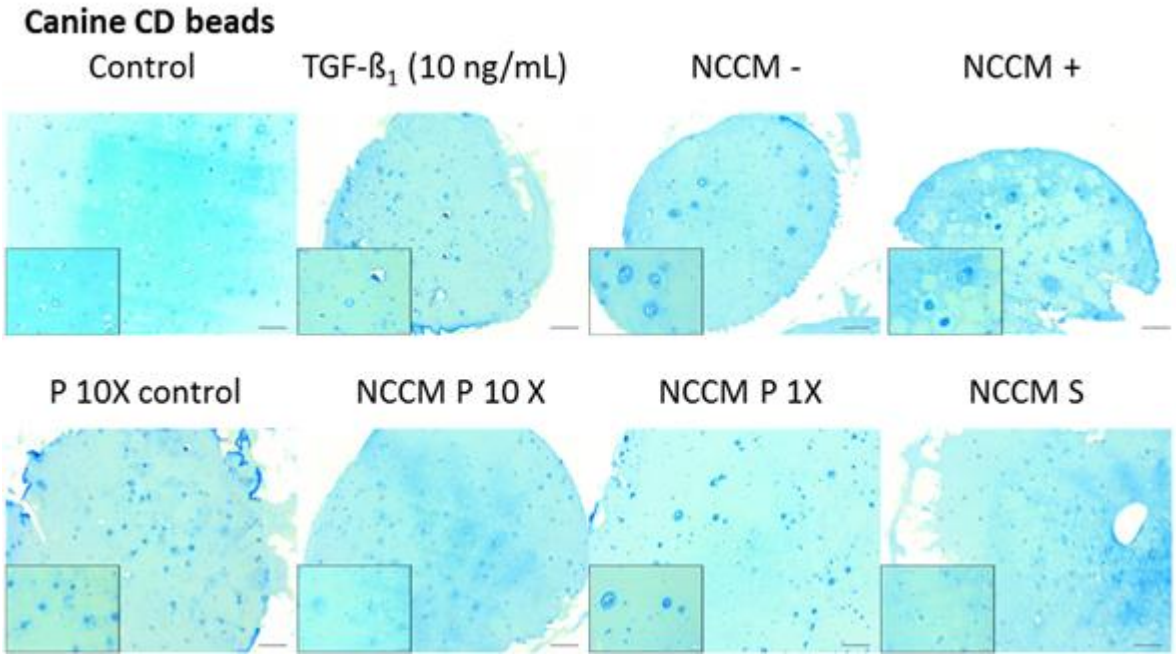


Figure 3. Overview of the Alcian Blue staining at day 28. The displayed pictures are representatives for all donors, scale bar represents 200 µm. Inset: scale bar represents 50 µm. Light blue is alginate, dark blue represents glycosaminoglycan (GAG) deposition.

Collagen type I and II immunohistochemistry

No collagen type I and II protein expression was detected by IHC.

Gene expression

Gene expression profiling was performed on day 7 (RT-qPCR), however reference and target gene expression was undetectably low, due to a technical limitation related to very low starting amounts of RNA. Therefore, results could not be further analyzed and are not reported here.

Discussion

Previous *in vitro* studies showed increased ECM production in human MSCs[14,18] and porcine and canine CLCs[19,22,50] upon NCCM treatment. However, the effect of the NCCM-derived pelletable and soluble fraction has not been tested previously. This study compared the effects of soluble and pelletable NCCM fractions on canine CD CLCs.

The 10X concentrated pelletable NCCM fraction significantly increased the CLC alginate bead DNA content compared with NCCM P 1X. However, NCCM P 10X did not differ from P 10X control, indicating that the augmenting effect could be attributed to the higher concentration of albumin in the culture medium[62]. Interestingly, the GAG content of the CLC-containing alginate beads treated with NCCM P 1X and P 10X was significantly increased compared with P 10X control-treated alginate beads. This indicates that the presence of pelletable fractions (1X or 10X) has a positive effect on GAG deposition. NCCM produced from dedifferentiated NCs (NCCM –) loses its DNA and GAG inductive potential, indicated by the lower DNA and GAG content of the alginate beads treated with NCCM – compared with the alginate beads treated with NCCM +, NCCM P 10X and NCCM S. This is in line with previous findings in which negative effects of bovine CLCs on bovine bone marrow MSCs (BMSCs) in coculture, compared with BMSC culture alone was observed; collagen type II and

aggrecan gene expression decreased more than 2-fold[22]. In contrast, NCCM + (derived from fresh NC-rich NP tissue) has positive effects on human MSCs in terms of GAG deposition. NCCM soluble and pelletable (P 1X) fractions were equally potent in stimulating the DNA and GAG content of the alginate beads but not as potent as NCCM +, suggesting that both soluble and pelletable fractions are responsible for the regenerative effects of NCCM + on CLCs. Future studies should focus on the individual fractions and concentration-dependent effects of NCCM P and S.

Limitations

In the present study, cell numbers significantly decreased during the culture period (about 60%), indicating massive cell death, while the same culture system has been employed with success[63]. Besides, RT-qPCR was not possible in the present study because of extremely low target and reference gene expression levels due to low RNA starting amounts. This all indicates that the canine CLCs did not thrive in the alginate bead culture system in the present study. Culturing canine CLCs in a pellet culture system would be preferred. In order to overcome the negative effects of NCCM in cell adhesion during pellet formation, growth factors like TGF-β_1 and BMPs could be employed to facilitate cell-cell adhesion[64-67]. Moreover, passage 2 cells were used, but dedifferentiation during the expansion phase is a common drawback[53,54]. Also, cells were cultured *in vitro* under normoxic conditions and a differential response may be expected when supplementing NCCM (fractions) in an explant system or *in vivo* to the hypoxic NP. Besides, the NCCM (fractions) were kept at -70°C for 6 months, which could have affected the regenerative potential compared with freshly added NCCM (fractions). Exosomes derived from several cell types have shown reactive potential in several studies, and even after multiple freezing and thawing, reactive potential, exosome size, and miRNA content remained stable[68-73]. Lastly, on Alcian Blue staining, considerable GAG deposition was encountered around the TGF-ß$_1$, NCCM –, NCCM +, P 10X control and NCCM P 1X-treated CLCs. This, however, only partially supports the results obtained from quantitative GAG analysis (DMMB assay). The discrepancy can be explained by the fact that the Alcian Blue staining is only a qualitative assessment of GAG deposition and that only some 5 µm-thick sections were stained and thus no overview of the whole bead is obtained. However, the middle (representative) part of the bead was stained, making this explanation less likely. Also limitations of the Alcian Blue staining itself can account for the observed discrepancies, because Alcian Blue is unstable in mild environments and does not bind to unsulfated GAGs[74-76], indicating that some GAGs are not detected by this staining. However, Alcian Blue staining and not Safranin O/Fast Green staining was performed in the present study, since the latter stains the alginate false positively red making visualization of GAG deposition impossible.

Clinical relevance and future research

In conclusion, NCCM + had a better regenerative effect than NCCM – in terms of inducing the DNA and GAG content of canine CLC alginate beads. Using an alginate bead culture system where CLCs did not thrive even in the presence of TGF-ß$_1$, we found that pelletable and soluble NCCM fractions (applied in the concentration as present in NCCM +) were equally potent in increasing the GAG and DNA content of the CLC alginate beads, but not as potent as NCCM +. This implies that both soluble and pelletable fractions are responsible for the regenerative effects of NCCM + on canine CLCs. Future studies should aim at (a) optimizing culture conditions for NCCM-treated canine CLCs with extended and detailed RT-qPCR and matrix analysis and (b) if it still appears that both soluble and pelletable NCCM fractions are responsible for the regenerative effects of NCCM +, both fractions should be further investigated for

bioactive NC-secreted factor identification. For identification of the bioactive factors in the soluble NCCM fraction, proteomic analysis is proposed. For identification of the bioactive factors in the pelletable NCCM fraction, RNA sequencing, proteomics and lipidomics are suitable. Ultimately, when the bioactive NC-secreted factor(s) with the best regenerative potency has/have been identified, it/they can be tested (in an optimal concentration) *in vitro*, and potentially *in vivo,* for the treatment of canine and/or human IVDD.

Chapter 3: Notochordal cell-conditioned medium does not exert regenerative effects in pretreated micro-aggregates: a pilot study

Abstract

Introduction. Intervertebral disc degeneration (IVDD) is one of the main causes of low back pain in both humans and dogs. Current treatment options do not cure the degenerated IVD and therefore notochordal cell-conditioned medium (NCCM) is proposed as a new regenerative therapy. However, NCCM-treated canine chondrodystrophic (CD) chondrocyte-like cells (CLCs) did not thrive in an alginate bead culture system. In addition, NCCM-treated canine CD CLC micro-aggregates disintegrated. NCCM thus contains fractions that inhibit canine CLC adhesion and therefore, optimization of NCCM-treated canine CLC culture conditions is necessary. The growth factors transforming growth factor beta 1 (TGF-ß$_1$) and bone morphogenetic protein 2 (BMP-2) are known to stimulate cell-cell adhesion. Therefore, the aim of this study was to determine whether growth factor (TGF-ß$_1$ and BMP-2) pretreatment prevents NCCM-induced canine CD CLC micro-aggregate disintegration. **Material and Methods.** CLCs were isolated from 4 canine CD donors and pooled. NCCM + was produced from one healthy nucleus pulposus (NP) donor. The CLCs were pretreated for 5 days with either 250 ng/mL BMP-2 or 10 ng/mL TGF-ß$_1$ after which they received chondrogenic control-, TGF-ß$_1$-, BMP-2- or canine NCCM + supplemented medium until day 28. Read out parameters were cell proliferation (DNA content) and extracellular matrix (ECM) production (glycosaminoglycan (GAG) content, Safranin O/Fast Green staining). **Results.** In NCCM-treated canine CD CLC micro-aggregates that were pretreated with TGF-ß$_1$ and BMP-2, disintegration was prevented. Both TGF-ß$_1$- and BMP-2-treated micro-aggregates showed an increased GAG/DNA content after 28 days compared with all other conditions, but 28 day TGF-ß$_1$ treatment increased the GAG/DNA content 2-fold compared with 28 day BMP-2 treatment. NCCM treatment was far less potent in enhancing the canine CD CLC micro-aggregates GAG and DNA content compared with TGF-ß$_1$- and BMP-2 treatment. **Conclusion.** Pretreatment with TGF-ß$_1$ or BMP-2 improves cell-cell adhesion and prevents the disintegration of NCCM-treated canine CLC micro-aggregates, indicating that this growth factor pretreatment is required to be able to test the regenerative effect of NCCM on canine CLCs *in vitro*. Canine NCCM was, however, not potent in inducing cell proliferation and ECM production by canine CLCs. Future studies should test more canine CLC and NCCM donors to determine whether canine NCCM can exert potent regenerative effects on canine CLCs, as also observed on human CLCs.

Introduction

Low back pain, common in both humans and dogs, has been related to degeneration of the intervertebral disc (IVD)[1,77]. Current treatments (surgery, medication), however, have serious drawbacks. Therefore, developing new treatment options for intervertebral disc degeneration (IVDD) requires fast, effective and applicable research. Growth factor- and cell-based treatments are proposed as regenerative strategies[4]. Transforming growth factor beta 1 (TGF-ß$_1$), bone morphogenetic protein 2 (BMP-2) and notochordal cell-conditioned medium (NCCM) stimulated extracellular matrix (ECM) production in canine chondrocyte-like cells (CLCs) derived from the nucleus pulposus (NP) (Chapter 2 and 4). However, NCCM-treated canine chondrodystrophic (CD) CLCs did not thrive in an alginate bead culture system (Chapter 2). Furthermore, treatment of canine CD CLCs with NCCM led to disintegration of the micro-aggregates (unpublished data). TGF-ß$_1$

stimulates cell-cell adhesion[64,65], indicating that this growth factor can potentially be used to prevent micro-aggregate disintegration. The same is proposed for BMP-2[66,67]. Therefore, the aim of this pilot study was to determine whether growth factor (TGF-ß$_1$ and BMP-2) pretreatment prevents NCCM-induced canine CD CLC micro-aggregate disintegration.

Materials and methods

Whole healthy canine spines were collected after euthanizing CD dogs for unrelated studies, as approved by the Animal Ethics Committee. Tissue from 4 canine CD donors was used for CLC isolation, after which the CLCs were pooled for use in culture (Table 1).

Culture

Culture and NCCM generation were performed as described previously (Chapter 1). Briefly, female, mixed breed donor 892190 was used to isolate healthy canine NP tissue (Thompson grade 1) and to produce NCCM. The nucleus pulposus (NP) was cultured under hypoxic conditions (37 °C, 5% O_2, 5% CO_2) in hgDMEM + 1% P/S (1 gram tissue per 30 mL medium). After 4 days, the NP tissue and culture medium was filtered through a 40 µm filter. Subsequently, the culture medium containing NC-secreted factors was centrifuged twice for 10 minutes (200 and 500 g) and the supernatant was collected after removal of cell debris. This supernatant was filtered again, over a 3 KDa filter tube (Amicon Ultra-15 Centrifugal filter) under simultaneous centrifugation for 45 minutes (4000 g, 4 °C) and the isolated NC-secreted factors were resuspended in fresh hgDMEM + 1% P/S (NCCM). All micro-aggregates were pretreated with 10 ng/mL TGF-ß$_1$ or 250 ng/mL BMP-2 for 5 days (Figure 1). Subsequently, from day 5 to 28, micro-aggregates were treated with control culture medium (T Control or B Control), chondrogenic medium supplemented with 10 ng/mL TGF-ß$_1$ (R&D Systems, 240-B-010) (T TGF), 250 ng/mL BMP-2 (TETEC) (B BMP) or NCCM (T NCCM or B NCCM). Expansion and chondrogenic culture medium were similar as described previously (Chapter 1).

Table 1. Overview of the used canine chondrodystrophic (CD) donors.

Canine	**Donor number**	**Age** (years)	**Gender**	**Breed**
CLC CD donor pool[1]				
	402932	10.1	Male	Beagle
	7140314	3.3	Male	Beagle
	7207859	4.8	Male	Beagle
	7199261	5.2	Male	Beagle

[1]Donor cells were pooled before use.

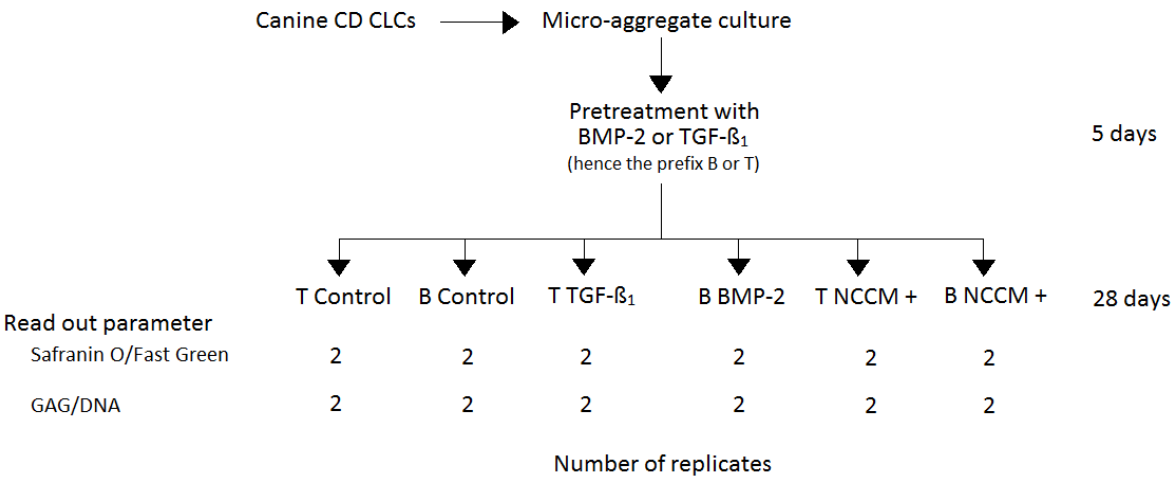


Figure 1. Canine chondrodystrophic (CD) chondrocyte-like cells (CLCs) were pretreated with either bone morphogenetic protein 2 (BMP-2) or transforming growth factor beta 1 (TGF-ß$_1$) for 5 days (hence the prefix B or T). After which micro-aggregates were treated with control chondrogenic medium (Chapter 1), TGF-ß$_1$-supplemented chondrogenic medium, BMP-2-supplemented chondrogenic medium or notochordal cell-conditioned medium (NCCM) for 28 days. Safranin O/Fast Green staining and GAG/DNA analysis were performed with 2 replicates per condition.

Read-out parameters

DNA content and GAG deposition

At day 28, micro-aggregates (n= 2 per condition) were harvested for measuring the DNA content and glycosaminoglycan (GAG) production. The DNA content was measured by the use of the Qubit dsDNA High Sensitivity Assay Kit (Qiagen, Valencia, CA) according to the manufacturers' protocol. The quantitative measurement of GAG deposition was performed as described previously (Chapter 1). GAG release into the culture medium was not evaluated due to the high concentration of GAGs in the NCCM itself, which would have given a biased comparison. Furthermore, Safranin O/Fast Green staining was performed as described previously (Chapter 1). Briefly, the sections were deparaffinized (xylene), dehydrated (ethanol) and underwent a citrate treatment (15 minutes). Sections were dipped in Mayers hematoxylin for 5 seconds and washed with demi-water. Subsequently, the sections were stained with 0.4% aqueous Fast Green for 4 minutes before dual treatment with fresh 1% acetic acid for 3 minutes. Next, sections were stained with 0.125% aqueous Safranin O for 7 minutes. All sections were dehydrated through ethanol and xylene. Next, sections were mounted and images were acquired using an Olympus BX60 microscope with colorview III digital camera and cell imaging software (Olympus, Zoeterwoude, the Netherlands).

Results

Pellet formation was maintained during the entire culture period in all TGF-β_1- and BMP-2-pretreated CD canine CLC micro-aggregates. Furthermore, both TGF-ß$_1$ and BMP-2 treatment increased the micro-aggregates' DNA and GAG content in the first 5 days (Figure 2). The DNA content of the T Control and T TGF-β_1-treated micro-aggregates – both pretreated for 5 days with TGF-β_1 – was not significantly different. The DNA content of micro-aggregates that were treated with BMP-2 for 28 days (B BMP) was, however, higher than the micro-aggregates which were only pretreated with

BMP-2 the first 5 days and thereafter received control culture medium (B Control). The GAG and GAG/DNA content of the micro-aggregates treated with BMP or TGF for 28 days (B BMP and T TGF, respectively) was increased compared with all other conditions with at least 2-fold. However, only 28 day TGF treatment increased the GAG/DNA content more than 2-fold compared with 28 day BMP-2 treatment. Additionally, NCCM was not as potent as TGF or BMP supplementation in inducing the DNA, GAG and GAG/DNA content of the CD canine CLC micro-aggregates.

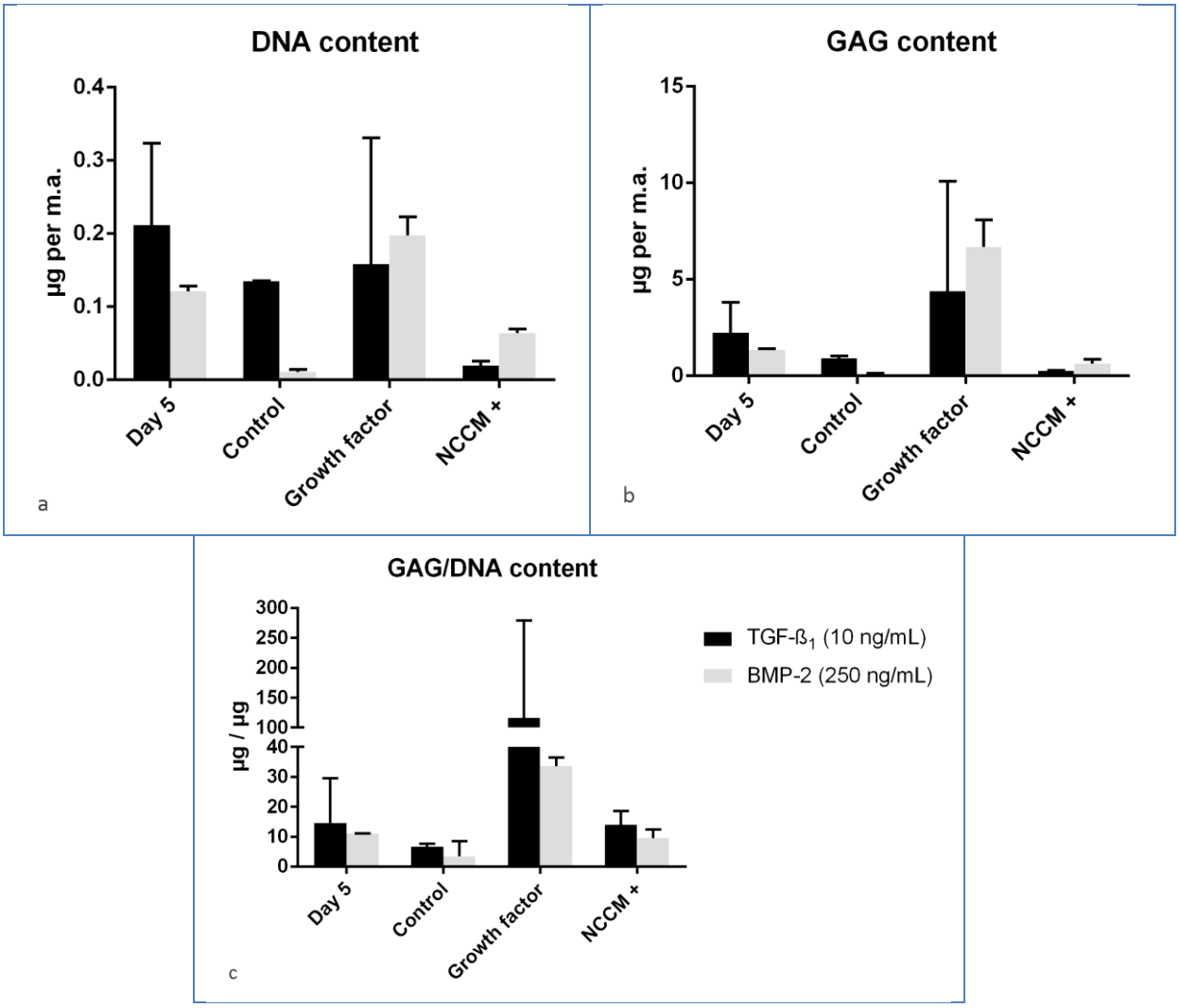


Figure 2. Mean DNA (a), GAG (b) and GAG/DNA (c) content ±S.D. of canine chondrodystrophic (CD) micro-aggregates at day 28. All conditions treated for 28 days, unless stated differently. TGF-$ß_1$: Transforming growth factor beta 1. BMP-2: Bone morphogenetic protein 2. NCCM: notochordal cell-conditioned medium.

Micro-aggregates pretreated with TGF-$ß_1$ for 5 days stained positively for GAGs, whereas this positive staining disappeared in the TGF-$ß_1$-pretreated control micro-aggregates (T Control), which received only control culture medium from day 5 until 28 (Figure 3). Micro-aggregates supplemented with TGF-$ß_1$ for 28 days (T TGF) showed a more intense staining than micro-aggregates supplemented with TGF-$ß_1$ for only 5 days. Additionally, there was no GAG staining observed in the NCCM-treated micro-aggregates pretreated with TGF-$ß_1$ (T NCCM), which had apoptotic cells in the center. Micro-aggregates pretreated with BMP-2 for 5 days stained positive for GAGs, whereas the BMP-2-pretreated control micro-aggregates (B Control) showed no GAG deposition. Treatment of BMP-2 for 28 days (B BMP) induced a more intense red staining compared with the micro-aggregates treated

with BMP-2 for 5 days. Also, NCCM-treated micro-aggregates pretreated with BMP-2 (B NCCM) did not show GAG deposition and had apoptotic cells in the center.

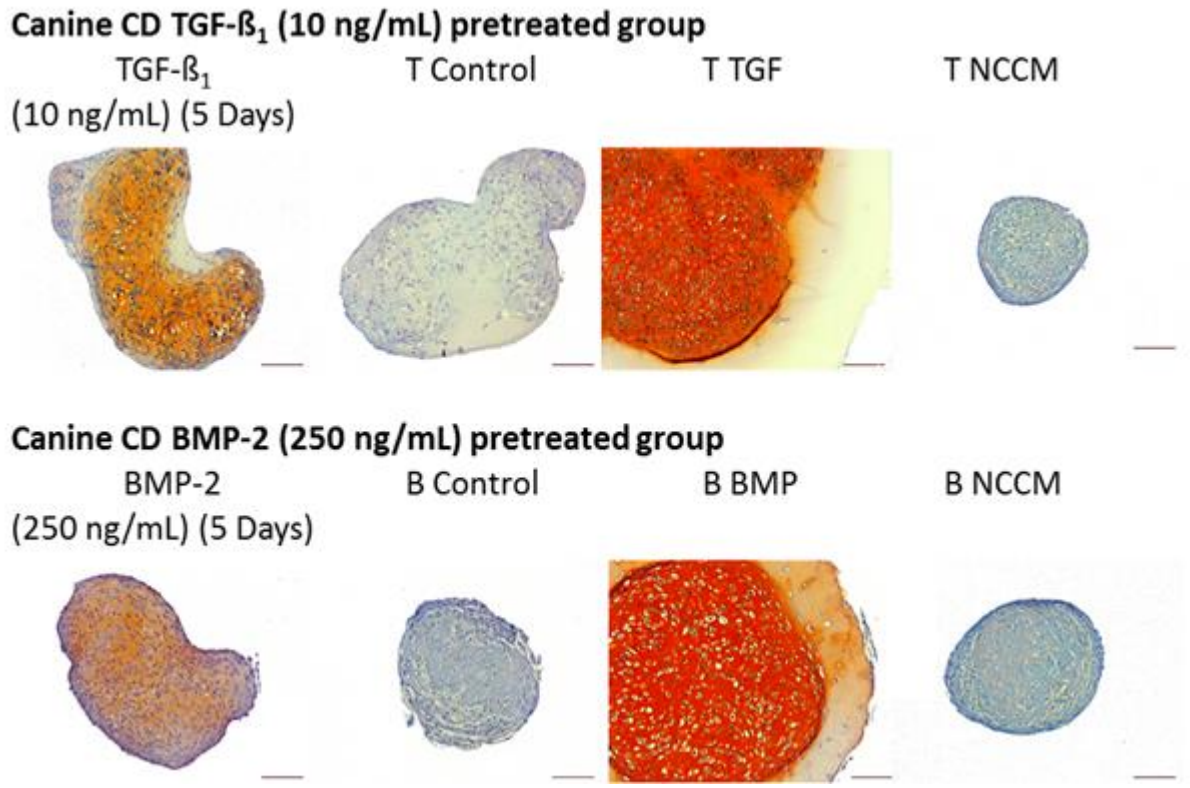


Figure 3. Overview of the canine chondrodystrophic (CD) chondrocyte-like cell (CLC) Safranin O/Fast Green staining at day 5 and 28. The displayed pictures are representatives for all donors. Scale bar represents 100 µm.

Discussion

Previous work by our group indicated that NCCM-treated canine CLC micro-aggregates lost coherence and disintegrated over time. TGF-$ß_1$ is a known stimulator of cell-cell adhesion[64,65], whereas BMP-2 is proposed to have a similar effect[66,67]. Therefore, the aim of this study was to determine whether TGF-$ß_1$ and/or BMP-2 pretreatment prevented NCCM-induced canine CD CLC micro-aggregate disintegration.

TGF-$ß_1$ or BMP-2 pretreatment inhibits NCCM-induced CLC micro-aggregate disintegration

Pellet formation was maintained during the entire culture period in all TGF-$β_1$- and BMP-2-pretreated CD canine CLC micro-aggregates, which was also observed by Purmessur *et al*. (2011) in TGF-$ß_3$-treated human BMSCs pellets (250,000 cells)[18]. Pan *et al.* showed that BMP-2-derived P24 proteins (coated onto cell membranes) were able to promote cell adhesion[67]. Additionally, also BMP-4 is suggested to be a potential mediator in cell adhesion[66]. The NCCM-treated canine CLC micro-aggregates remained very small, showed signs of apoptosis and had a considerably lower GAG/DNA content than micro-aggregates treated with TGF-$β_1$ and BMP-2 for 28 days. The results from the current pilot study indicates that BMP-2 and TGF-$ß_1$ (supplemented for either 5 or 28 days) are more potent in inducing cell proliferation and ECM deposition than canine NCCM and that 5 day TGF-$ß_1$ or BMP-2 pretreatment does not increase canine CD CLC susceptibility to canine NCCM. However, the current study was only a pilot experiment with one NCCM donor and one CLC donor pool, and future

studies should test more canine CLC and NCCM donors to determine whether canine NCCM can induce canine CLC cell proliferation and ECM deposition as also observed in human CLCs.

Continuous BMP-2 stimulation was required to maintain the high BMP-2-induced DNA content, indicating that the proliferative effect of BMP-2 is lost when CLCs are not continuously stimulated with this growth factor. In contrast, the inductive effect of TGF-ß$_1$ on the canine CLC micro-aggregates DNA content was maintained throughout the entire culture period, whether TGF-β_1 was added the entire culture period or only the first 5 days. Continuous BMP-2/TGF-ß$_1$ supplementation, however, induced a more potent regenerative effect in terms of ECM production than pretreatment for only the first 5 days. This was supported by Wang *et al*. (2013) who showed that proliferation in human CLCs increased when TGF-ß$_1$ was supplemented for a longer period of time[26] and by Van Beuningen *et al*. (2000) and Blaney Davidson *et al.* (2007) who showed that multiple injections with TGF-ß in mice knee joints increased proteoglycan production more than a single injection[78,79]. The continuous effect of BMP-2 was shown by Yoon *et al.* in rat CLCs *in vitro*, where an increased proliferation and GAG content was observed over time[80].

Limitations, clinical relevance and future research

This study had several limitations. First of all, no statistical analysis was performed in this pilot study due to the low number of biological replicates. Future studies should test more canine CLC and NCCM donors (and also perform statistical analysis) to determine whether canine NCCM can induce canine CLC proliferation and ECM deposition as also observed in human CLCs (Chapter 1). Furthermore, the addition of micro-aggregates that were not pretreated with growth factors would provide more insight into the disintegration process. Additionally, RT-qPCR should be performed to determine target gene expression levels during pretreatment and the rest of the culture period. Moreover, passage 2 cells were used, but dedifferentiation during the expansion phase is a common drawback[53,54]. Also, cells were cultured *in vitro* under normoxic conditions and a differential response may be expected when supplementing NCCM (fractions) in an explant system or *in vivo* to the hypoxic NP.

In conclusion, 5 day pretreatment of canine CD CLC micro-aggregates with either 10 ng/mL TGF-ß$_1$ or 250 ng/mL BMP-2 completely counteracted the disintegration of NCCM-treated micro-aggregates. This indicates that TGF-ß$_1$/BMP-2 pretreatment is required to be able to test the regenerative effect of NCCM on canine CLCs *in vitro* in a 3D micro-aggregate culture system. In this pilot study, canine NCCM appeared to only marginally induce canine CLC proliferation and ECM production. Future studies should test more canine CLC and NCCM donors to determine whether canine NCCM can exert regenerative effects on canine CLCs, as also observed on human CLCs.

Chapter 4: The effect of transforming growth factor beta 1 and bone morphogenetic protein 2 on chondrocyte-like cells derived from degenerated human and canine intervertebral discs

Abstract

Introduction. Current treatments for intervertebral disc degeneration (IVDD) are not satisfactory since they do not repair the degenerated Intervertebral disc (IVD) and, therefore, new, regenerative therapies are being studied. Transforming growth factor beta (TGF-ß) exerts chondrogenic and anabolic effects on human chondrocyte-like cells (CLCs), however, it can induce undesirable fibrotic (re)differentiation. Bone morphogenetic protein 2 (BMP-2) also exerts regenerative effects on human CLCs in terms of proteoglycan and collagen production. The aforementioned growth factors have been studied separately on human CLCs, but until now their regenerative potential on canine chondrodystrophic (CD), non-chondrodystrophic (NCD) and human CLCs has not been compared. Therefore, the aim of this study was to compare the regenerative potential of TGF-$ß_1$ and BMP-2 on canine CD, NCD and human CLCs and to determine which growth factor would be the best candidate for the treatment of IVDD *in vivo*. **Material and Methods.** CLCs from degenerated human, canine CD and NCD IVDs (Thompson score 3) were cultured in micro-aggregates in negative control, TGF-$ß_1$ (10 ng/mL)- or BMP-2 (100 and 250 ng/mL)-supplemented culture medium for 28 days. Read out parameters were extracellular matrix (ECM) production (RT-qPCR, glycosaminoglycan (GAG) content and release, Safranin O/Fast Green staining and immunohistochemistry (IHC)), cell proliferation (DNA content, RT-qPCR) and apoptosis (RT-qPCR)). **Results.** 250 ng/mL BMP-2 was more potent in increasing GAG deposition and DNA content in canine CD, NCD and human CLC micro-aggregates compared with 100 ng/mL BMP-2. TGF-$ß_1$ and 250 ng/mL BMP-2 treatment both induced GAG deposition. However, only TGF-$ß_1$ induced increased *COL1A1* expression and a fibrotic rim surrounding the micro-aggregates. NCD CLCs treated with 250 ng/mL BMP-2 incorporated more GAGs into their ECM, whereas CD CLCs treated with 250 ng/mL BMP-2 secreted more GAGs into the culture medium. **Conclusion.** BMP-2 has a regenerative potential and is in a concentration of 250 ng/mL more potent in canine CD, NCD and human CLCs than 100 ng/mL BMP-2. Additionally, BMP-2 does not induce fibrotic (re)differentiation as observed with TGF-$ß_1$ treatment. This indicates that 250 ng/mL BMP-2 can be used in future studies in the search for novel treatment mediators for IVDD in canine and human patients.

Introduction

Intervertebral disc degeneration (IVDD) is one of the main causes of low back pain and accounts for billions of costs. Several studies have been performed with growth factors in order to create a satisfactory regenerative therapy for IVDD. One of the most widely used growth factors *in vitro* is transforming growth factor beta (TGF-ß). TGF-ß has tremendous chondrogenic and anabolic effects on chondrocyte-like cells (CLCs) via Smad-dependent or –independent pathways[12,23,30,81]. However, a major drawback of TGF-ß treatment is the fibrotic (re)differentiation it can induce (Chapter 1)[52]. Bone morphogenetic proteins (BMPs) can also work via Smad-dependent pathways and have stimulating effect on chondrogenesis[82]. BMP-2, one of the most clinically used BMPs, has already been used in vertebrae fusion[31]. However, it also has positive effects on proteoglycan production in rabbit, bovine and human CLCs, with maintenance of the CLC phenotype[12]. This suggests that BMP-2 could be a

candidate for regenerative IVDD *in vitro* studies, but more importantly, as a regenerative treatment for IVDD *in vivo*[12,56].

An ideal/optimal regenerative therapy *in vitro* and *in vivo* will ideally have no potential to induce fibrotic (re)differentiation, will stimulate extracellular matrix (ECM) production, can restore the phenotype of degenerated intervertebral disc (IVD) cells, and/or can restore the declined cell numbers of the degenerated IVD. The separate effects of TGF-ß$_1$ and BMP-2 on human CLCs have already been studied, but they have not been compared. Also, they have not been tested on canine CLCs. Therefore, the aim of this study was to investigate and compare the effects of TGF-ß$_1$ and BMP-2 on human, canine chondrodystrophic (CD) and non-chondrodystrophic (NCD) CLCs to determine their regenerative potential as an IVDD treatment.

Materials and methods

For the canine CLCs, whole canine spines were collected after euthanizing CD and NCD dogs in unrelated research studies (approved by the Animal Ethics Committee). Tissue from 4 canine CD and NCD donors was used for CLC isolation (Table 1). Regarding the human CLCs, the same set up was used as displayed in Chapter 1. Degenerated IVD tissue from human donors (Thompson grade 3, kindly provided by the Department of Orthopaedics, University Medical Centre Utrecht, the Netherlands) obtained during a standard post mortem diagnostic procedure was used for CLC isolation (L2-L5).

Table 1. Overview of the used canine chondrodystrophic (CD) and non-chondrodystrophic (NCD) donors.

Canine	**Donor number**	**Age** (years)	**Gender**	**Breed**
CLC CD donor pool[1]				
	402932	10.1	Male	Beagle
	7140314	3.3	Male	Beagle
	7207859	4.8	Male	Beagle
	7199261	5.2	Male	Beagle
CLC NCD donor pool[1]				
	879061	2.2	Male	Mixed breed
	121114	10	Male	German Shepard
	77662	1.2	Male	Mixed breed
	100913	11	Male	Jack Russell

[1]Donor cells were pooled before use.

Culture

Nucleus pulposus (NP) tissue from canine CD and NCD donors was collected as described previously (Chapter 1). Cells were expanded in expansion medium in T175 culture flasks (Greiner bio-one, Cellstar, 660175) until passage 2 was reached (Chapter 1). Next, the cells were counted, pooled and plated with a density of 35.000 cells/well in a Ultra Low Cluster, Round Bottom, Ultra Low Attachment, 96 Well plate (Corning Costar, 7007). Micro-aggregates were formed after centrifugation at 500 rpm for 5 minutes with 50 µL culture medium per well. Human CLCs were cultured as described previously (Chapter 1).

Micro-aggregates were treated with chondrogenic control culture medium (control), chondrogenic culture medium with supplementation of TGF-ß$_1$ (10 ng/mL, R&D Systems, 240-B-010) (TGF-ß$_1$), or

two different concentrations of BMP-2 (100 ng/mL and 250 ng/mL, TETEC) for 28 days. Composition of expansion medium and chondrogenic medium was similar as described previously (Chapter 1).

Read-out parameters

Gene expression profiling

Table 2. Information of the canine primers used in the current study.

Primer name	Sequence[1]	Number of steps	Amplicon size	Temperature annealing (°C)
GAPDH[2]	5' TGTCCCCACCCCCAATGTATC 3' 5' CTCCGATGCCTGCTTCACTACCTT 3'	2	100	57
RPS19[2]	5' CCTTCCTCAAAAAGTCTGGG 3' 5' GTTCTCATCGTAGGGAGCAAG 3'	2	95	58
HPRT[2]	5' AGCTTGCTGGTGAAAAGGAC 3' 5' TTATAGTCAAGGGCATATCC 3'	2	104	56 + 58
SDHA[2]	5' GCCTTGGATCTCTTGATGGA 3' 5' TTCTTGGCTCTTATGCGATG 3'	2	92	61
ACAN	5' GGACACTCCTTGCAATTTGAG 3' 5' GTCATTCCACTCTCCCTTCTC 3'	2	111	63.5
COL2A1	5' GCAGCAAGAGCAAGGAC 3' 5' TTCTGAGAGCCCTCGGT 3'	2	151	62 – 63.5
CCND1	5' GCCTCGAAGATGAAGGAGAC 3' 5' CAGTTTGTTCACCAGGAGCA 3'	2	117	65
ID1	5' CTCAACGGCGAGATCAG 3' 5' GAGCACGGGTTCTTCTC 3'	2	135	62 – 66
PAI1	5' AAACCTGGCGGACTTCTC 3' 5' ACTGTGCCACTCTCATTCAC 3'	2	98	64 – 65.5
ALK5	5' GAGGCAGAGATTTATCAGACC 3' 5' ATGATAATCTGACACCAACCAG 3'	2	116	61
COL1A1	5' GTGTGTACAGAACGGCCTCA 3' 5' TCGCAAATCACGTCATCG 3'	2	109	58 – 64
MMP13	5' CTGAGGAAGACTTCCAGCTT 3' 5' TTGGACCACTTGAGAGTTCG 3'	2	250	64
BCL2	5' TGGAGAGVGTCAACCGGGAGATGT 3' 5' AGGTGTGCAGATGCCGGTTCAGGT 3'	2	87	64
BAX	5' CCTTTTGCTTCAGGGTTTCA 3' 5' CTCAGCTTCTTGGTGGATGC 3'	2	108	58 – 64
CASP3	5' ATCACTGAAGATGGATGGGTTGGGTT 3' 5' TGAAAGGAGCATGTTCTGAAGTAGCACT 3'	2	139	55.5 – 59
ADAMTS5	5' CTACTGCACAGGGAAGAG 3' 5' GAACCCATTCCACAAATGTC 3'	2	149	62.5
ALK1	5' CCTTTGGTCTGGTGCTGTG 3' 5' CGAAGCTGGGATCATTGGG 3'	2	107	62.5
SOX9	5' CGCTCGCAGTACGACTACAC 3' 5' GGGGTTCATGTAGGTGAAGG 3'	2	105	65.5
AXIN2	5' GGACAAATGCGTGGATACCT 3' 5' TGCTTGGAGACAATGCTGTT 3'	2	141	57
TIMP1	5' GGCGTTATGAGATCAAGATGAC 3' 5' ACCTGTGCAAGTATCCGC 3'	2	120	66

[1]Forward and reverse primer, respectively.

[2]Reference genes.

At day 7, micro-aggregates were harvested (n=7 for each condition). RNA isolation, cDNA synthesis and RT-qPCR was performed described previously (Chapter 1). The used canine target and reference gene primers are depicted in Table 2. Human target and reference genes are shown in Table 3.

Table 3. Information of the human primers used in the current study.

Primer name	Sequence[1]	Number of steps	Amplicon size	Temperature annealing (°C)
TBP[2]	5' TGCACAGGAGCCAAGAGTGAA 3' 5' CACATCACAGCTCCCCACCA 3'	2	132	63.5
YWHAZ[2]	5' ACTTTTGGTACATTGTGGCTTCAA 3' 5' CCGCCAGGACAAACCAGTAT 3'	2	94	64
HPRT[2]	5' TATTGTAATGACCAGTCAACAG 3' 5' GGTCCTTTTCACCAGCAAG 3'	2	192	60
SDHA[2]	5' TGGGAACAAGAGGGCATCTG 3' 5' CCACCACTGCATCAAATTCATG 3'	3	86	58
ACAN	5' CAACTACCCGGCCATCC 3' 5' GATGGCTCTGTAATGGAACAC 3'	2	160	63.5
COL2A1	5' AGGGCCAGGATGTCCGGCA 3' 5' GGGTCCCAGGTTCTCCATCT 3'	2	195	63.5
CCND1	5' AGCTCCTGTGCTGCGAAGTGGAAAC 3' 5' AGTGTTCAATGAAATCGTGCGGGGT 3'	2	480	65
ID1	5' CTCTACGACATGAACGGCTGT 3' 5' TGCTCACCTTGCGGTTCTG 3'	2	76	65
PAI1	5' GCTGGTGAATGCCCTCTAC 3' 5' GGCAGCCTGGTCATGTTG 3'	2	318	65
ALK5	5' GCCGTTTGACTGAAGGCTG 3' 5' GGGCATCCCAAGCCTCATC 3'	2	146	61
COL1A1	5' TCCAACGAGATCGAGATCC 3' 5' AAGCCGAATTCCTGGTCT 3'	2	191	61
MMP13	5' TCCCAGGAATTGGTGATAAAGTAGA 3' 5' CTGGCATGACGCGAACAATA 3'	2	123	64
BCL2	5' ATCGCCCTGTGGATGACTGAG 3' 5' CAGCCAGGAGAAATCAAACAGAGG 3'	2	125	64
BAX	5' GGACGAACTGGACAGTAACATGG 3' 5' GCAAAGTAGAAAAGGGCGACAAC 3'	2	150	60
CASP3	5' CAGTGGAGGCCGACTTCTTG 3' 5' TGGCACAAAGCGACTGGAT 3'	2	102	58
ADAMTS5	5' GCCAGCGGATGTGCAAGC 3' 5' ACACTTCCCCCGGACGCAGA 3'	3	130	62.5
ALK1	5' GCAACCTGCAGTGTTGCATC 3' 5' CGGATCTGCTCGTCCAGCAC 3'	3	139	62.5
SOX9	5' CCCAACGCCATCTTCAAGG 3' 5' CTGCTCAGCTCGCCGATGT 3'	3	242	65.5
AXIN2	5' GGTAGGCATTTTCCTCCATCAC 3' 5' AGCCAAAGCGATCTACAAAAGG 3'	3	104	57
TIMP1	5' CTTCTGGCATCCTGTTGTTG 3' 5' GGTATAAGGTGGTCTGGTTG 3'	2	153	64
COL10A1	5' CACTACCCAACACCAAGACA 3' 5'CTGGTTTCCCTACAGCTGAT 3'	2	225	61

[1]Forward and reverse primer, respectively.

[2]Reference genes.

GAG deposition and GAG release in the medium

At day 28, micro-aggregates (n= 7 per condition) were harvested for the quantitative measurement of glycosaminoglycan (GAG) production as described previously (Chapter 1). Total canine GAG production was calculated by additional measurement of the GAG release into the culture medium. For this purpose, we collected culture medium per week and calculated the total GAG release. This medium was diluted (1:33) with PBS-EDTA and processed as described previously (Chapter 1).

DNA content

The DNA content was measured by use of the Qubit dsDNA High Sensitivity Assay Kit (Qiagen, Valencia, CA) according to the manufacturers' protocol.

Histology analysis

Safranin O/Fast Green staining and collagen type I and II immunohistochemistry (IHC) were performed as described previously (Chapter 1). Briefly, for Safranin O/Fast Green staining sections were deparaffinized (xylene), dehydrated (ethanol) and underwent a citrate treatment (15 minutes). Sections were dipped in Mayers hematoxylin for 5 seconds and washed with demi-water. Subsequently, the sections were stained with 0.4% aqueous Fast Green for 4 minutes before dual treatment with fresh 1% acetic acid for 3 minutes. Next, sections were stained with 0.125% aqueous Safranin O for 7 minutes. For collagen I and II IHC, sections were deparaffinized (xylene), dehydrated (ethanol) and blocked with 0.3% H_2O_2 for 10 minutes and washed with 0.1% PBST. Antigen retrieval was performed with 1 mg/mL pronase (Roche Diagnostics 11459643001) and 10 mg/mL hyaluronidase (Sigma-Aldrich, H3506) both for 30 minutes at 37 °C. Blocking was performed with 5% PBS/BSA for 30 minutes at 37 °C. The sections were then incubated with collagen type II mouse monoclonal antibody (DSHB, II-II6B3) (1:2000 in PBS 5%) or with the collagen type I (ab6308, Abcam) mouse primary monoclonal antibody (1:1500 in 5% BSA/PBS 5%) overnight at 4 °C. As a negative control, Mouse IgG_1 (3877, Santa Cruz Biotechnology) was used; no aspecific staining was observed in these sections. The next day, the sections were washed with 0.1% PBST and incubated with the secondary antibody (EnVision+ System-HRP Goat anti-Mouse, DAKO, K4001). DAB peroxidase substrate (DAKO, K3468) was used for visualization of staining and sections were counterstained with Hematoxylin QS solution (both 1 minute). All sections were dehydrated through ethanol and xylene. Next, sections were mounted and images were acquired using an Olympus BX60 microscope with colorview III digital camera and cell imaging software (Olympus, Zoeterwoude, the Netherlands).

Statistical analysis

Statistics were performed using IBM SPSS Statistics 22. First, normality was checked by the Shapiro Wilks test. Subsequently, a Kruskal Wallis and a Mann Whitney U test were performed on all non-normally distributed data. All normally distributed data was tested by a One-Way Anova. Afterwards, post hoc corrections for multiple comparisons were performed by Benjamini & Hochberg False Discovery Rate. A p-value < 0.05 was considered significant for all tests. Canine CD and NCD CLC micro-aggregate conditions were statistically compared within and between groups, whereas human CLC micro-aggregates were statistically evaluated within the human CLC conditions.

Results

Gene expression

Matrix production and chondrocyte differentiation

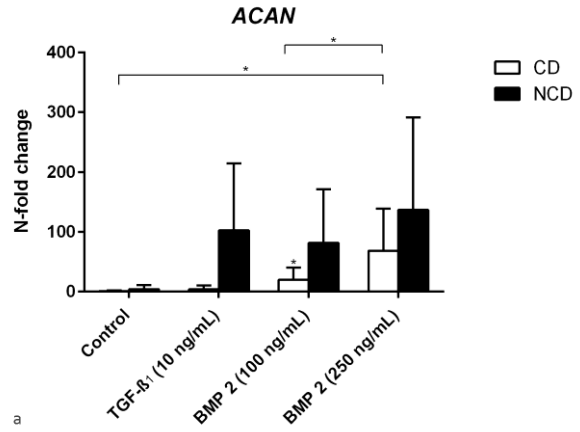
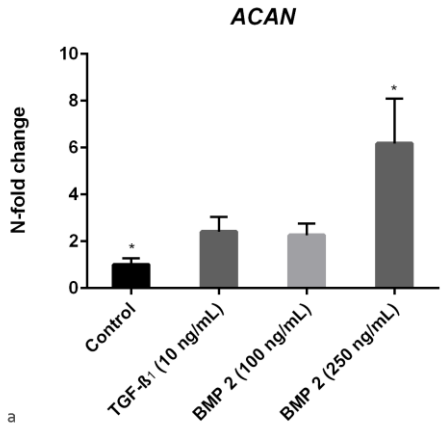
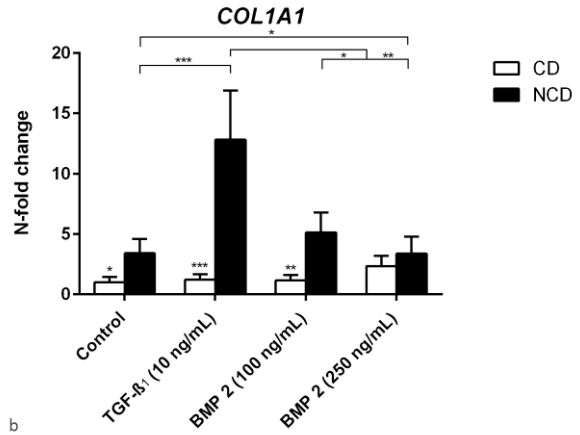
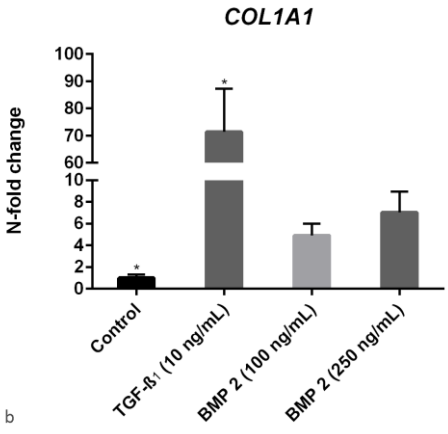
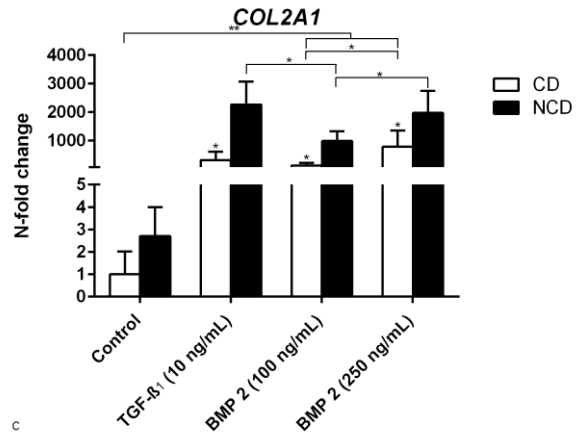
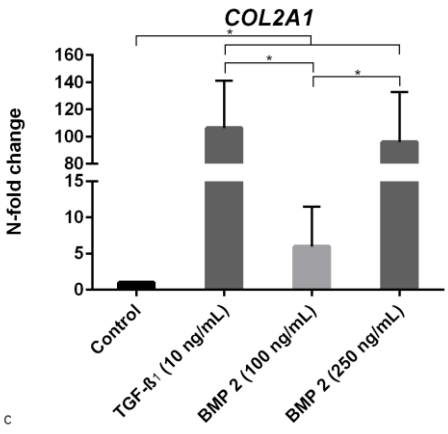
Gene expression of *ACAN* was significantly upregulated in the CD micro-aggregates treated with 250 ng/mL BMP-2 compared with micro-aggregates treated with 100 ng/mL BMP-2 and the negative controls (Figure 1a). 100 ng/mL BMP-2-treated CD micro-aggregates showed lower *ACAN* gene expression levels than NCD CLCs treated with 100 ng/mL BMP-2. All human CLCs showed a significant increase in *ACAN* gene expression compared with controls. 250 ng/mL BMP-2 was more potent in inducing *ACAN* expression than TGF-ß$_1$ and 100 ng/mL BMP-2 in human CLCs.

CD micro-aggregates treated with 100 and 250 ng/mL BMP-2 showed significantly upregulated *COL2A1* gene expression compared with the negative controls (Figure 1c). *COL2A1* gene expression was significantly upregulated for NCD micro-aggregates treated with TGF-ß$_1$ and 250 ng/mL BMP-2 compared with 100 ng/mL BMP-2, whereas there was no significant difference between these conditions for the CD micro-aggregates. For both CD and NCD aggregates, a dose-dependent significant upregulation of *COL2A1* gene expression was found between the low and the high BMP concentration. Both TGF-ß$_1$ and BMP-2 250 ng/mL upregulated *COL2A1* gene expression in similar levels in human CLCs compared with controls.

COL1A1 gene expression was significantly increased in NCD micro-aggregates treated with TGF-ß$_1$ compared with both BMP-2 concentrations (Figure 1b), which were in turn both upregulated compared with the negative controls. In addition, NCD micro-aggregates treated with negative control culture medium, TGF-ß$_1$ and 100 ng/mL BMP-2 showed significantly upregulated *COL1A1* gene expression compared with corresponding conditions in CD micro-aggregates. In canine CD CLCs, *COL1A1* expression was increased by BMP-2 treatment, but this was only significant at a concentration of 100 ng/mL. *COL1A1* expression was significantly higher in TGF-ß$_1$-treated human CLCs compared with 100 and 250 ng/mL BMP-2-treated human CLCs, whereas *COL1A1* expression was lowest in the negative controls.

COL10A1 gene expression was only detected in TGF-ß$_1$-treated human CLC micro-aggregates (data not shown). No *COL10A1* expression was detected after 40 RT-qPCR cycles in canine CD and NCD micro-aggregates.

CD and NCD micro-aggregates treated with TGF-ß$_1$ showed significantly increased *SOX9* gene (a chondrocyte differentiation marker) expression compared with controls (Figure 1d). *SOX9* gene expression was significantly downregulated in CD micro-aggregates treated with 100 and 250 ng/mL BMP-2 compared with TGF-ß$_1$ supplementation. Human CLC *SOX9* gene expression was downregulated in TGF-ß$_1$- and 250 ng/mL BMP-2-treated CLCs compared with controls.

Canine CD and NCD CLCs	Human CLCs
 a	 a
 b	 b
 c	 c

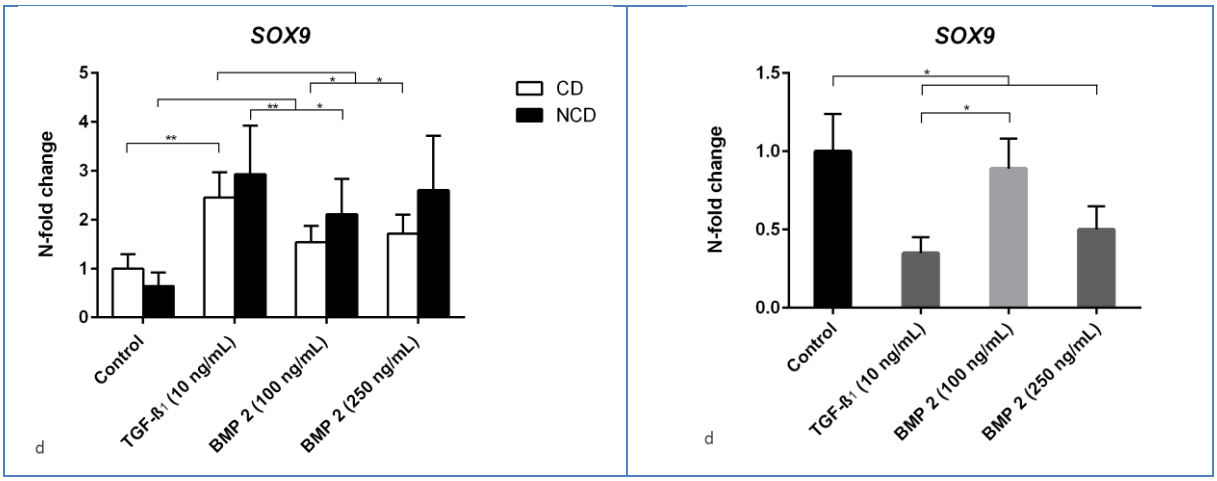


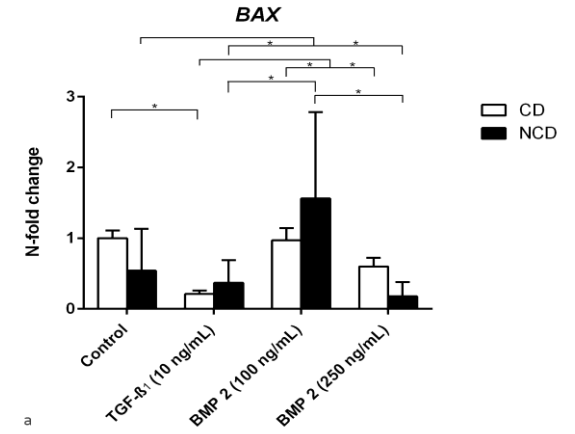
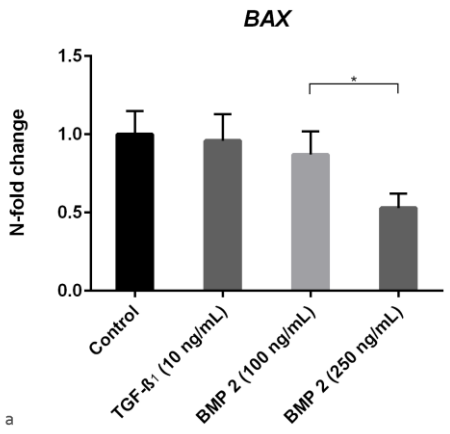
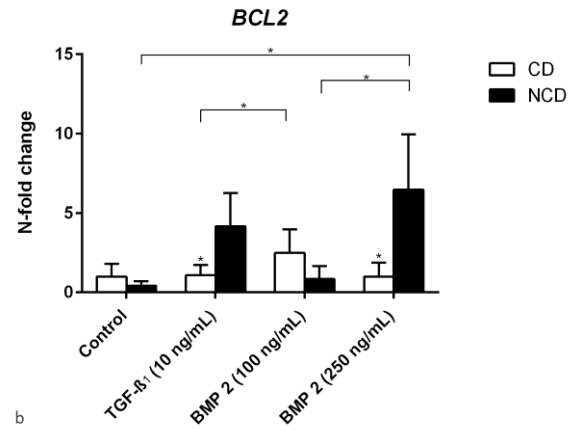
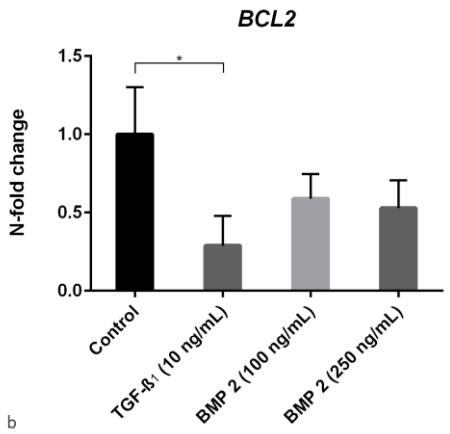
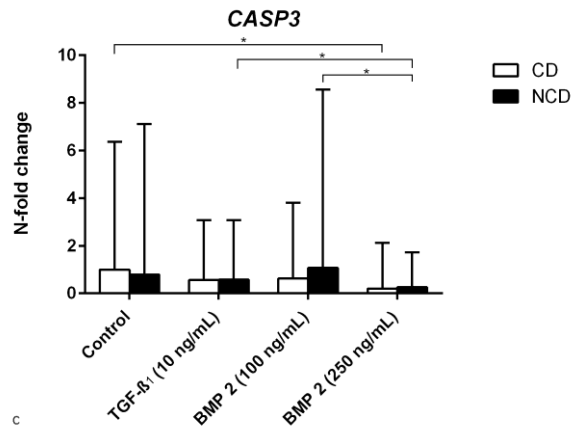
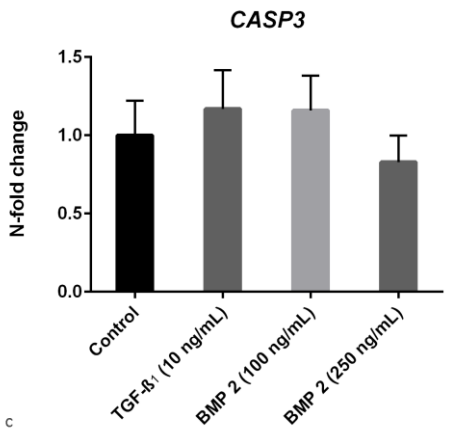
Figure 1. Relative gene expression levels ±S.D. of *ACAN* (a), *COL1A1* (b), *COL2A1* (c), and *SOX9* (d). Left column: canine chondrodystrophic (CD) and non-chondrodystrophic (NCD) chondrocyte-like cells (CLCs). Right column: human CLCs. * = $p<0.05$, ** = $p<0.01$ and *** = $p<0.001$. N-fold gene expression in the control cultures (CD and human) was set at 1.

Cell proliferation and apoptosis

Both CD and NCD micro-aggregates treated with TGF-$ß_1$ and NCD micro-aggregates treated with 250 ng/mL BMP-2 showed decreased *BAX* gene expression compared with controls (Figure 2a). An upregulation of *BAX* gene expression, an apoptotic gene, was found in the CD micro-aggregates treated with 250 ng/mL BMP-2 compared with the micro-aggregates treated with TGF-$ß_1$. *BAX* gene expression was downregulated by 250 ng/mL BMP-2 compared with 100 ng/mL BMP-2 in human CLCs. Moreover, *BCL2* gene expression of TGF-$ß_1$- and 250 ng/mL BMP-2-treated NCD micro-aggregates was significantly upregulated compared with CD micro-aggregates of the same conditions (Figure 2b). 100 ng/mL BMP-2 significantly increased *BCL2* gene expression, an anti-apoptotic gene, of CD micro-aggregates compared with TGF-$ß_1$ supplementation, whereas 250 ng/mL BMP-treated NCD CLCs showed a higher *BCL2* gene expression compared with 100 ng/mL BMP-2 CLCs. Human CLCs cultured in TGF-$ß_1$ supplemented culture medium showed significantly downregulated *BCL2* gene expression compared with controls.

The canine *BAX*/*BCL2* ratio was significantly higher in NCD versus CD 250 ng/mL BMP-2-treated micro-aggregates (Figure 2d). Additionally, the ratio was higher in both CD and NCD micro-aggregates treated with TGF-$ß_1$ than in micro-aggregates treated with 100 ng/mL BMP-2. Only for NCD micro-aggregates, the *BAX*/*BCL2* ratio in the 250 ng/mL BMP-2-treated CLCs was significantly higher than in the 100 ng/mL BMP-2-treated CLCs.

CCND1 gene expressing, a proliferation marker, was upregulated for NCD TGF-$ß_1$-treated micro-aggregates compared with CD TGF-$ß_1$-treated micro-aggregates (Figure 2e). Human CLCs showed significantly increased *CCND1* expression after 100 and 250 ng/mL BMP-2 treatment compared with TGF-$ß_1$ supplementation, however only 100 ng/mL BMP-2 significantly increased *CCND1* gene expression compared with controls.

Canine CD and NCD CLCs	Human CLCs
	
	
	

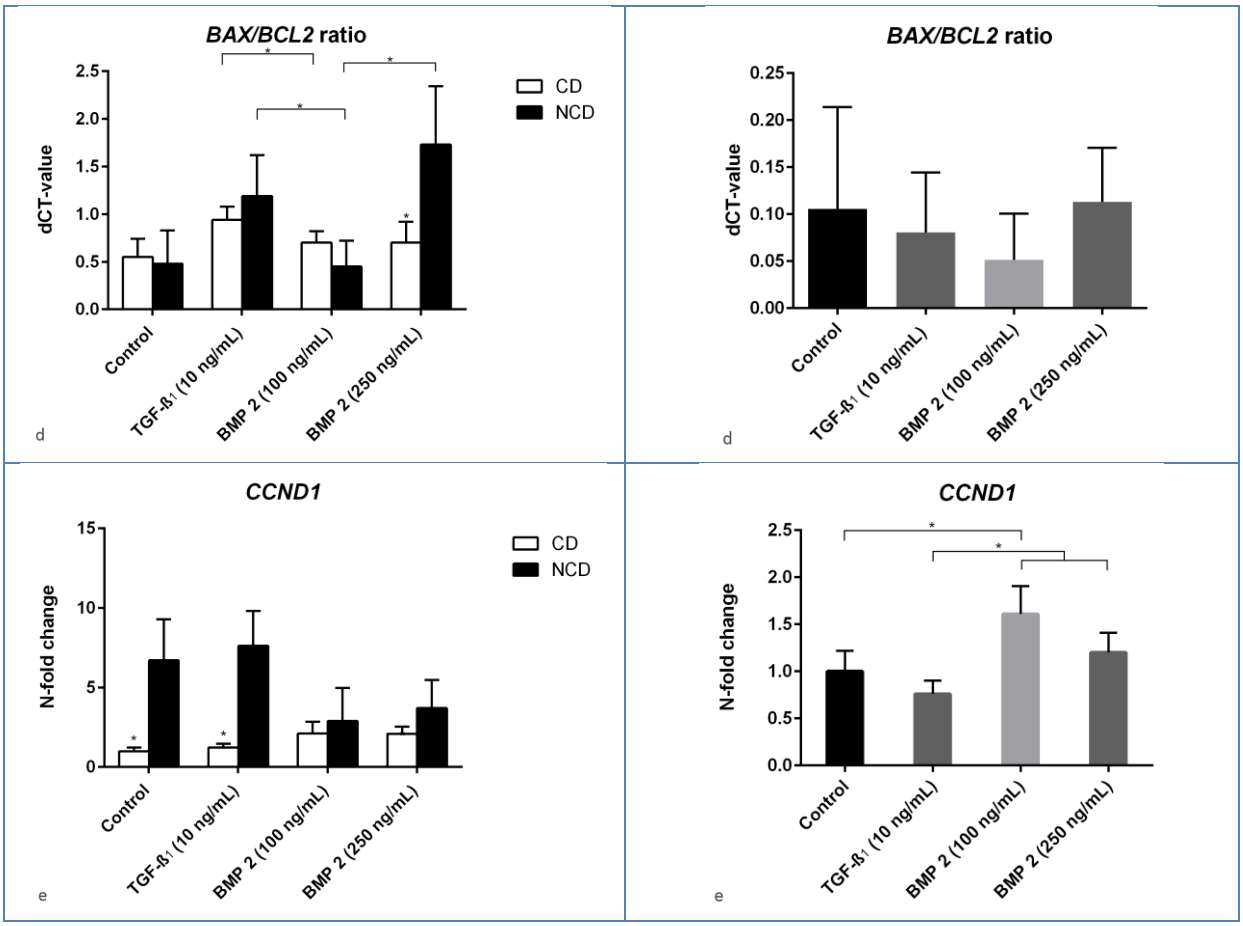


Figure 2. Relative gene expression levels ±S.D. of *BAX* (a), *BCL2* (b), *CASP3* (c), and *BAX*/*BCL2* ratio (d). Left column: canine chondrodystrophic (CD) and non-chondrodystrophic (NCD) chondrocyte-like cells (CLCs). Right column: human CLCs. * = $p<0.05$, ** = $p<0.01$ and *** = $p<0.001$. N-fold gene expression in the control cultures (CD and human) was set at 1.

Matrix remodeling

ADAMTS5 gene expression, responsible for cleavage of aggrecan, was not upregulated in the canine and human CLCs and a high standard deviation was encountered (Figure 3a). *MMP13*, responsible for collagen degradation, was significantly upregulated in CD micro-aggregates treated with both BMP-2 conditions compared with TGF-ß$_1$ supplementation, while *MMP13* gene expression of NCD micro-aggregates treated with 100 ng/mL BMP-2 was significantly upregulated compared with micro-aggregates treated with TGF-ß$_1$ and 250 ng/mL BMP-2 (Figure 3b). In general, CD micro-aggregates showed a significantly higher *MMP13* gene expression than NCD micro-aggregates. Gene expression of *MMP13* in human CLCs was significantly upregulated by TGF-ß$_1$ treatment compared with all other conditions. 250 ng/mL BMP-2 stimulated gene expression of *MMP13* more than 100 ng/mL BMP-2.

There was a significant difference in *TIMP1* gene expression (inhibitor of MMPs) between CD and NCD micro-aggregates treated with TGF-ß$_1$ and 100 ng/mL BMP-2 within the same condition, but not in the control and 250 ng/mL BMP-2-treated micro-aggregates (Figure 3c). TGF-ß$_1$ supplementation to NCD micro-aggregates significantly upregulated *TIMP1* gene expression compared with both BMP-2 conditions. 100 ng/mL BMP-2, in turn, stimulated *TIMP1* gene expression more than 250 ng/mL BMP-2 in NCD micro-aggregates. In human CLCs, all conditions except 100 ng/mL BMP-2 downregulated gene expression of *TIMP1* compared with controls.

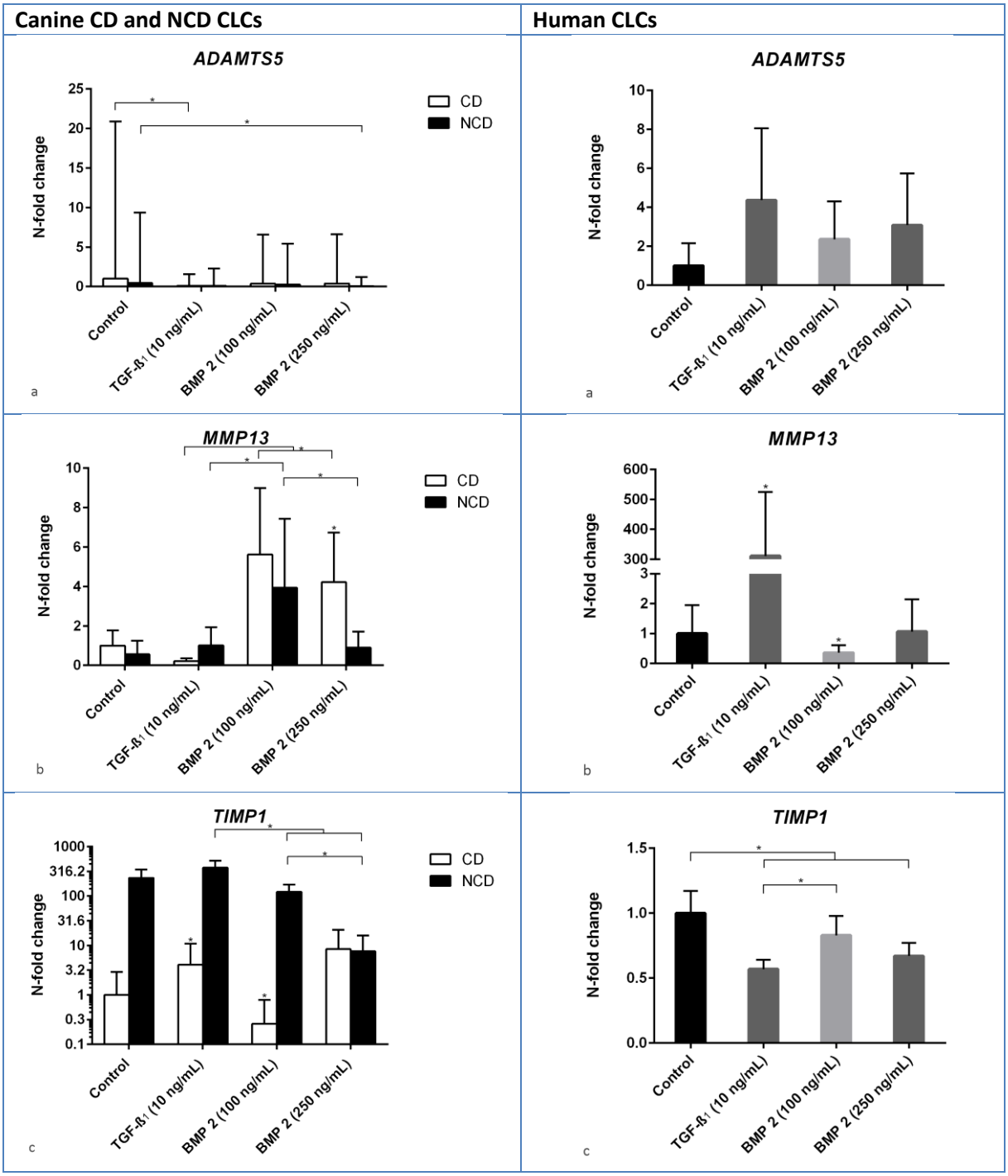


Figure 3. Relative gene expression levels ±S.D. of *ADAMTS5* (a), *MMP13* (b), and *TIMP1* (c). Left column: canine chondrodystrophic (CD) and non-chondrodystrophic (NCD) chondrocyte-like cells (CLCs). Right column: human CLCs. * = $p<0.05$, ** = $p<0.01$ and *** = $p<0.001$. N-fold gene expression in the control cultures (CD and human) was set at 1.

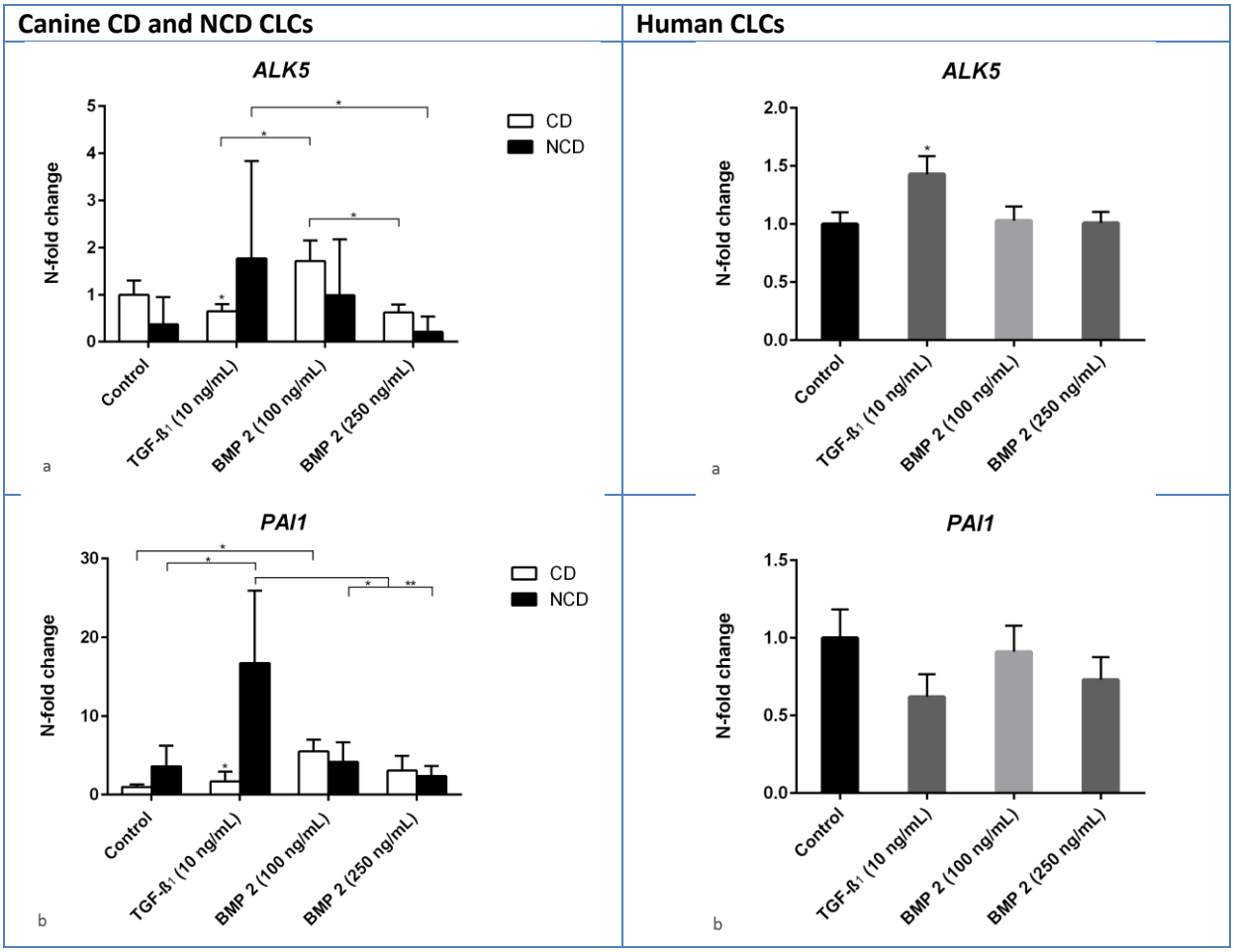
TGF-ß$_1$/BMP-2 pathway

ALK5 gene expression (receptor of TGF-ß family) of NCD micro-aggregates treated with TGF-ß$_1$ was significantly downregulated compared with those treated with 250 ng/mL BMP-2 (Figure 4a). *ALK5* gene expression was significantly upregulated in TGF-ß$_1$-treated NCD micro-aggregates compared with corresponding CD micro-aggregates. In human CLCs, *ALK5* gene expression was significantly upregulated by TGF-ß$_1$ treatment compared with all other conditions. In NCD CLCs, the expression of

the ALK5 downstream target *PAI1* was significantly higher after TGF-ß$_1$ supplementation compared with supplementation of both BMP-2 concentrations and the negative controls (Figure 4b). TGF-ß$_1$-treated NCD CLCs showed a significantly higher *PAI1* expression than CD CLCs treated with TGF-ß$_1$.

ALK1 gene expression (receptor of TGF-ß family) of NCD micro-aggregates treated with TGF-ß$_1$ or 250 ng/mL BMP-2 significantly decreased compared with controls (Figure 4c). 100 ng/mL BMP-2 increased *ALK1* gene expression compared with TGF-ß$_1$ treatment. No *ALK1* expression was detected in human CLCs after 40 RT-qPCR cycles.

CD micro-aggregates treated with 250 ng/mL BMP-2 showed a significantly increased *ID1* gene expression (downstream target of ALK1) compared with controls (Figure 4d). *ID1* gene expression was significantly downregulated in NCD micro-aggregates treated with TGF-ß$_1$ compared with those treated with both BMP-2 concentrations. Additionally, TGF-ß$_1$-treated NCD micro-aggregates showed a significantly upregulated *ID1* gene expression compared TGF-ß$_1$-treated CD micro-aggregates. Treatment of the human CLCs with 100 and 250 ng/mL BMP-2 significantly increased *ID1* gene expression compared with TGF-ß$_1$ treatment and controls.



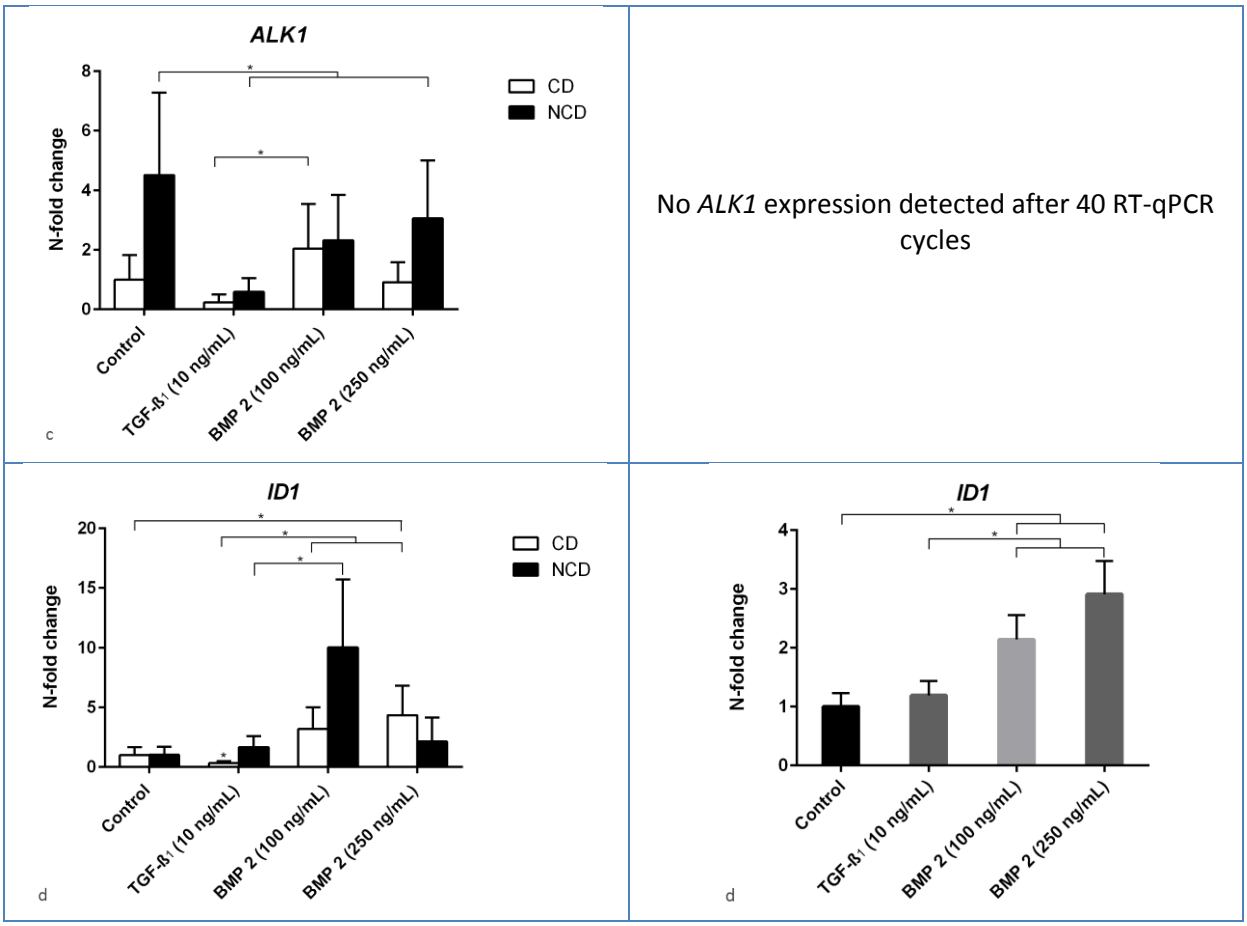


Figure 4. Relative gene expression levels ±S.D. of *ALK5* (a), *PAI1* (b) and *ALK1* (c), and *ID1* (d). Left column: canine chondrodystrophic (CD) and non-chondrodystrophic (NCD) chondrocyte-like cells (CLCs). Right column: human CLCs. * = $p<0.05$, ** = $p<0.01$ and *** = $p<0.001$. N-fold gene expression in the control cultures (CD and human) was set at 1.

DNA, GAG and GAG/DNA content of CD and NCD micro-aggregates

The DNA content of CD and NCD micro-aggregates treated with TGF-ß$_1$ and 100 and 250 ng/mL BMP-2 was significantly increased compared with controls (Figure 5a). In CD micro-aggregates, all culture conditions decreased the DNA content compared with day 0, whereas in NCD micro-aggregates, only the control and 100 ng/mL BMP-2 treatment significantly decreased the DNA content compared with day 0. In NCD dogs, TGF-ß$_1$-treated micro-aggregates showed the highest DNA content. However, the DNA content was not significantly different between the TGF-ß$_1$ and BMP treated CD micro-aggregates (both concentrations). Moreover, the DNA content of every CD micro-aggregate condition was significantly higher than the corresponding NCD micro-aggregate condition.

250 ng/mL BMP-2 significantly increased the GAG content of both CD and NCD micro-aggregates, where it was significantly higher in the NCD than the corresponding CD micro-aggregates (Figure 5b). TGF-ß$_1$ supplementation to CD and NCD micro-aggregates significantly increased the GAG content compared with negative controls and 100 ng/mL BMP-2.

The 250 ng/mL BMP-2-treated canine CD and NCD micro-aggregates also showed the highest GAG/DNA content. The NCD micro-aggregates treated with 250 ng/mL BMP-2 showed a significantly higher GAG/DNA content than the CD micro-aggregates of the same condition (Figure 5c).

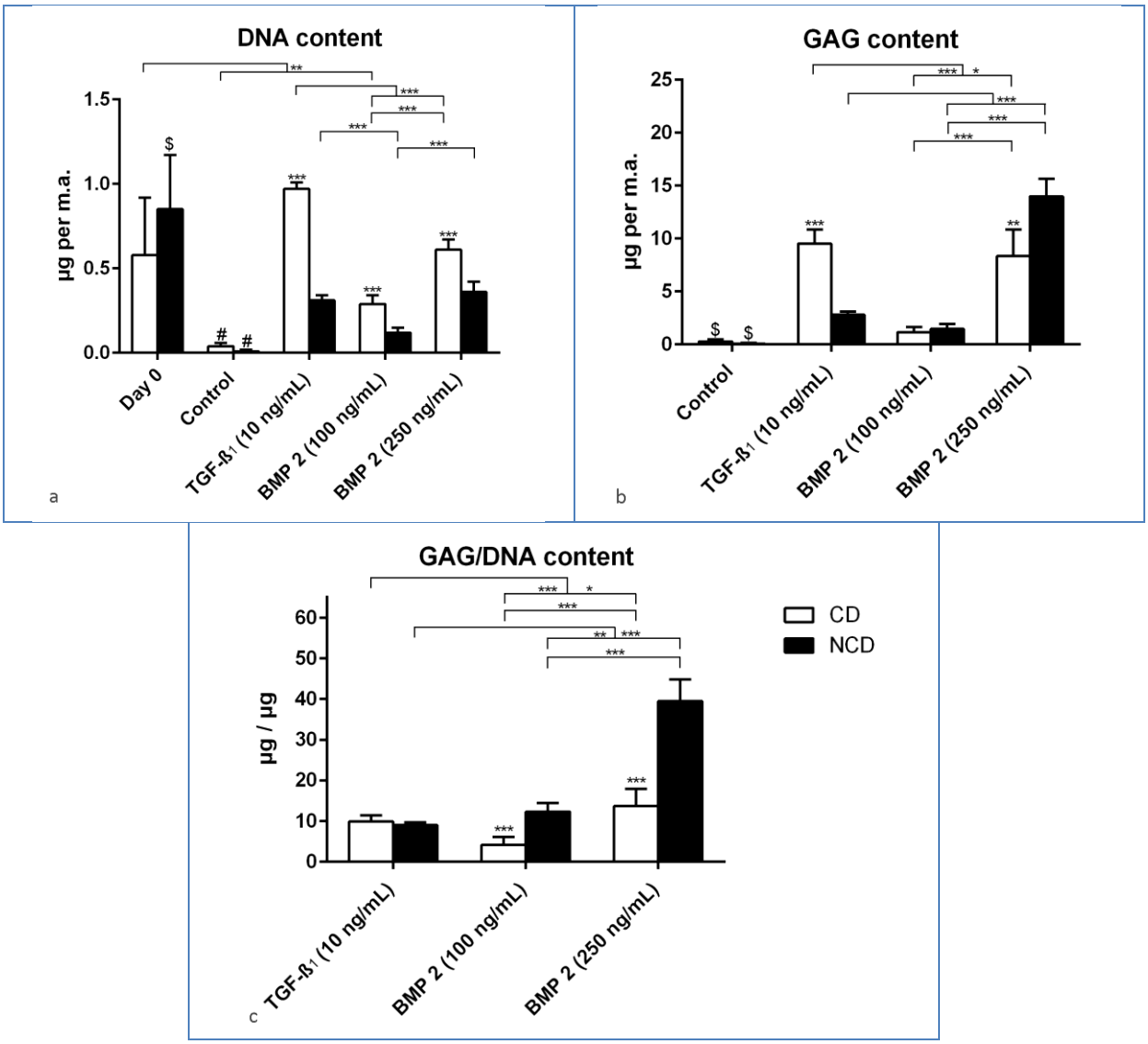


Figure 5. Mean DNA (a), GAG (b) and GAG/DNA (c) content ±S.D. of canine chondrodystrophic (CD) and non-chondrodystrophic (NCD) canine chondrocyte-like cells (CLCs) micro-aggregates at day 28. $: significantly different from all other conditions within the group CD or NCD. #: significantly different from all other conditions but day 0 within the group CD or NCD. * = p<0.05, ** = p<0.01 and *** = p<0.001. M.a. = micro-aggregates.

DNA, GAG and GAG/DNA content micro-aggregate human CLCs

The DNA content of human CLC micro-aggregates treated with TGF-ß$_1$ and 100 and 250 ng/mL BMP-2 was significantly increased compared with controls (Figure 6a). TGF-ß$_1$-treated micro-aggregates showed the highest DNA content on both time points with a drop in DNA content between day 14 and 28. The DNA content of the 250 ng/mL BMP-2-treated micro-aggregates stayed constant over time.

All treatments stimulated GAG production at both time points compared with controls (Figure 6b). TGF-ß$_1$ and 250 ng/mL BMP-2 treatment significantly increased the GAG content of human CLC micro-aggregates equally at day 28, whereas TGF-ß$_1$-treated micro-aggregates had a higher GAG content at day 14.

The micro-aggregates treated with 250 ng/mL BMP-2 showed the highest GAG/DNA content at day 28 (Figure 6c). However, TGF-ß$_1$ supplementation increased the GAG/DNA content more than 100 ng/mL BMP-2 supplementation at both time points.

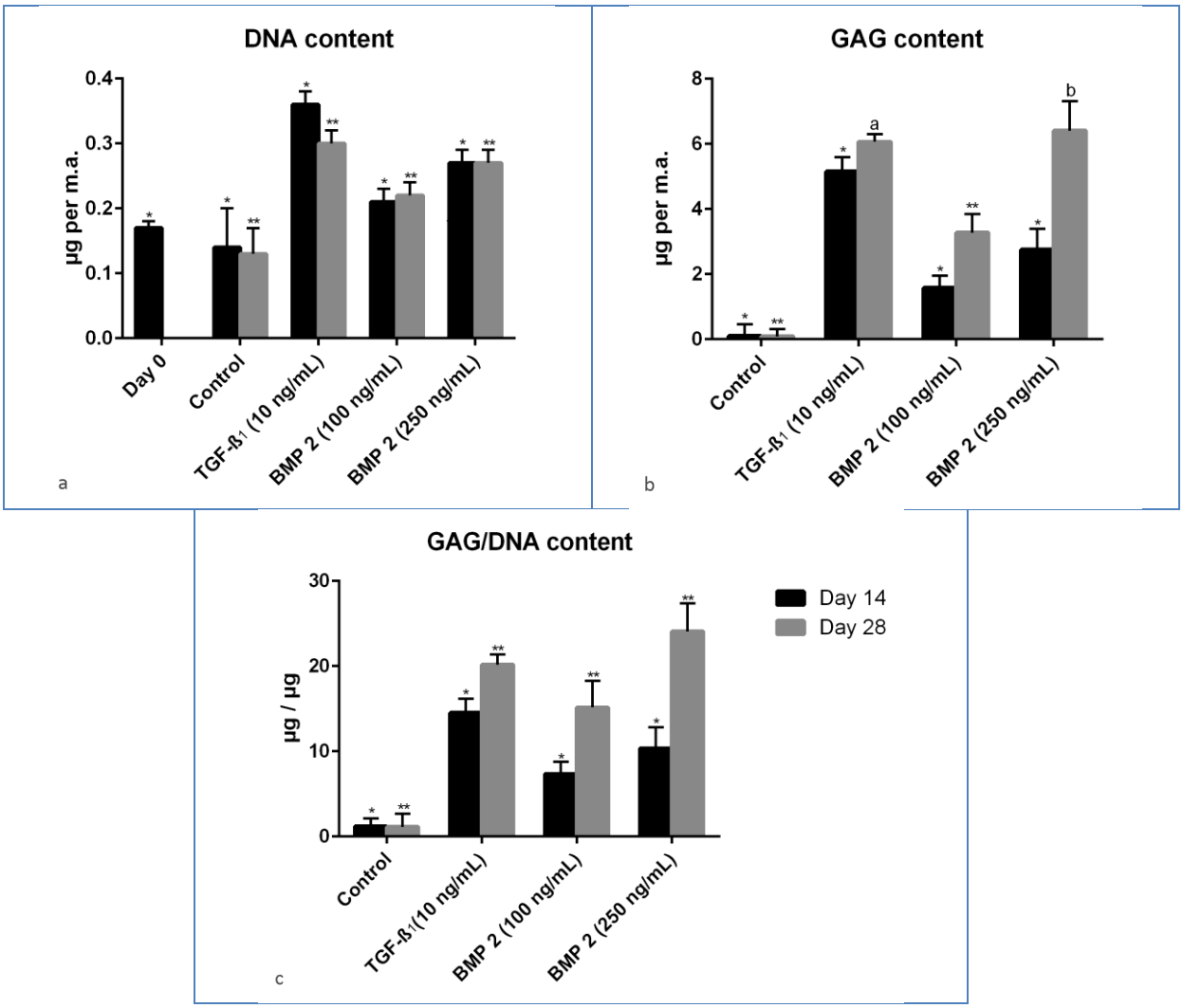


Figure 6. Mean DNA (a), GAG (b) and GAG/DNA (c) content ±S.D. of human chondrocyte-like cells (CLCs) micro-aggregates at day 14 and 28. *,**: significantly different with all other conditions at that time point. a: significantly different from all other conditions but BMP-2 (250 ng/mL) at that time point. b: significantly different from all other conditions but TGF-ß$_1$ (10 ng/mL) at that time point. M.a. = micro-aggregates. Significance level of p<0.05.

GAG release medium CD and NCD

Every week, GAG release into the culture medium by CD micro-aggregates was significantly upregulated for the 250 ng/mL BMP-2 versus TGF-ß$_1$- and 100 ng/mL BMP-2 treated micro-aggregates (Figure 7a). Moreover, every week, TGF-ß$_1$- and 250 ng/mL BMP-2-treated micro-aggregates excreted more GAGs into the culture medium than the 100 ng/mL BMP-2-treated micro-aggregates.

NCD micro-aggregates treated with TGF-ß$_1$ released more GAGs into the culture medium in week 1 and 2 than the other conditions, however in week 3 and 4 there was no significant difference anymore between TGF-ß$_1$ and 250 ng/mL BMP-2 treatment (Figure 7b). Moreover, every week, TGF-

ß$_1$- and 250 ng/mL BMP-2-treated NCD micro-aggregates excreted more GAGs into the culture medium than the 100 ng/mL BMP-2-treated micro-aggregates.

CD micro-aggregates treated with 250 ng/mL BMP-2 showed the highest total GAG release into the culture medium, whereas there was no significant difference between the total GAG released into the culture medium by TGF-ß$_1$- and 250 ng/mL BMP-2-treated NCD micro-aggregates (Figure 7c). There was no significant difference between total released GAG content between CD and NCD in the TGF-ß$_1$-treated micro-aggregates, and there was a significantly higher excretion of total GAG into the culture medium in the CD than in the NCD micro-aggregates treated with 250 ng/mL BMP-2.

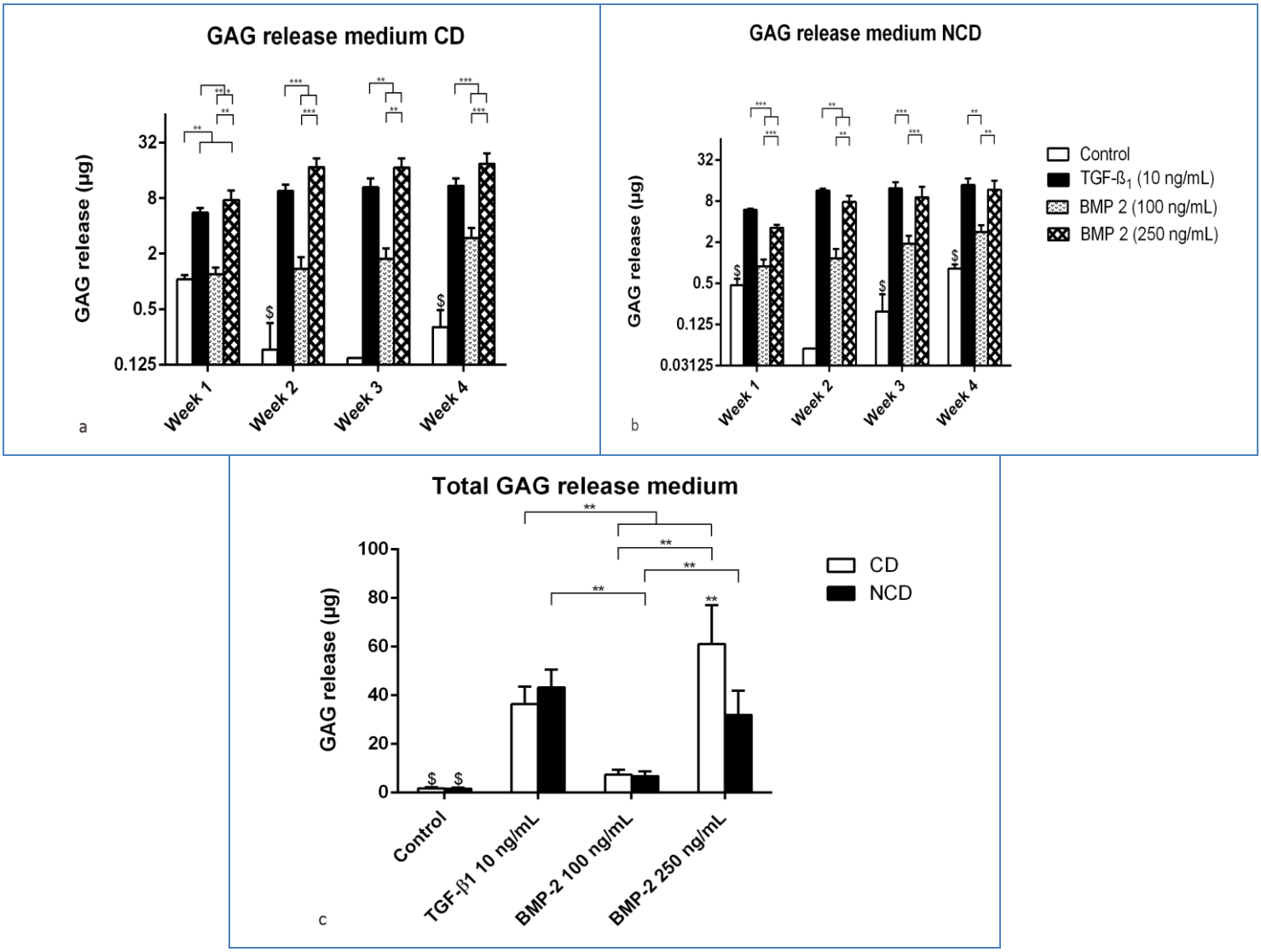


Figure 7. GAG release per week in medium ±S.D. for chondrodystrophic (CD)- (a) and non-chondrodystrophic (NCD)- (b) treated micro-aggregates and total GAG release (c). $: significantly different from all other conditions within the group CD or NCD. * = p<0.05, ** = p<0.01 and *** = p<0.001. M.a. = micro-aggregates.

Safranin O/Fast Green staining

All treatments induced an increase in size compared with the control canine CD micro-aggregates (Figure 8). Both TGF-ß$_1$ and BMP-2-treated canine CD micro-aggregates showed a positive red staining indicating GAG deposition. While TGF-ß$_1$-treated canine CD micro-aggregates showed a homogeneous staining (except for the outer rim), the BMP-2-treated canine CD aggregates

presented a spotted red pattern. Additionally, the BMP-2-treated canine CD micro-aggregates showed a higher cell density at the center of the aggregates, whereas TGF-ß$_1$ treatment provided a more homogeneous cell density (except for the outer rim). A rim of GAG- and cell depleted ECM was formed around the TGF-ß$_1$-treated canine CD micro-aggregates. Lastly, the 100 ng/mL BMP-2 treated canine CD micro-aggregates showed a center of apoptotic cells with less GAGs present, which was not seen in the other conditions.

Canine NCD CLC micro-aggregates showed the same characteristics as described for the canine CD aggregates (Figure 8). However, 250 ng/mL BMP-2 treatment did not induce a high cell density center as seen in CD dogs. Both TGF-ß$_1$- and BMP-2-treated human CLC micro-aggregates showed an intense red staining (Figure 8). A less distinctive, fibrocyte-like cell containing rim with a decreased GAG amount was formed around the TGF-ß$_1$-treated human micro-aggregates.

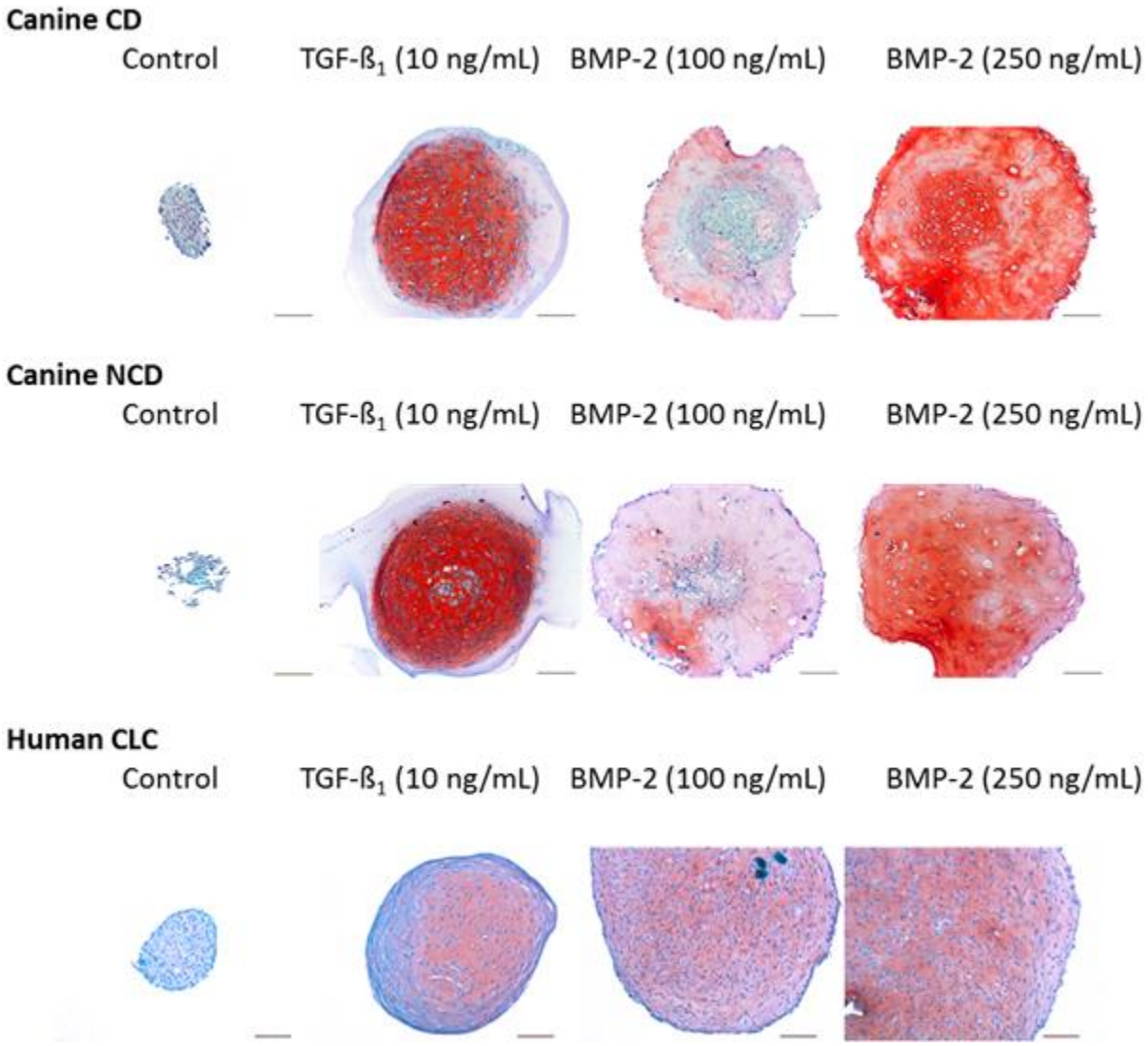


Figure 8. Overview of the canine chondrodystrophic (CD), non-chondrodystrophic (NCD) and human chondrocyte-like cell (CLC) micro-aggregate Safranin O/Fast Green staining at day 28. The displayed pictures are representatives for all donors. Scale bar represents 100 µm.

Collagen type I IHC

A positive brown staining was observed in TGF-ß$_1$- and BMP-2-treated canine CD micro-aggregates. TGF-ß$_1$ supplementation, however induced the darkest staining (Figure 9). In the canine CD micro-aggregates treated with both BMP concentrations, the most intense staining was seen in the center of the micro-aggregates. 250 ng/mL BMP-2 appeared to decrease collagen type I deposition compared with 100 ng/mL BMP-2 and TGF-ß$_1$ in canine CD CLCs. Canine NCD micro-aggregates showed less dark brown staining in all culture conditions compared to their corresponding CD micro-aggregates (Figure 9). The canine NCD micro-aggregates treated with both BMP-2 concentrations stained less positive for collagen type I than the TGF-ß$_1$-treated micro-aggregates. A diffuse positive brown staining for collagen type I was observed in the human CLC micro-aggregates treated with TGF-ß$_1$ and (to a lesser extent) 100 ng/mL BMP-2, whereas the 250 ng/mL BMP-2-treated human micro-aggregates showed hardly any collagen type I deposition (Figure 9).

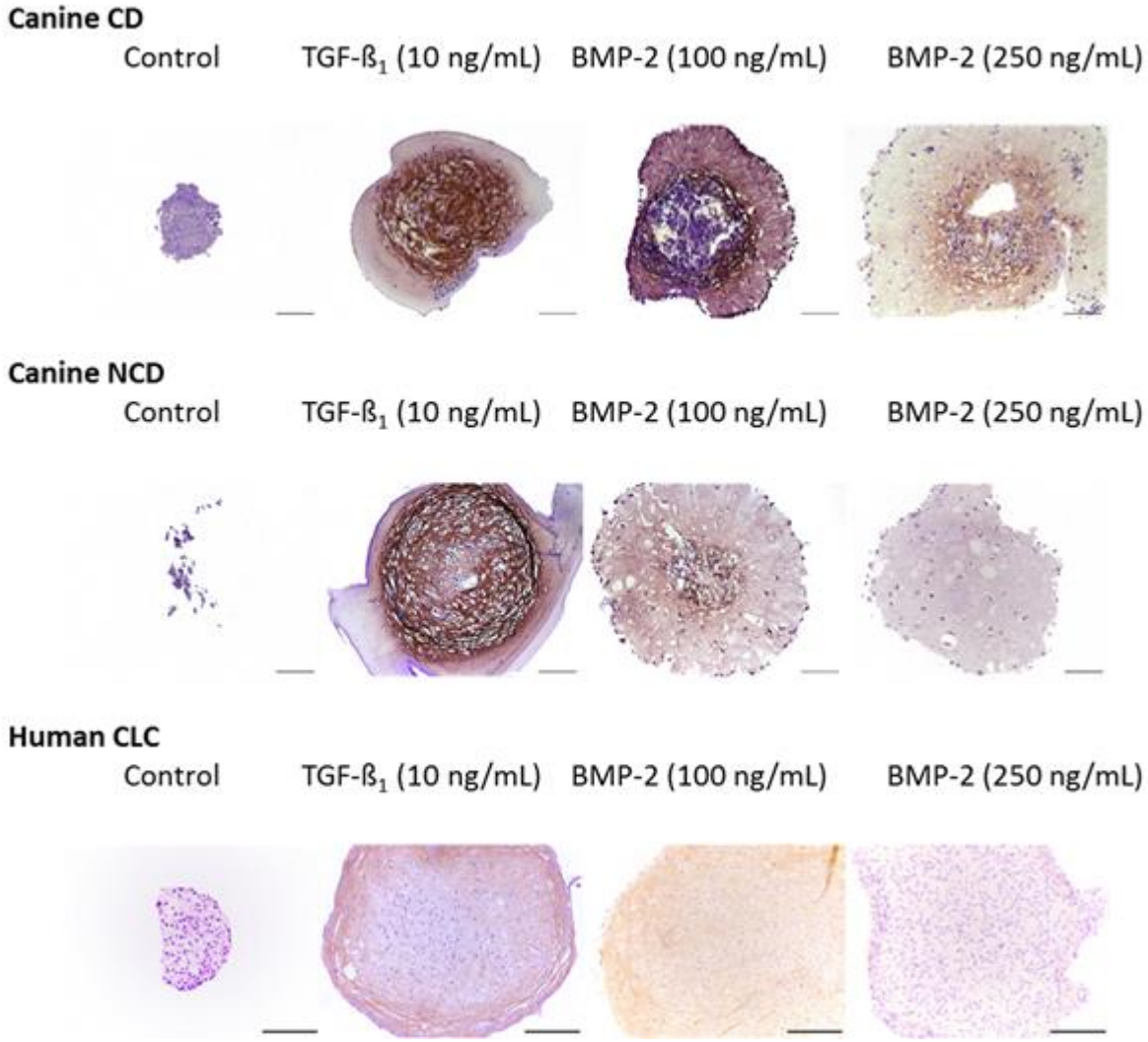


Figure 9. Overview of the collagen type I immunohistochemistry for canine chondrodystrophic (CD), non-chondrodystrophic (NCD) and human chondrocyte-like cells (CLCs) at day 28. The displayed pictures are representatives for all donors. Scale bar represents 100 µm.

Collagen type II IHC

Collagen type II IHC showed an intense brown staining in all culture conditions for canine CD CLC micro-aggregates. TGF-ß$_1$-treated canine CD micro-aggregates were diffusely stained (Figure 10). Also the outer rim, which was negative in the Safranin O/Fast Green staining and the collagen type I IHC, stained positive for collagen type II. 250 ng/mL BMP-2-treated canine CD micro-aggregates showed slightly less collagen type II deposition than 100 ng/mL BMP-2- and TGF-ß$_1$-treated micro-aggregates. Canine NCD CLCs showed the same pattern as described for the collagen type II IHC of the CD micro-aggregates (Figure 10). Collagen type II was less present in 250 ng/mL BMP-2-treated NCD micro-aggregates than in 250 ng/mL BMP-2-treated CD micro-aggregates. Collagen type II was only distinctly present in human CLC micro-aggregates treated with 250 ng/mL BMP-2 (Figure 10).

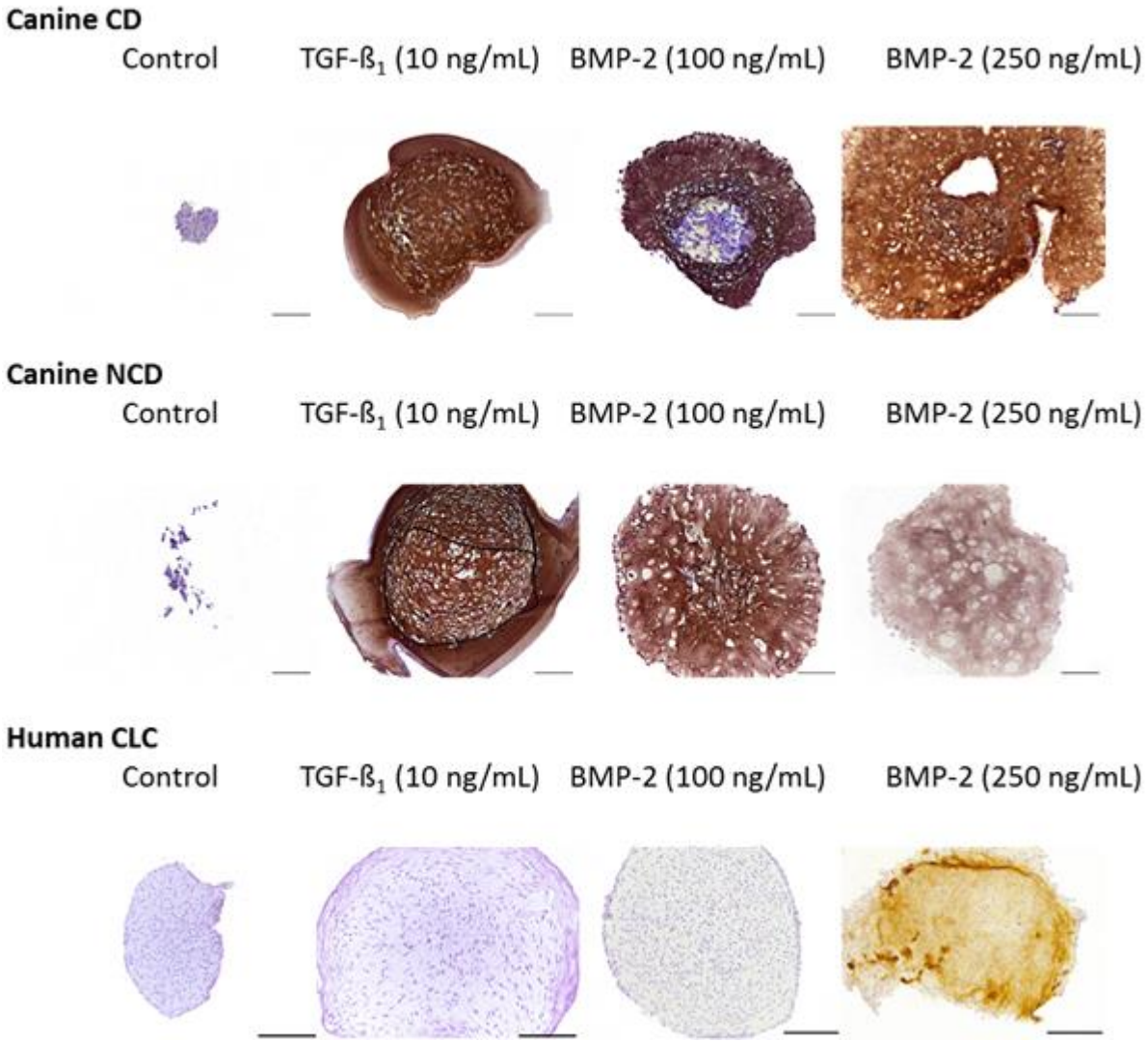


Figure 10. Overview of the collagen type II human immunohistochemistry for canine chondrodystrophic (CD), non-chondrodystrophic (NCD) and human chondrocyte-like cells (CLCs) at day 28. The displayed pictures are representatives for all donors. Scale bar represents 100 µm.

Discussion

TGF-ß$_1$ is known to stimulate chondrogenic ECM production, but it can also induce an undesirable fibrotic phenotype of human CLCs as also shown in Chapter 1[53]. BMP-2 is also known to induce chondrogenic ECM production[12], possibly without the undesirable side effects that TGF-β$_1$ can provoke[52]. The current study is the first to report the comparative and differential effects of TGF-ß$_1$ and BMP-2, where TGF-ß$_1$ is shown to induce chondrogenesis and fibrosis, while BMP-2 does not cause the latter. Additionally, both TGF-ß$_1$ and BMP-2 were able to stimulate their Smad-pathway. Even more so, NCD CLCs incorporated more newly produced GAGs in the ECM, whereas CD CLCs secreted more GAGs into the culture medium, suggesting a difference in response to external stimuli. CD and NCD CLCs can be used as a model for regenerative treatment for human IVDD.

Optimal BMP-2 concentration in canine and human CLCs

The present study shows a dose-response effect of BMP-2 on canine CD and NCD and human CLCs derived from degenerated IVDs (Thompson grade 3): 250 ng/mL BMP-2 induced optimal regenerative effects *in vitro* compared with the lower (100 ng/mL) concentration of BMP-2. Mainly in human CLCs, a higher BMP-2 concentration is favorable: it induced a higher DNA, GAG and GAG/DNA content as well as higher collagen type II and lower collagen type I deposition. The results of the present study are in line with previous work showing that proteoglycan synthesis and collagen type I, II and aggrecan gene expression is increased in human CLCs treated with high BMP-2 concentrations (300 and 1500 ng/mL)[83,84]. Also, Gilbertson *et al.* showed that 200 and 300 ng/mL BMP-2 induces more proteoglycan synthesis than lower BMP-2 concentrations and that collagen synthesis in human CLCs (Thompson grade 3) peaks with 200 ng/mL BMP 2 treatment[85]. The present study is the first to report BMP-2 effects on canine CLCs. In canine CLCs, in contrast to human CLCs, collagen type II deposition was slightly decreased by the higher concentration of BMP-2. Additionally, 250 ng/mL BMP-2-treated CD and NCD micro-aggregates showed less apoptotic CLCs in their center compared with 100 ng/mL BMP-2-treated micro-aggregates, indicating that 250 ng/mL BMP-2 was able to stimulate CLCs in the center of the micro-aggregates, whereas 100 ng/mL BMP-2 was not. Altogether this indicates that BMP-2 dose-dependently induces regenerative effects in canine and human CLCs and that a higher BMP-2 concentration (in the present study 250 ng/mL) is most optimal in establishing these beneficial effects.

BMP-2 has a qualitatively better regenerative effects compared with TGF-ß$_1$ in canine and human CLCs

The present study shows that both TGF-ß$_1$ and BMP-2 stimulated their Smad-pathways (via upregulation of *PAI1* and *ID1*)[86,87] and that 250 ng/mL BMP-2 has an even higher regenerative potential than TGF-ß$_1$ supplemented at 10 ng/mL on canine CD, NCD and human CLCs: it increased CLC GAG deposition, whereas the DNA content of the BMP-2-treated CLC micro-aggregates was similar compared with TGF-ß$_1$-treated micro-aggregates. Wei *et al*. suggested anti-apoptotic effect of BMP-7 on human CLCs by inactivation of *CASP3*[88]. However, in this study in canine CLCs, both gene expression markers of proliferation and apoptosis did not differ between treatment groups, indicating that the anabolic effects are primarily at the matrix level. Furthermore, in human CLCs, the *BAX/BCL2* ratio did not differ between the growth factor-treated groups, whereas *CCND1*, a marker for proliferation, was significantly higher in the BMP-2 treated micro-aggregates compared with the TGF-ß$_1$-treated micro-aggregates. Additionally, TGF-ß$_1$ induced fibrotic (re)differentiation according

to the fibrotic rim surrounding the canine and human micro-aggregates and the increased collagen type I expression, whereas BMP-2 did not induce this. Collagen type I content is increasing in the degenerative IVD and is the main ECM component in scar formation of the skin[89,90]. Collagen type I is therefore not desired in the IVD, because it stiffens the NP which would limit the biomechanical function of the disc[91]. Altogether, BMP-2 exerts a better matrix anabolic effect, quantitatively and qualitatively, than TGF-ß$_1$ on CD, NCD and human CLCs *in vitro*, and hence a more suitable growth factor *in vivo* for the treatment of IVDD.

Comparison canine CD with NCD, and within human CLCs

Although in dogs and humans the IVDD process is similar[8], human, canine CD and NCD CLCs can respond differently to growth factors. Generally, the NCD CLC response towards growth factors appeared to be more pronounced at gene expression and protein level than the CD CLC response, which indicates that NCD CLCs are more metabolically active. Furthermore, canine CD micro-aggregates treated with 250 ng/mL BMP-2 released more GAGs into the culture medium than TGF-ß$_1$-treated micro-aggregates. In NCD micro-aggregates, however, CLCs treated with TGF-ß$_1$ released more GAGs into the culture medium than CLCs treated with 250 ng/mL BMP-2 in week 1 and 2, but this difference disappeared in week 3 and 4. This suggests that CD micro-aggregates treated with 250 ng/mL BMP-2 secrete and incorporate more GAGs than TGF-ß$_1$-treated micro-aggregates. However, canine NCD micro-aggregates treated with 250 ng/mL BMP-2 react differently by incorporating more and releasing less GAGs into the culture medium than TGF-ß$_1$-treated micro-aggregates. Based on the difference in *COL2A1* gene expression between 250 ng/mL BMP-2-treated CD and NCD micro-aggregates, it could be that NCD micro-aggregates create a better and abundant collagen scaffold. This could be an explanation that NCD micro-aggregates create a better support matrix for the GAGs to grasp. Altogether, this indicates that CD CLCs have less ability to incorporate GAGs in their ECM. These results are supported by Cappello *et al*. (2006), who found that CD and NCD NP cells assemble ECM in a different manner[92]. Altogether, NCD CLCs are more metabolically active, which suggests that their ability to respond to a regenerative stimulus is more suitable than responses exerted by CD CLCs. This will increase matrix production, which supports the difference in clinical sign development in NCD vs CD dogs; CD dogs show clinical signs earlier in life than NCD dogs.

Collagen type II protein was present in 250 ng/mL BMP-2-treated human micro-aggregates. *COL2A1* gene expression was, however, similar in TGF-ß$_1$- and 250 ng/mL BMP-2-treated micro-aggregates. This discrepancy between mRNA and protein expression of collagen type II could be explained by (a) post-transcriptional regulation of *COL2A1*, (b) the highly increased *MMP13* gene expression (collagen degradation) in the TGF-ß$_1$-treated CLCs, which indicates increased degradation of collagen type II or (c) that *COL2A1* gene expression peaked around day 7 after TGF-ß$_1$-treatment, whereas *COL2A1* gene expression peaked at a later time point after 250 ng/mL BMP-2 treatment (and therefore the *COL2A1* gene expression after 250 ng/mL BMP-2 treatment was missed at day 7). The most obvious difference between the canine CD and NCD CLC response to 250 ng/mL BMP-2 treatment was seen in collagen type II mRNA and protein expression. NCD CLCs treated with BMP-2 significantly increased *COL2A1* gene expression compared with the same CD CLCs-treated condition, whereas the collagen type II IHC was more positive in CD CLCs treated with BMP-2. The TGF-ß$_1$-treated canine CD and NCD micro-aggregates showed more collagen type II deposition than the 250 ng/mL BMP-2-treated micro-aggregates, which can possibly also be explained by upregulated *MMP13* expression in the BMP-2-treated canine CD micro-aggregates.

Interestingly, CD an NCD CLCs treated with either growth factor showed a similar gene expression response, GAG and collagen type I deposition as corresponding human CLCs. Collagen type II deposition was, however, different between the species: canine CLCs treated with TGF-β_1 and 100 ng/mL BMP-2 showed most collagen type II deposition, whereas human CLCs treated with 250 ng/mL BMP-2 showed the highest collagen type II deposition. In addition, human CLCs proliferated more than canine CLCs when both were treated with 250 ng/mL BMP-2. The latter could be explained by difference in donor age and affected IVDD grade, a discrepancy in species comparison regarding IVDD. Thus, CD and NCD CLCs can be used as a model for regenerative treatment for human IVDD, while same responses on gene expression level, GAG and collagen type I deposition were observed. However, to complete this comparison and map changes in the ECM, elaborated and detailed ECM analysis over a longer time period has to be performed.

Study limitations, suggested improvements for the study design and read out parameters

There are several limitations to this study. First of all, passage 2 cells were used and cultured under normoxic conditions, but dedifferentiation during the expansion phase is a common drawback[53,54]. Also, studies in (hypoxic) explant systems are beneficial for mimicking *in vivo* effects. On top of that, as showed by Wei *et al.* (2008)[88], even higher doses of BMP-2 also induced regenerative effects, which should also be studied on canine CLCs. Furthermore, GAG release into the culture medium was only measured in canine CD and NCD micro-aggregates, making the comparison with GAG incorporation and GAG release by human micro-aggregates impossible. Future studies on human CLC micro-aggregates should also collect culture medium for GAG release analysis after TGF-β_1 and BMP-2 treatment. Furthermore, measuring gene expression levels on multiple time points with more replicates and detailed ECM analysis is recommended. Moreover, analysis of cell senescence markers will make a comparison between (re)active and inactive cells possible. In addition, Carreira *et al.* summarized that BMP-2, as an early mediator in fracture repair, is able to induce production of other BMPs[39]. This could mean that BMP-2 treatment has a lasting effect via other members of the BMP sub-family, which can be determined at gene (RT-qPCR) and protein (WB, IHC, ELISA) expression level. Additionally, BMP-2 has been shown to have an osteogenic potential and to induce osteophyte formation at the IVD level [13,40,41,82]. However, BMP-2 does not always induce osteogenic differentiation[93], and can also drive human CLCs towards a chondrogenic phenotype[56]. Moreover, TGF-ß$_3$ supplementation can also induce disk calcification[94]. Osteogenic markers should be measured to elucidate this in future studies, since in the present study only hypertrophic markers (*COLX, MMP13*) were evaluated. In addition, CLCs derived from more severely degenerated IVDs (Thompson grade 4 or 5) should be tested as well in future studies, since these CLCs have more tendency to undergo hypertrophic/osteogenic differentiation with calcification[95].

Conclusion

In conclusion, 250 ng/mL BMP-2 had a more potent regenerative effect on CD, NCD and human CLCs in terms of GAG production, DNA content and collagen type II production (the latter only in human CLCs) compared with the lower 100 ng/mL BMP-2 concentration. TGF-ß$_1$ and 250 ng/mL BMP-2 both showed a considerable regenerative potential, but the fibrotic (re)differentiation caused by TGF-ß$_1$ treatment was not seen in the 250 ng/mL BMP-2-treated CLC micro-aggregates. This indicates that 250 ng/mL BMP-2 is a potent regenerative growth factor for use in *in vitro* IVDD studies. The most intriguing difference in reactive capacity between NCD and CD CLCs was observed in the assemblage of newly produced GAGs. NCD CLCs incorporated more newly produced GAG in the ECM, whereas CD

CLCs secreted more GAGs into the culture medium. This distinction in GAG processing suggests that there is a difference in canine CD and NCD breeds regarding CLC response to external stimuli. Future research should aim at (a) optimizing the conditions for the use of BMP-2 as a regenerative (combination) treatment for IVDD *in vitro* and later on *in vivo* and (b) at elucidating differences among CD, NCD and human CLCs in the response to external stimuli over a longer culture period.

General discussion

Low back pain is a major health issue for people all over the world and accounts for economic costs of €3.5 billion in 2007 in the Netherlands alone[11]. Low back pain has been strongly related to intervertebral disc degeneration (IVDD)[77]. Not only humans, but also dogs suffer from low back pain due to IVDD, with similar characteristics[8]. Current human and canine treatment options for IVDD include surgery, physiotherapy and/or medication, but they do not repair the IVD and can have serious side effects. Regenerative medicine-based research strategies try to biologically repair the degenerated IVD and include cell-, growth factor- or gene-based therapies *in vitro*, with the possible extension towards animal models. The ultimate aim is to produce an effective, minimally invasive and widely applicable new treatment for IVDD.

The main aim of this project was to evaluate the potency of regenerative treatment strategies on chondrocyte-like cells (CLCs) derived from degenerated human or canine IVDs (Thompson grade 3) *in vitro*. The regenerative effect of notochordal cell-conditioned medium (NCCM) and NCCM fractions was determined in Chapter 1 and 2, respectively, whereas the regenerative effect of the growth factors transforming growth factor beta 1 (TGF-ß$_1$) and bone morphogenetic protein 2 (BMP-2) was determined in Chapter 4. Canine, porcine and human NCCM induced a significant cross-species regenerative effect on human CLCs in terms of cell proliferation and ECM (glycosaminoglycan (GAG) and collagen type II) deposition. In addition, canine NCCM induced a less pronounced regenerative effect in canine CLCs compared with human CLCs, which could be due to the higher activity of canine CLCs. Future studies should confirm the regenerative NCCM effect on canine CLCs using more donors and optimal culture conditions: with TGF-β_1/BMP-2 pretreated canine CLC micro-aggregates (Chapter 3). Also, CLCs cultured under hypoxic conditions increased collagen type II content more than CLCs cultured in normoxic conditions[96,97]. Detailed analysis (gene expression profiling (RT-qPCR) on multiple time points, ECM analysis (GAG content, RT-qPCR, immunohistochemistry (IHC) and Safranin O/Fast Green staining), proliferative effect (DNA content, RT-qPCR) and apoptosis (RT-qPCR)) will provide more insight into the effects of NCCM on canine CLCs. These additional studies are indicated because besides regenerative effects, NCCM also induced some undesirable effects on canine and human CLCs: porcine and canine NCCM induced mild signs of fibrosis in human CLCs, as also shown by Portier *et al*. (2014)[22]. Interestingly, besides fibrosis-inductive factors, canine NCCM also contains factors that prevent canine CLC adhesion, resulting into micro-aggregate disintegration (Chapter 3). This all indicates that a cocktail of different (bioactive) factors is present in NCCM in suboptimal concentrations and that the notochordal cell (NC)-secreted factor(s) with the most potent regenerative effect should be identified and tested *in vitro* under optimal conditions. In this way, undesirable NCCM side effects – mentioned above – are circumvented and optimal regenerative effects are obtained.

For the identification of NC-secreted bioactive factors, NCCM was separated in a soluble (peptides and small proteins) and pelletable (micro-vesicles and large protein aggregates) fraction by ultracentrifugation. Since both fractions exerted regenerative effects on canine CLCs (but not as potent as NCCM itself), both fractions should be investigated in future studies for the identification of the most potent bioactive NC-secreted factor(s), e.g. by proteomic analysis, RNA sequencing, proteomics and lipidomics. Lai *et al*. hypothesized that exosomes derived from mesenchymal stem cells (MSCs) function as tissue stromal supporters[58]. This includes involvement in communication

processes and metabolism, but also in tissue repair and regeneration[58]. Generally, exosomes and cellular vesicles are considered to mediate cellular communication. They can be isolated from several cell types and consist of a distinct set of proteins which can be located inside the exosomes or on its membrane. Several mechanisms of intercellular communication by exosomes are proposed. These include exosomal membrane protein interaction with cleavage of soluble ligands and exosomes-target cell fusion. Subsequently, a reaction is trigged in the target cell which may include numerous pathways[59]. Since human NCCM induced more collagen type II deposition and canine and porcine NCCM induced more GAG deposition by human CLCs, combinatory strategies with an optimal balance between different bioactive fractions may induce optimal ECM production. With respect to this, we have shown in Chapter 4 that both 250 ng/mL BMP-2 and 10 ng/mL TGF-ß$_1$ exerted regenerative effects on canine chondrodystrophic (CD), non-chondrodystrophic (NCD) and human CLCs, but fibrosis was only induced by TGF-ß$_1$ and not by BMP-2 treatment. Most similar were the DNA and GAG content of canine CD and human micro-aggregates, however collagen type II deposition of canine and human CLCs was different. Additionally, NCD CLCs showed to be more metabolically active, suggesting that they respond better to a regenerative stimulus than CD CLCs. Increased matrix production follows and this supports the difference in clinical sign development in NCD vs CD dogs; CD dogs show clinical signs earlier in life than NCD dogs. Altogether, this indicates that canine CD and NCD CLCs can serve as an *in vitro* model to test regenerative treatment strategies on human CLCs.

The degenerated IVD may contain too less cells for the growth factor-based (combinatory) therapy to stimulate and have a proper regenerative response *in vivo* (depending on the degenerative grade). Therefore, it is proposed to test this growth factor-based regenerative treatment in combination with stem cells. MSCs are widely studied for the use in regenerative medicine because of their availability and differential potential. MSCs have the ability to differentiate into CLCs, they have anti-inflammatory properties and secrete trophic fractions which stimulate surrounding cells[4]. Thus, MSCs can replenish the depleted CLC pool in the degenerated IVD and can have a regenerative effect on native CLCs. In this way, the bioactive NCCM component and/or BMP-2 will have the appropriate amount of cells to exert its effects on. In line with this hypothesis, a hydrogel co-culture of canine BMP-2-treated MSCs and CLCs (1:1) appeared to have deposited more GAG and collagen type II than BMP-2-treated CLC cultures alone (unpublished data from our group). Altogether, a growth factor (combinatory) treatment in combination with the supplementation of MSC is proposed to exert an additive regenerative effect on the degenerated IVD *in vivo* (Figure 1).

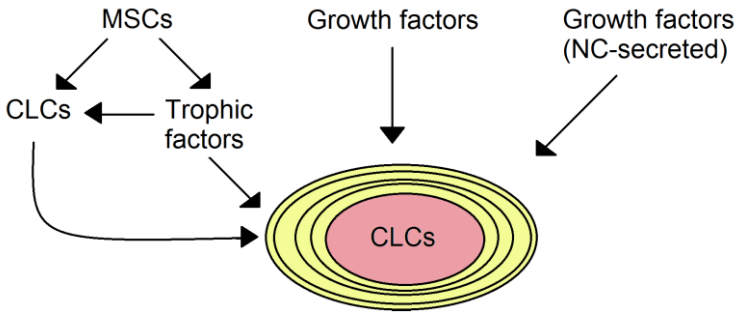


Figure 1. Proposed future research: a regenerative treatment combination of mesenchymal stem cells (MSCs), growth factors (e.g. bone morphogenetic protein 2 (BMP-2)) and the bioactive growth factors isolated (and concentrated) from notochordal cell (NC)-conditioned medium exerts a positive effect on the degenerated intervertebral disc with chondrocyte-like cells (CLCs).

Courses

My Honours Programme year gave me the possibility to successfully participate in multiple courses and to visit several academic conferences.

Courses:

- *Introduction to biomolecular methods*
    - Dr. P.A.J. Leegwater and Dr. L.C. Penning
- *Introductory Statistics course*
    - J. van den Broek and J.C.M. Vernooij
    - Two-days course (December 8 and 9, 2014)
    - Faculty of Veterinay Medicine, Utrecht
- *Modern methods in Data Analysis*
    - J. van den Broek and C. Kruitwagen
    - 3 weeks full time course (January 5 – 23 January ,2015)
    - MSC Epidemiology, 4.5 ECTS
- *Writing a Scientific Paper*
    - A. van der Zeeuw
    - 6 3-hour meetings (March 27 – August 7, 2015)
    - Graduate School of Life Sciences, 3 ECTS
- *Presenting in English for PhD researchers*
    - M. de Wolf
    - 3 3-hour meetings (July 30 – August 13, 2015)
    - Graduate School of Life Sciences, 1 ECTS

Conferences:

- *RM PhD Program Theme Day: MSCs*
    - October 13, 2014
    - UMC, Utrecht
- *International Stem Cell Forum Symposium. Regenerative Medicine and Stem Cells: Translational Challenges*
    - KNAW, ZonMw
    - November 18, 2014
    - Het Trippenhuis, Amsterdam
- *Veterinary Science day*
    - November 20, 2014
    - Antropia, Driebergen
- *European Veterinary Conference Voorjaarsdagen*
    - Presented my own research
    - April 9 – April 11, 2015
    - RAI, Amsterdam
- *Dutch Society for Matrix Biology*
    - FMWV
    - May 28, 2015
    - Conference Center De Werelt, Lunteren

Acknowledgements

I would like to thank my supervisors Frances Bach and Marianna Tryfonidou for their endless support, sharing of knowledge and enjoyable conversations. Also thanks to Anna Tellegen, Alberto Miranda Bedate, Jeanette Wolfswinkel, Saskia Plomp, Frank Riemers, Eyleen de Poel, Margot Müller and the rest of the orthopedics research team for their support, advice and for making this year an unforgettable experience for me. We shared a lot of laughter and nice conversations. Thank you all for making me part of this great team. During this year, I've learned a lot, not only in the lab, but also during the joined conferences and even during the joint drinks. Thank you all for this great opportunity and wonderful year.

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