THE EFFICACY OF LINK-N AS NEW REGENERATIVE TREATMENT FOR CANINE INTERVERTEBRAL DISC DEGENERATION

HONOURS PROGRAMME RESEARCH PROJECT 2015-2016

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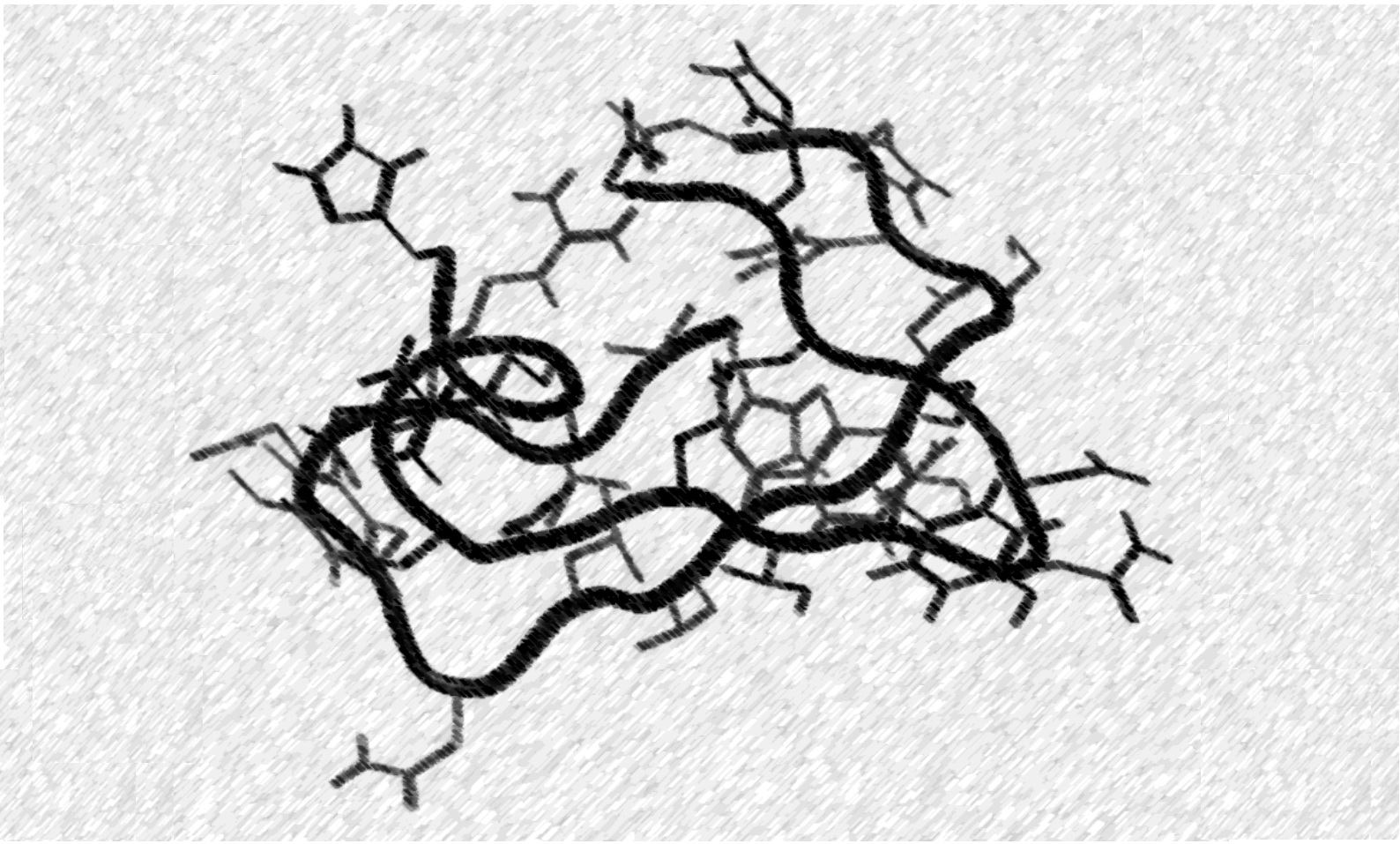

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GENERAL SUMMARY

Background. Intervertebral disc (IVD) degeneration is a major medical issue and causes back pain in humans and dogs. New regenerative treatments are desirable, since current treatments are invasive, expensive and only available for end-stage disease. Link-N is the N-terminal peptide of the link protein that stabilizes non-covalent proteoglycan interactions. Human Link-N is considered to be a promising peptide with growth factor-like properties, and has been shown to repair degenerated rabbit IVDs *in vivo* by direct binding to the bone morphogenetic protein receptor type II, to stimulate the expression of collagen type I and II in human IVD cells, and to restore the glycosaminoglycan (GAG) and collagen type II content in degenerated bovine discs. Additionally, Link-N has been shown to inhibit osteogenesis and increase the chondrogenic differentiation of human mesenchymal stromal cells (MSCs) *in vitro*. This suggests that combined treatment with Link-N and MSCs could be a promising new regenerative strategy for IVD degeneration.

Objectives. The main aim of this project was to determine the *in vitro* efficacy of Link-N alone or combined with MSCs as new regenerative treatment for canine patients that suffer from back pain due to IVD degeneration. Additionally, the experiments attempted to find a suitable large animal model to facilitate the translation of this new treatment strategy into human patients with IVD disease.

Methods. Several culture experiments were performed using nucleus pulposus cells (NPCs) from different species (canine, human, bovine, ovine), whereby the regenerative effects of the human and canine variant of Link-N and canine MSCs were tested. Extracellular matrix production and cell proliferation were determined by gene expression profiling, histological evaluation and measuring the DNA and GAG content. Activated signaling pathways were determined by measuring pSMAD1 and 2 levels and gene expression profiling.

Results. Human Link-N induced a concentration-dependent increase in GAG deposition and release in human and bovine NPCs. Canine and human Link-N induced a small, significant, concentration-independent increase in GAG deposition in canine NPCs, but this appeared not biologically relevant. Moreover, the addition of canine MSCs did not result in an increased GAG and/or DNA content. Although not significant, a moderate increase in SMAD1 and 2 signaling of human and bovine NPCs was detected after treatment with human and canine Link-N. Both human and canine Link-N significantly increased phosphorylated (p)SMAD1 levels in canine NPCs, whereas only canine Link-N treatment significantly increased pSMAD2 levels.

Conclusions. These studies imply that both canine and human Link-N alone or combined with canine MSCs lack the potency to be used as new regenerative therapy for IVD degeneration in dogs. Experiments investigating the species differences in Link-N-induced activated signaling pathways did not result in an explanation for this mild response of canine NPCs, so this remains to be elucidated in future studies. Furthermore, our results indicate that the dog cannot serve as a suitable large animal model for translation of Link-N treatment into human patients. However, since Link-N remains a promising peptide to be used as treatment for IVD regeneration in human patients, the search for a suitable large animal model should continue.

GENERAL INTRODUCTION

THE HEALTHY CANINE IVD

During embryologic development of the spine, the somites give rise to the axial skeleton. Each somite is divided into a dermatome forming the dermis, a myotome forming the musculature, and a sclerotome forming the vertebral bodies. Once formed, the spine consists of 7 cervical, 13 thoracic, 7 lumbar, 3 sacral, and a variable number of coccygeal vertebrae.[1] These vertebral bodies (from C2 until S1 and all coccygeal vertebrae) are connected via intervertebral discs (IVDs).[2] The main function of these IVDs is to distribute pressure over the vertebrae and provide mobility as well as stability to the spine.[2] The healthy IVD consists of a nucleus pulposus (NP), annulus fibrosus (AF), transition zone (TZ), and vertebral endplates (EPs) (Fig. 1).[1, 2]

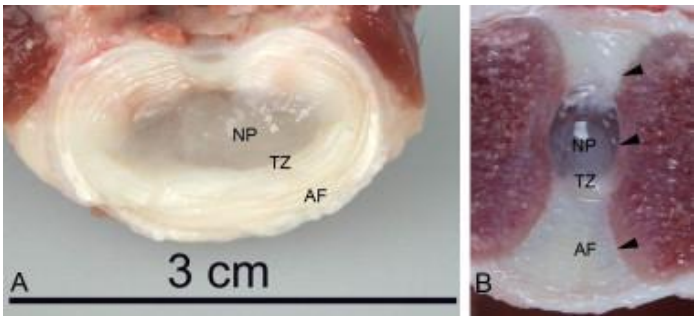

Fig. 1: Images of a transverse (A) and sagittal (B) section through a healthy intervertebral disc of a mature non-chondrodystrophic dog, showing the nucleus pulposus (NP), transition zone (TZ), annulus fibrosus (AF), and endplates (arrowheads). Reprinted from Bergknut *et al.* (2013).[2]

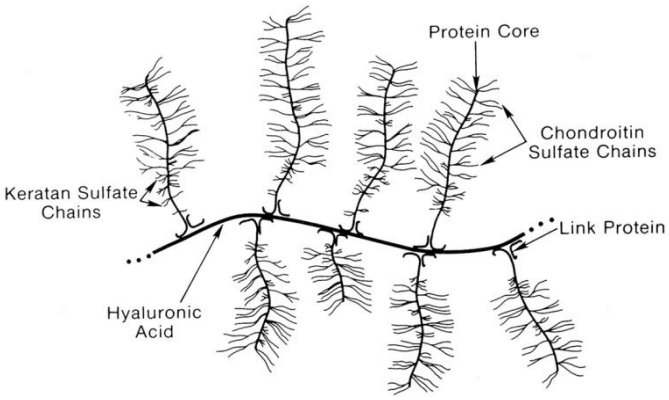

Fig. 2: Drawing of a proteoglycan aggregate composed of a central hyaluronic acid chain, to which the proteoglycan monomers are attached by link proteins. Each monomer consists of a protein core, with multiple glycosaminoglycans bound to it. Reprinted from Newman *et al.* (1998).[11]

The NP is a bean-shaped structure in the center of the IVD.[3] The main cell type of the healthy NP is the notochordal cell (NC).[4, 5] NCs are found in clusters and contain characteristic cytoplasmic vesicles.[6, 7] They produce an amorphous basophilic extracellular matrix (ECM).[4, 6] As a result, the NP is – in a healthy state – composed of proteoglycans intermixed with mainly collagen type II fibers, which are present at high ratio (> 20:1).[8, 9] The proteoglycan molecules are composed of negatively charged glycosaminoglycan (GAG) side-chains, mostly chondroitin and keratin sulfate, connected to a protein core (Fig. 2).[8] In the healthy NP, the major proteoglycan present is aggrecan.[9, 10] The negatively charged proteoglycans are, in their turn, connected with hyaluronic acid by link proteins (Fig. 2).[11] In this way, they form proteoglycan aggregates which together create a strong osmotic gradient. As a result, over 80% of the NP is composed of water.[12] This high water content causes a strong intradiscal pressure enabling the NP to function as a hydraulic cushion.[8]

The AF surrounds the NP and has a more dense network of multiple organized fibrous lamellae, composed of collagen fibrils aggregated with elastic fibers and coated by proteoglycans.[8, 10] The AF consists of 60% of water and its ventral part is three times thicker than its dorsal part (Fig. 1).[12] The outer layers of the AF are more fibrous and contain mainly collagen type I, whereas the inside of the AF has a more cartilaginous character and contains mostly collagen type II.[4] This inner part forms the

connection between the NP and the AF and is called the transitional zone (TZ).[4, 6] The fibers of the outer AF are connected to the bony vertebral body epiphyses, called Sharpey's fibres.[4] The fibers of the inner AF are connected with the endplates (EPs), which form the cranial and caudal borders of the IVD. The EPs regulate the transport of nutrients into the IVD through diffusion and osmosis. [13] These are very essential structures since innervation and vascularization of the IVD tissue is sparse in the outer layers of the AF and totally lacking in the TZ and NP.[4,14] Biochemically, the EPs are very comparable with articular cartilage.[15]

CANINE IVD DEGENERATION

It has been established that the dog can serve as a suitable animal model for studying human IVD degeneration, since IVD degeneration develops spontaneously in dogs and shows alterations and pathology patterns similar to reported in human IVD degeneration.[16] IVD degeneration is a multifactorial process that is characterized by a cell-mediated response to progressive structural failure of the IVD. Examples of factors that are identified to contribute to the degenerative process include trauma, chronic loading or abnormal biomechanical stresses and genetic predisposition (Fig. 3).[17, 18]

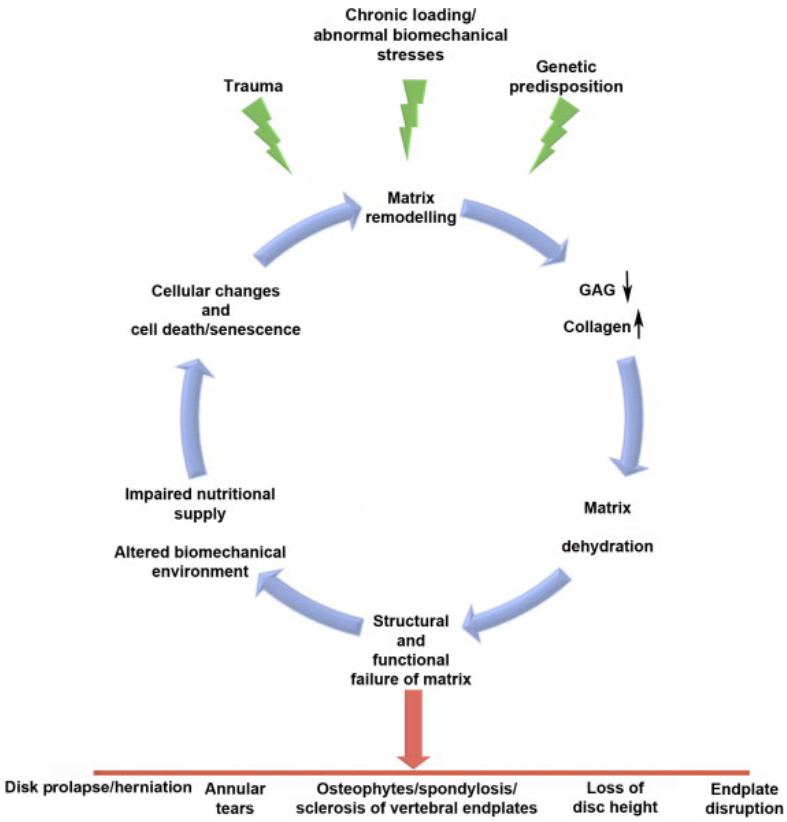

Fig. 3: Schematic overview of the pathophysiology of intervertebral disc (IVD) degeneration. The green symbols illustrate the factors that initiate the degenerative process leading to a vicious circle of inadequate repair of the IVD matrix. The structural and/or functional failure may lead to several pathological changes (red arrow) of the IVD and vertebrae. Modified from Bergknut *et al.* (2013).[2]

The macroscopic grading of human IVD degeneration is described by Thompson *et al.*[19] This five-category grading scheme has been validated for the grading of IVD degeneration in dogs as well.[20] The grading starts with a completely healthy disc (grade I) and ends with a severely degenerated IVD (grade V) (Fig. 4). The degenerative process frequently starts in the NP, which changes from a shining translucent mucoid structure to a dull more rigid structure. The lamellar structure of the AF becomes disorganized and the TZ widens and becomes irregular. At the end of the degenerative process, the NP and AF become more similar in composition, which makes it harder to distinguish these two components from each other macroscopically. During the IVD degeneration process, the EPs thicken and become irregular. Ultimately, due to new bone formation, osteophytes can develop and ventral spondylosis can occur at the peripheral margins of the vertebrae.[2, 4]

Histologically, the onset of the degeneration process is characterized by cellular changes within the NP. The large NC clusters become replaced by smaller clusters or single NCs.[4] Essentially, the NCs are all replaced by nucleus pulposus cells (NPCs), which have chondrocyte-like characteristics, together with their ECM mainly consisting of disorganized collagen fibers. This process is known as *chondrification*.[4] In the AF, the degenerative process leads to an ingrowth of NPCs from the TZ and disorganization of the fibers. The EPs become thicker, irregular and breaches can occur.[2]

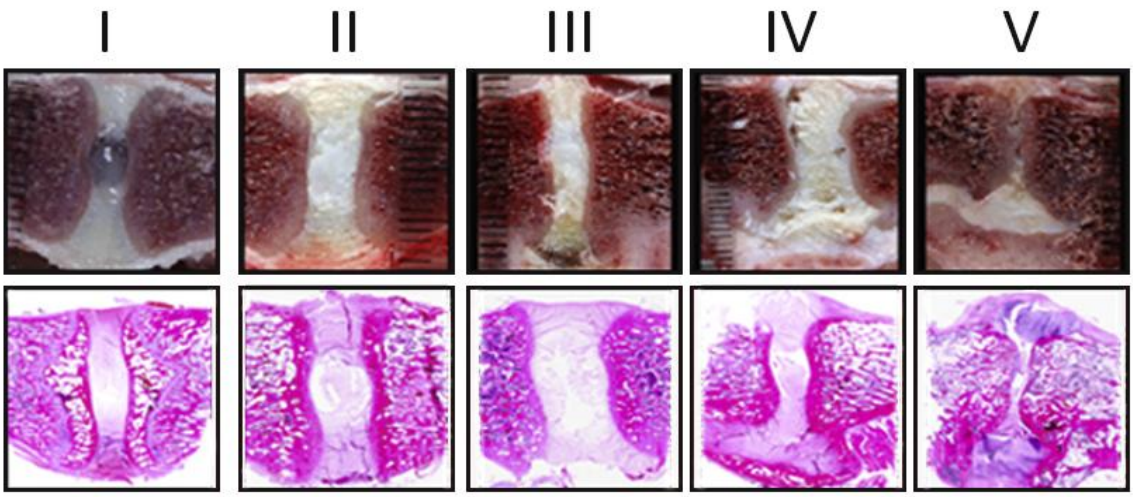

Fig. 4: Images of mid-sagittal sections through canine intervertebral discs illustrating the different stages (I to V) of degeneration. The top pictures show the macroscopic grading scheme and the bottom pictures the histopathological one, depicting a healthy intervertebral disc at the far left and increasing grade of degeneration ongoing from left to right. Modified from Bergknut *et al*. (2013).[2]

The remodeling and catabolism of ECM components of the IVD is controlled by regulatory enzymes like metalloproteinase (MMPs) and a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS).[21] The balance between the synthesis and breakdown of ECM components determines the quality and integrity of the ECM, and thus the mechanical behavior of the IVD.[22] MMPs are mainly responsible for collagen break-down, while ADAMTS is mostly involved in the break-down of aggrecan.[23] In a physiological state, the activity of MMPs is regulated by endogenous tissue inhibitors of metalloproteinases (TIMPs).[2] During IVD degeneration, a disbalance between MMPs and TIMPs occurs, which finally results in a loss of ECM.[24] It has been established that the increase in MMPs is correlated with the grade of IVD degeneration of the canine spine.[16] The decrease in GAG content and relative increase in collagen type I content results in the production of a more rigid ECM. Additionally, the overall loss of GAGs and the replacement of long chondroitin sulphate side chains by shorter keratin sulphate side chains result in a decrease in osmotic gradient which leads to less water attraction.[8, 10] This process can be accelerated by several inflammatory mediators such as interleukin-1β, and -6 and tumor necrosis factor-α.[25]

Dehydration of the NP might cause a decrease in disc height resulting in non-physiological loading of the AF and EP that take over the compressive load-bearing function. Subsequently, this altered loading can lead to a stiffer and weaker AF and annular tearing and fracturing of the EPs. As a consequence, bulging or herniation of the IVD can occur.[2] The degenerative process can also lead to an increased ingrowth of nerves and blood vessels into the IVD[26], which may result into chronic back pain.[27] Altogether, the IVD can no longer properly fulfill its role as shock absorber and diffusion of nutrients becomes impaired. This suboptimal biomechanical function and lack of nutrients cause a

vicious circle which further deteriorates the state of degeneration (Fig. 3).[2] All these factors may eventually lead to clinical signs known as IVD disease.

NCD VS. CD DOGS

Based upon the predisposition to chondrodystrophy, a classification of two groups of dog breeds can be made, *i.e.* between chondrodystrophic (CD) and non-chondrodystrophic (NCD) breeds. Beside showing differences in the process of endochondral ossification, CD and NCD dogs also differ in the age of onset, location and frequency of IVD degeneration.[4, 28]

CD dog breeds are characterized by disproportionally short limbs due to the disturbed endochondral ossification. In CD dogs, IVD degeneration is more common and is typically seen in the cervical or thoracolumbar spine at about 3-7 years of age.[4] At the age of three months, their NCs are already replaced by NPCs.[4, 6, 16] IVD degeneration progresses rapidly in CD dogs and may, at very young age, result in dorsal herniation of the NP. This herniation, known as Hansen type I herniation, typically has a sudden and explosive appearance with complete rupture of the AF leading to extrusion of the NP (Fig. 5).[4] Hansen type II herniation can also occur in CD dogs, but this is less common.[28]

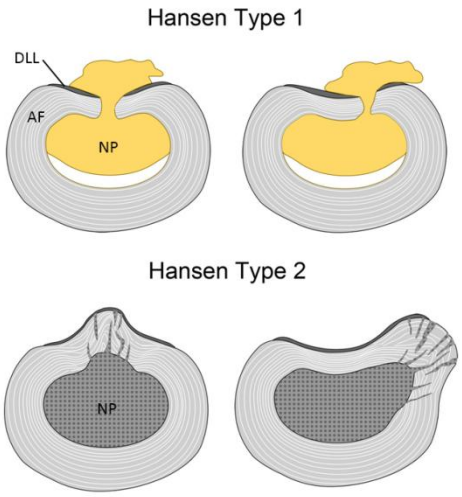

Fig. 5: Schematic figure of Hansen type I and type II herniation of a canine intervertebral disc. Hansen type I herniation is characterized by complete rupture of the dorsomedial (left) or dorsolateral (right) annulus fibrosus (AF) and dorsal longitudinal ligament (DLL). Consequently degenerated nucleus pulposus (NP) tissue is extruding. Hansen type 2 herniation involves disorganized and ruptured AF and consequently bulging of the NP, AF, and DLL the dorsomedial (left) or dorsolateral (right) side. Modified from Smolders *et al.* (2013).[28]

NCD dog breeds can also suffer from IVD degeneration, but more frequently in the caudal cervical or lumbosacral region at about 6-8 years of age. In contrast with CD dogs, the NCD dogs retain the NCs as predominant cell type of the NP during their lives. Whereas IVD degeneration mostly occurs spontaneously in CD dogs, in NCD dogs trauma or 'wear and tear' are the main causes. IVD degeneration in NCD dogs proceeds more gradually and mostly consists of partial rupture of AF fibers. This may result in Hansen type II herniation, characterized by partial NP herniation through a defect in the AF, leading to intradiscal protrusions and bulging of the IVD and dorsal longitudinal ligament (Fig. 5).[28] This usually occurs in the caudal cervical and lumbosacral spine. Hansen type I herniation is also reported in NCD dogs, but is less common.[4]

The macroscopic, histopathological and biochemical changes seen in IVD degeneration are comparable between CD and NCD dog breeds.[13]

CURRENT TREATMENTS VS. REGENERATIVE STRATEGIES

Currently, no treatment is available that has the potential to actually cure degenerated IVDs, since current treatments mainly aim at treating the symptoms of the disease. These therapies can be either surgical or non-surgical. Surgical procedures that are performed on dogs suffering from IVD degeneration are based on decompression. Thus, the aim of these surgeries is to alleviate the compression of neural structures which can be present due to herniation of the NP. Examples of such procedures are nucleotomy, laminectomy and a ventral slot procedure.[29, 30] Non-surgical therapies include alteration of exercise patterns, weight loss, and the prescription of drugs that relief the pain such as corticosteroids and non-steroidal anti-inflammatory drugs. Since none of these therapies eliminate the cause of the disease, but only reduce the pain and/or slow down the degenerative process, all current treatments can be classified as palliative care.

A more effective way to treat IVD disease would be to biologically repair the degenerated IVDs. It would be beneficial to tackle the degenerative disease in its initial phase by correcting the altered cell population. For this reason, there is increasing interest in the development of regenerative therapies.[29] Regenerative strategies aimed at treating orthopedic disorders mainly focus on combining specific cell types with certain growth-factors. Cell-based strategies are necessary because relatively few cells remain in the degenerated IVD and the viability of these remaining cells is impaired.[29] So stimulation of only these remaining cells may be insufficient to achieve adequate repair.[31] Potential candidates for these cell-based therapies include NPCs, NCs, and mesenchymal stromal cells (MSCs).[29] Additionally growth factors can be used to stimulate cell proliferation and ECM synthesis by inhibiting catabolic and/or stimulating anabolic processes.[32] Several growth factors have been shown to successfully decrease cell apoptosis, stimulate chondrogenic ECM production, and to enhance MSC differentiation to a NP-like phenotype.[29] Growth-factors that are commonly used are transforming growth factor beta (TGF-β) and bone morphogenetic proteins (BMPs).[33, 34] However, disadvantages of growth-factors include their high costs and potential side-effects.[35, 36] Therefore, there is a need to identify new molecules that can induce IVD matrix regeneration. A promising alternative agent with the ability to stimulate ECM synthesis, and therefore possessing growth factor-like properties, is a peptide known as Link-N. Link-N is a 16 amino acid sequence (DHLSDNYTLDHDRAIH) and is the N-terminal peptide of the link protein that stabilizes the proteoglycan aggregates in both the intervertebral disc and cartilage.[37-39] Human Link-N has already been shown to exert regenerative effects on degenerated IVD cells of several species (rabbit, bovine, human) and is thus considered as a promising candidate to be used for IVD repair.[38, 40-43] Although canine patients would also benefit from such a new regenerative treatment and are considered a suitable animal model for human IVD degeneration[16], the effect of Link-N on canine IVD cells has not been delineated yet.

AIMS AND STRUCTURE OF THE RESEARCH PROJECT

The experiments described in this paper mainly aimed at determining the efficacy of Link-N alone or combined with mesenchymal stromal cells as a new regenerative treatment for canine patients that suffer from back pain due to IVD degeneration. Additionally, the experiments attempted to find a suitable large animal model to facilitate the translation of this new treatment strategy into human patients. To accomplish this, several culture experiments were performed using NPCs from different species (canine, human, bovine) targeting the following aims:

Aim 1: Validating the culture system (using human and bovine NPCs) and delineating the regenerative effects of human Link-N on canine NPCs in both normoxic and hypoxic culture conditions.

Aim 2: Delineating whether the combined treatment of human Link-N and canine MSCs results in optimal regenerative effects on canine NPCs.

Aim 3: Investigating the regenerative effects of the canine variant of Link-N on human, bovine, and canine NPCs.

Aim 4: Analyzing whether human/canine Link-N induced the activation of SMAD signaling pathways in human, bovine and canine NPCs.

This report consists of four chapters, each focusing on one of the aforementioned aims.

EMBEDDING OF THE RESEARCH PROJECT

This project is part of the 'Regenerative Medicine and Stem cells' program of the faculty of Veterinary Medicine of Utrecht University. It was funded by a grand from the AO spine research network (in collaboration with the department of Biomechanical Bioengineering of the Technical University of Eindhoven). Furthermore, this work was funded by an additional award focusing on translating Link-N towards the clinic in collaboration with Dr. Fackson Mwale, McGill University, Canada.

CHAPTER 1: THE REGENERATIVE EFFECTS OF HUMAN LINK-N ON HUMAN, BOVINE AND CANINE NUCLEUS PULPOSUS CELLS

SUMMARY

Background. Intervertebral disc (IVD) degeneration is a major medical issue and causes back pain in humans and dogs. New regenerative treatments are desirable, since current treatments are invasive, expensive and only available for end-stage disease. Link-N is the N-terminal peptide of the link protein that stabilizes non-covalent proteoglycan interactions. Link-N is considered to be a promising peptide with growth factor-like properties, and has been shown to repair degenerated rabbit IVDs *in vivo*, to stimulate the expression of collagen type I and II in human IVD cells, and to restore the glycosaminoglycan (GAG) and collagen type II content in degenerated bovine discs. The first 8 amino-acids of Link-N, called short Link-N (sLink-N), presumably form the biologically active part of Link-N. It is reported that sLink-N stimulates matrix production to a similar extent as the 16 amino acid structure.

Objective. This study aimed at delineating the regenerative effects of human Link-N and sLink-N on human, bovine, and especially canine nucleus pulposus cells (NPCs), since the effect of Link-N on the latter species has not been studied yet.

Methods. NPCs from degenerated human, bovine and canine IVDs were cultured in 3D micro-aggregates (35,000 cells) for 28 days in basal culture medium with and without supplementation of human Link-N (1 µg/mL and 10 ng/mL) and sLink-N (0.5 µg/mL and 5 ng/mL). Extracellular matrix production and cell proliferation were determined by gene expression profiling, histological evaluation and measuring the DNA and GAG content.

Results. Supplementation of human (s)Link-N resulted in a small concentration-dependent increase in DNA content of the bovine micro-aggregates compared with untreated controls. Furthermore, (s)Link-N induced a concentration-dependent increase in GAG deposition and release of human and bovine NPC micro-aggregates. Collagen type I deposition of the bovine and human NPCs was stimulated by (s)Link-N, whereas collagen type II deposition was not affected. A small, but significant, concentration-independent increase in GAG deposition of the canine micro-aggregates was induced by (s)Link-N, whereas GAG release and total GAG production were not influenced.

Conclusions. The present study showed that human (s)Link-N stimulated extracellular matrix production of human and bovine NPCs, but exerted only a mild effect on canine NPCs. The biological relevance of these moderate regenerative effect of (s)Link-N on canine NPCs is debatable. Therefore, follow up studies will focus on optimizing this treatment strategy by using canine mesenchymal stromal cells as cell supplement and additional trophic factor.

INTRODUCTION

Intervertebral disc (IVD) degeneration is considered as a major medical issue and causes back pain in humans and dogs.[2] During IVD degeneration, the glycosaminoglycan (GAG) content decreases and catabolic matrix metalloproteinase (MMP) activity and denatured collagen content increases.[2] Therefore, the stimulation of repair of the degenerated IVD requires both increased production of extracellular matrix (ECM) and downregulated proteinase activity. ECM synthesis can be stimulated using exogenous growth factors, such as transforming growth factor beta (TGF-β) and bone morphogenetic protein-2 (BMP2).[33, 34] These growth-factors are involved in the regulation of the transcription of *SOX9*, which is the master regulator of chondrogenic differentiation and essential for ECM specific gene expression, such as *COL2A1* (collagen type II) and a *ACAN* (aggrecan).[44] The use of growth factors in clinical practice is, however, limited because of their high cost and potential side effects.[35, 36] In principle, this can be circumvented using synthetic peptides, which are relatively cheap to produce. A promising candidate with the ability to stimulate ECM synthesis, and therefore possessing growth factor-like properties, is a peptide known as Link-N. Link-N is a 16 amino acid sequence (DHLSDNYTLDHDRAIH) and is the N-terminal peptide of the link protein that stabilizes the proteoglycan aggregates in both the IVD and cartilage.[37-39] It is generated *in vivo* by cleavage of the link protein between His-Ile residues.[37] Link-N is again cleaved in the AF creating an even shorter peptide, called short Link-N (sLink-N), which consist of only the first 8 amino acids (DHLSDNYTL).[45]

Link-N has already been shown to stimulate the expression of aggrecan, collagen type I and II in human IVD cells *in vitro* and to restore the GAG and collagen type II content of degenerated bovine IVDs *ex vivo*.[38, 41, 46] Furthermore, a study from Mwale *et al.* (2011) demonstrated that, when administered to degenerated rabbit IVDs *in vivo*, Link-N stimulated *ACAN* gene expression and down regulated *MMP* expression, leading to an increased proteoglycan content of both the NP and AF.[42] The results of the study from Gawri *et al.* (2014) revealed that only the first 8 amino acids, called short Link-N (sLink-N), induced proteoglycan synthesis in both the NP and AF cells to a similar extent as full-length Link-N.[45] Therefore, they suggest that the biologically active part is preserved within this fragment.

Since Link-N is a degradation product of the link protein generated by MMPs, it was suggested that it can 'feed-back' and therefore stimulate ECM production. In that sense, the presence of Link-N could be an indicator that ECM degradation is taking place, signaling the need for increased synthesis of ECM components to compensate for degradation.[43] A study of Wang *et al*. (2013) showed that Link-N exerts its effect by direct interaction with the BMP receptor type II, at least in rabbit IVD cells.[40] This interaction initiates SMAD signaling and upregulates BMP proteins, including BMP-4 and BMP-7. Through a cell-autonomous loop, BMP-4 and BMP-7 promote expression of *SOX9* and downstream *ACAN* and *COL2A1* genes by binding to the BMP receptor type I which sustains or even amplifies SMAD 1/5/8 signal transduction (Fig. 6).[40]

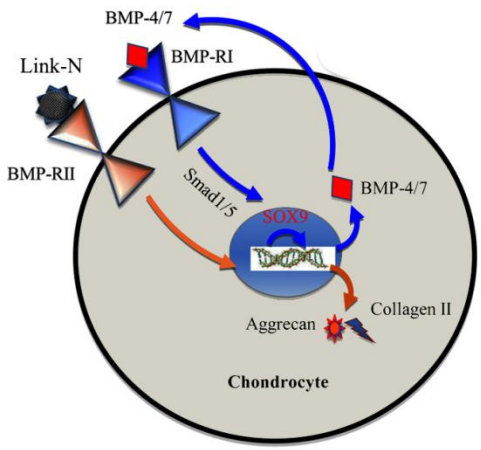

Fig. 6: Proposed model for the induction of matrix production by of Link-N in rabbit intervertebral discs. Modified from Wang *et al.* (2013).[40]

The effects of the human variant of Link-N have been tested on IVD cells of different species, but its regenerative effect on canine IVD cells has not been investigated yet. Therefore, the aim of the research presented here was to delineate the regenerative effects of human Link-N and sLink-N on human, bovine, and especially canine NPCs. By including human and bovine NPCs, this study aimed to reproduce the work of other research groups and thereby validate our used culture system and Link-N batch. Additionally, by culturing in both normoxia and hypoxia, the optimal culture conditions for the NPCs were determined. The ultimate goal was to investigate the potential of human Link-N to be used as new treatment for IVD degeneration in canine patients and to determine whether the dog could serve as a suitable large animal model for the translation of this treatment into human patients.

MATERIALS AND METHODS

HARVEST AND ISOLATION OF THE NPCS

Degenerated IVDs from available spines of canine donors (Table 1) were opened in a sterile manner, where after the NP tissue was sampled and put in hgDMEM+Glutamax (31966, Invitrogen, Paisley, UK) with 2% P/S (P11-010, GE Healthcare Life Sciences, Eindhoven, the Netherlands). Next, the tissue was brought over to a clean conical 50 mL tube and hgDMEM+Glutamax with 1% P/S was added until full. After removing the supernatant, the complete sample was digested using a 0.15% pronase (11459643001, Roche Diagnostics, IN, USA) solution in hgDMEM+Glutamax with 1% P/S during 45 minutes at 37°C. Thereafter, hgDMEM+Glutamax with 1% P/S was added to the tube until full to dilute the pronase. Next, the supernatant was removed and a 0.15% collagenase II (4176, Worthington, Lakewood, NJ, USA) solution in hgDMEM+Glutamax with 1% P/S was added. The sample was digested overnight at 37°C. The next day the NPCs were filtered over a 70-µm cell strainer (BD Biosciences). hgDMEM+Glutamax with 10% fetal calf serum (FCS) (high performance 16000–044, Gibco, Bleiswijk, The Netherlands) and 1% P/S was added to the 50 mL tube until full, where after the tube was centrifuged at 500 *g* for 5 minutes and the supernatant was removed (twice). The sample was resuspended in hgDMEM+Glutamax with 20% FCS. The cells were counted using an automatic cell counter. Next, hgDMEM+Glutamax with 20% dimethyl sulfoxide (DMSO) was added, thereby creating a solution with hgDMEM+Glutamax with 10% FCS and 10% DMSO. The NPCs were placed in cryogenic vials at -196°C until further use. Human and bovine NPCs were supplied by other research institutes, but were harvested in a similar manner as the canine NPCs.

MICRO-AGGREGATE CULTURE OF THE NPCS

The NPCs from degenerated IVDs of human, canine and bovine donors (Table 1) were expanded in expansion medium containing hgDMEM+Glutamax with 10% FCS, 1% P/S, 0.1 mM Ascorbic acid 2-phosphate (A8960, Sigma-Aldrich, Saint Louis, USA), 10^{-9} M dexamethasone (AD1756, Sigma-Aldrich, Saint Louis, USA) and 1 ng/mL bFGF (PHP105, AbD Serotec, Puchheim, Germany) in an incubator under normoxic conditions (Nx) (21% O_2, 5% CO_2, 37°C) or hypoxic conditions (Hx) (5% O_2, 5% CO_2, 37°C). The media were changed twice a week. After expansion, micro-aggregates of 35,000 cells were formed. For this purpose, the NPCs were plated in low-adherence cell repellent surface 96-well plates (650970, CELLSTAR® Greiner Bio-one, Alphen a/d Rijn, the Netherlands) in 50 µL basal culture medium: hgDMEM+Glutamax with 1% P/S, 1% ITS + premix (354352, Corning Life Sciences,

Amsterdam, the Netherlands), 0.04 mg/mL L-proline (P5607, Sigma-Aldrich, Saint Louis, USA), 0.1 mM Ascorbic acid 2-phosphate, and 1.25 mg/mL Bovine Serum Albumin (Sigma Aldrich, A9418). To induce micro-aggregate formation, the 96-well plates were centrifuged at 50 *g* for 5 minutes. The next day (day 0), basal culture medium was replaced by either basal culture medium for the negative control micro-aggregates, basal culture medium supplemented with 10 ng/mL human recombinant TGF-β_1 (240-B, R&D Systems, Inc., Minneapolis, USA) for the positive control micro-aggregates, basal culture medium supplemented with 1 µg/mL or 10 ng/mL (the low concentration was not tested on the canine NPCs in Hx) human Link-N (DHLSDNYT-LDHDRAIH; CanPeptide, Montreal, Canada), or 0.5 µg/mL or 5 ng/mL human sLink-N (DHLSDNYT; CanPeptide, Montreal, Canada). sLink-N was used in half the concentration compared with Link-N, because it consists of only half the amount of amino acids as Link-N, so thereby a comparable concentration was created. The micro-aggregates were cultured for 28 days under normoxic or hypoxic conditions. Media were changed two times a week.

Table 1: Overview of the used donors in the study and their characteristics

Number	Gender	Species	Thompson score	Age (years)
S14-80	Female	Human	III	63
S13-142	Male	Human	III	47
S13-23	Male	Human	III	66.5
B1	N.A.	Bovine	I/II	± 2
B2	N.A.	Bovine	I/II	± 2
B3	N.A.	Bovine	I/II	± 2
B4	N.A.	Bovine	I/II	± 2
B5	N.A.	Bovine	I/II	± 2
B6	N.A.	Bovine	I/II	± 2
1399617	Male	Canine (Beagle)	III	4.5
7140151	Male	Canine (Beagle)	III	5.5
7283679	Male	Canine (Beagle)	III	2.5
7207859	Male	Canine (Beagle)	III	5
0906743	Female	Canine (Beagle)	III	5.5
1305503	Female	Canine (Beagle)	III	1.5
7200013	Male	Canine (Beagle)	III	5
1208156	Male	Canine (Beagle)	III	1.5

GENE EXPRESSION PROFILING

RNA ISOLATION

At day 7, RNA-isolation of the NPC micro-aggregates was performed using the RNeasy® Micro Kit (74004, Qiagen, Valencia, USA). Firstly, 10 µL β-mercaptoethanol (β-ME) was added to 1 mL Buffer RLT to effectively inactivate RNAses in the lysate. 4 volumes of ethanol (96 - 100%) were added to Buffer RPE to create the working solutions. The DNase I stock solution (RNAse-Free DNase Set, 79254, Qiagen, Valencia, USA) was prepared by dissolving the lyophilized DNase I in 550 µL RNase-free water.

The micro-aggregates were frozen in liquid nitrogen and crushed using pestles (P9951-901, Argos Technologies, Elgin, USA). 350 µL Buffer RLT was added to the lysate to support the binding of RNA to the silica membrane of the RNeasy MinElute spin columns. Next, 350 µL of 70% ethanol was added to precipitate the RNA and the solution was mixed well by pipetting. Thereafter, the samples were transferred to a RNeasy MinElute spin column in 2 mL collection tubes and centrifuged for 30 seconds at 16100 *g* to separate the precipitated RNA from the rest of the solution. The flow-through was discarded. 350 µL Buffer RW1 was added to the RNeasy MinElute spin columns to remove non-specifically bound biomolecules to the silica membrane and then the samples were centrifuged for 30 seconds at 16100 *g*. The flow-through was discarded. 45 µL DNase I stock solution was added to 315 µL Buffer RDD and mixed by inverting the tube. Next the DNase I incubation mix (80 µL) was added directly to the RNeasy MinElute spin columns membrane and incubated for 15 minutes to successfully digest the genomic DNA. Thereafter, again 350 µL Buffer RW1 was added to the RNeasy MinElute spin columns and the samples were centrifuged for 30 seconds at 16100 *g*. The collection tubes were discarded where after the RNeasy MinElute spin columns were placed in new 2 mL collection tubes (supplied). 500 µL Buffer RPE was added to the spin columns for washing the membrane-bound RNA and the samples were centrifuged for 30 seconds at 13,200 rpm. The flow-through was discarded. 500 µL of 80% ethanol was added to the RNeasy MinElute spin columns and the samples were centrifuged for 2 minutes at 16100 *g*. The collection tubes were discarded. The RNeasy MinElute spin columns were placed in new 2 mL collection tubes. After opening the lids, the samples were centrifuged at full speed for 5 minutes to dry the membrane. The flow-through and collection tubes were discarded. The RNeasy MinElute spin columns were placed in new 1.5 mL collection tubes. 17 µL RNase-free water was added directly to the center of the spin columns membrane and the samples were centrifuged for 1 minute at full speed to elute the RNA. The flow-through was added again directly to the center of the spin columns membrane and for the last time, the samples were centrifuged for 1 minute at full speed. The spin columns were discarded and the samples were stored at -70°C.

CDNA SYNTHESIS

cDNA synthesis was performed by using the iScript™cDNA Synthesis Kit (170-8891, Bio-Rad, Veenendaal, the Netherlands). 4 µL of iScript Reaction Mix and 1 µL of iScript Reverse Transcriptase were added to 15 µL of RNA to get a final volume of 20 µL per sample. The complete reaction mix was incubated for 5 minutes at 25°C, 30 minutes at 42°C, and 5 minutes at 85°C and stored at -20°C until further use.

QUANTITATIVE RT-PCR

HUMAN NPCS

Four stably expressed reference genes (TATAA-box binding protein (*TBP*), hypoxanthine-guanine phosphoribosyltransferase (*HPRT*), succinate dehydrogenase subunit A (*SDHA*) and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide *(YWHAZ*)) were used to normalize gene expression of the target genes (aggrecan (*ACAN*), collagen type I (*COL1A1*), collagen type II (*COL2A1*), collagen type X (*COL10A1*), a disintegrin and metalloproteinase with thrombospondin motifs 5 (*ADAMTS5*), matrix metalloproteinase 13 (*MMP13*), Tissue inhibitor of metalloproteinases 1 (*TIMP1*), cyclin D1 (*CCND1*), Bcl2- like-protein (*BAX*), B-cell CLL/lymphoma 2 (*BCL2*), caspase-3 (*CASP3*), inhibitor of DNA binding-1 (*ID1*), plasminogen activator inhibitor-1 (*PAI1*),

activin receptor-like kinase 1 (*ALK1*), TGF-β receptor type I (*ALK5*), SRY-box 9 (*SOX9*) and axin 2 (*AXIN2*)) (Table 2).

Table 2: Overview of the primers used to asses gene expression of the human NPCs

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

BOVINE NPCS

Three stably expressed reference genes (Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), Ribosomal Protein L13a (*RPL13a*) and hypoxanthine phosphoribosyltransferase 1 (*HPRT1*)) were used to normalize gene expression of the target genes (aggrecan (*ACAN*), a disintegrin and metalloproteinase with thrombospondin motifs 5 (*ADAMTS5*), collagen type I (*COL1A1*), collagen type II (*COL2A1*), collagen type X (*COL10A1*), matrix metalloproteinase 13 (*MMP13*), Tissue inhibitor of metalloproteinases 1 (*TIMP1*)) (Table 3).

Table 3: Overview of the primers used to asses gene expression of the bovine NPCs

Genes	Forward sequence 5' → 3'	Reverse sequence 5' → 3'	Amplicon size	Annealing temp (°C)
Reference genes				
GAPDH	GGCGTGAACCACGAGAAGTATAA	CCCTCCACGATGCCAAAGT	119	60
RPL13a	CTGCCCCACAAGACCAAG	TTGCGAGTAGGCTTCAGAC	140	60
HPRT1	GAGGCATTGTGTCAGAGAGA	CTGTATTGAAAAGGAACTGTTGAC	129	60
Target genes				
ACAN	CCAACGAAACCTATGACGTGTACT	GCACTCGTTGGCTGCCTC	107	60
ADAMTS5	TCACTGCCTACTTAGCCCTGAA	GCTCCAACCGCTGTAGTTCAT	125	60
COL1A1	TGAGAGAGGGGTTGTTGGAC	AGGTTCACCCTTCACACCTG	142	60

COL2A1	TGGCTGACCTGACCTGAC	GGGCGTTTGACTCACTCC	187	60
COL10A1	TGAGCGATACCAAACACCTACAG	ACCTTTACCCTTTATGGCATACGG	91	60
MMP13	CTTGTTGCTGCCCATGAGTT	TTGTCTGGCGTTTTGGGATG	197	60
TIMP1	GTCAATGAAACTGCCTTATACC	TTCTGGGACCTGTGGAAG	149	60
SOX9	ACGCCGAGCTCAGCAAGA	CACGAACGGCCGCTTCT	70	60

CANINE NPCS

Four stably expressed reference genes (Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), Hypoxanthine-guanine phosphoribosyltransferase (*HPRT*), Ribosomal protein S19 (*RPS19*) and succinate dehydrogenase complex subunit A (*SDHA*)) were used to normalize gene expression of the target genes (aggrecan (*ACAN*), collagen type I (*COL1A1*), collagen type II (*COL2A1*), collagen type X (*COL10A1*), a disintegrin and metalloproteinase with thrombospondin motifs 5 (*ADAMTS5*), matrix metalloproteinase 13 (*MMP13*), Tissue inhibitor of metalloproteinases 1 (*TIMP1*), cyclin D1 (*CCND1*), Bcl2- like-protein (*BAX*), B-cell CLL/lymphoma 2 (*BCL2*), caspase-3 (*CASP3*), inhibitor of DNA binding-1 (*ID1*)), plasminogen activator inhibitor-1 (*PAI1*), activin receptor-like kinase 1 (*ALK1*), TGF-β receptor type I (*ALK5*), SRY-box 9 (*SOX9*), bone morphogenetic protein receptor type II (*BMPR2*)) (Table 4).

Table 4: Overview of the primers used to asses gene expression of the canine NPCs

Genes	Forward sequence 5' → 3'	Reverse sequence 5' → 3'	Amplicon size	Annealing temp (°C)
Reference genes				
GAPDH	TGTCCCCACCCCCAATGTATC	CTCCGATGCCTGCTTCACTACCTT	100	58
HPRT	AGCTTGCTGGTGAAAAGGAC	TTATAGTCAAGGGCATATCC	104	58
RPS19	CCTTCCTCAAAAAGTCTGGG	GTTCTCATCGTAGGGAGCAAG	95	61
SDHA	GCCTTGGATCTCTTGATGGA	TTCTTGGCTCTTATGCGATG	92	56.5
Target genes				
ACAN	GGACACTCCTTGCAATTTGAG	GTCATTCCACTCTCCCTTCTC	111	62
ADAMTS5	CTACTGCACAGGGAAGAG	GAACCCATTCCACAAATGTC	149	61
ALK1	CCTTTGGTCTGGTGCTGTG	CGAAGCTGGGATCATTGGG	107	61
ALK5	GAGGCAGAGATTTATCAGACC	ATGATAATCTGACACCAACCAG	116	59.5
BAX	CCTTTTGCTTCAGGGTTTCA	CTCAGCTTCTTGGTGGATGC	108	58
BCL2	TGGAGAGVGTCAACCGGGAGATGT	AGGTGTGCAGATGCCGGTTCAGGT	87	62
BMP2R	GTCTTCACAGTATGAACATGATGG	AACACTTTCACAGCAACTGG	150	65
CASP3	ATCACTGAAGATGGATGGGTTGGGTT	TGAAAGGAGCATGTTCTGAAGTAGCACT	139	58
COL1A1	GTGTGTACAGAACGGCCTCA	TCGCAAATCACGTCATCG	109	61
COL2A1	GCAGCAAGAGCAAGGAC	TTCTGAGAGCCCTCGGT	151	62
Col10A1	CCAACACCAAGACACAG	CAGGAATACCTTGCTCTC	80	61
CCND1	GCCTCGAAGATGAAGGAGAC	CAGTTTGTTCACCAGGAGCA	117	60
ID1	CTCAACGGCGAGATCAG	GAGCACGGGTTCTTCTC	135	59.5
MMP13	CTGAGGAAGACTTCCAGCTT	TTGGACCACTTGAGAGTTCG	250	65
PAI1	AAACCTGGCGGACTTCTC	ACTGTGCCACTCTCATTCAC	98	61.5
SOX9	CGCTCGCAGTACGACTACAC	GGGGTTCATGTAGGTGAAGG	105	62
TIMP1	GGCGTTATGAGATCAAGATGAC	ACCTGTGCAAGTATCCGC	120	66

The RT-qPCR was performed by using the iQT™ SYBR Green Supermix Kit (Bio-Rad, Veenendaal, the Netherlands) and the CFX384 Touch™ Real-Time PCR Detection System (Bio-Rad, Veenendaal, the Netherlands). An internal standard was used to show that the PCR reaction was working optimal

(efficiency is 95-105%). Furthermore, the standard will show the detection limit of the PCR reaction. The standard samples ranged from 1 till 7 and consisted of a serial dilution (2 times dilution for human samples and 4 times dilution for canine samples) of a pool of 10 times diluted cDNA from all the samples. The remaining 10 times diluted cDNA was diluted another 5 times to yield 50 times diluted cDNA. A master mix was made containing SYBR Green, forward and reverse primer in concentrations adjusted to the experiment. 6 µL master mix and 4 µL 50 times diluted cDNA was pipetted in a 384 wells plate according to the designed plate set-up. The plate was centrifuged for 1 minute at 593 *g* before it was put in the PCR machine at the right temperature and protocol (2 or 3 steps).

The Normfirst (EΔΔCq) method was used to determine the relative quantitative gene expression. For each target gene, the Cq-value of the test sample and the calibrator sample was normalized to the mean Cq-value of the reference genes using the following formula: $\Delta Cq = Cq_{mean\ ref} - Cq_{target}$. As calibrator the Cq-values of the negative control micro-aggregates were used. Thereafter, the EΔCq – value for the test and calibrator sample was calculated. In this formula, E indicates the amplification efficiency of the target/reference gene. $E^{\Delta\Delta Cq}$ was calculated by normalizing the EΔCq-value of the test sample to the one of the calibrator: $E^{\Delta\Delta Cq} = E^{\Delta Cq\ test} - E^{\Delta Cq\ calibrator}$. For each target gene, the mean n-fold change and standard deviation in gene expression were calculated. Target gene expression of the control samples was set at 1.

GAG AND DNA MEASUREMENTS

In order to measure the GAG and DNA content of the NPC micro-aggregates, they were first digested. Therefore, 75 µL of papain digestion solution (pH 6, 200 mM H2 NaPO4 *2 H_2O (21254, Boom B.V., Meppel, the Netherlands), 10 mM EDTA (100944, Merck Millipore, Amsterdam, the Netherlands), 10 mM cysteine HCl (C7880, Sigma-Aldrich, Saint Louis, USA), and 10 mM papain (P3125, Sigma-Aldrich, Saint Louis, USA)) was added to each micro-aggregate, followed by overnight incubation at 60°C. The next day the samples were vortexed followed by another hour of incubation at 60°C.

Each medium change, 25 µL culture medium of each micro-aggregate, used for GAG and DNA measurements, was collected and pooled. Before quantification of the GAG content by the dimethyl methylene blue (DMMB) assay, the digested micro-aggregates and culture medium were diluted (5x for canine samples and 10x for bovine and human samples). Immediately after DMMB (341088, SigmaAldrich, Saint Louis, USA) was added, the absorbance (540/595 nm) was measured using a microplate reader. The GAG content was calculated using a chondroitin sulphate (C4384, Sigma-Aldrich, Saint Louis, USA) standard line with polynomic properties.

The DNA content per micro-aggregate was measured using the Qubit® dsDNA High Sensitivity Assay Kit (Q32851, Invitrogen, Paisley, UK). 200 µL of the Qubit™ working solution was prepared for each standard and sample by diluting the Qubit™ reagent 1:200 in Qubit™ buffer. The standard assay tubes were prepared by adding 10 µL standard from the kit to 190 µL of working solution. The sample assay tubes were prepared by adding 5 µL of sample to 195 µL of working solution. All tubes were vortexed for 2-3 seconds. After determining the standard line using the standard assay tubes, the assay tubes were inserted in Qubit® 2.0 and readings were taken.

HISTOLOGY

At day 28, the micro-aggregates were fixed in 1% neutral buffered formaldehyde (4286, Klinipath B.V., Duiven, the Netherlands) with 10% eosin for 24 hours at room temperature (RT). The eosin was used to improve the visibility of the micro-aggregates within the paraffin blocks. Next, the samples were embedded in 2.4% alginate (Sigma Aldrich, A2033) which was cross-linked by adding formalin with 100 mM $CaCl_2$. The samples were stored in embedding cassettes in 70% alcohol until they were dehydrated through graded alcohol steps. After dehydration, samples were embedded in paraffin and 5 µm sections were mounted on Microscope KP+ slides (KP-3056, Klinipath B.V., Duiven, The Netherlands).

SAFRANIN O/FAST GREEN STAINING

For the Safranin O/Fast Green staining, the sections were deparaffinised through xylene (two times 5 minutes) and graded ethanol (96%, 80%, 70%, 60%; 5 minutes each), followed by one PBS rinse. The sections were subjected to Mayer's haematoxylin (3870, J.T.Baker® Chemicals - Avantor Performance Materials, Center Valley, USA) for 10 seconds and rinsed with demineralized water for 5 minutes. Subsequently, the sections were counterstained with filtered 0.4% Fast Green (F7252, Sigma-Aldrich, Saint Louis, USA) for 4 minutes, subjected to 1% acetic acid for two times 3 minutes, and stained with 0.125% aqueous Safranin O (58884, SigmaAldrich, Saint Louis, USA) for 7 minutes. The sections were then dehydrated with 100% ethanol (2 minutes) and xylene (two times 5 minutes) and mounted (Vectamount, H5000, Vector Laboratories, Burlingame, USA).

COLLAGEN TYPE I AND II IMMUNOHISTOCHEMISTRY

For collagen type II immunohistochemistry (IHC), the sections were deparaffinised (see Safranin O/Fast Green staining). Thereafter, they were put in 0.3% H_2O_2 (51008600.9025, Boom B.V., Meppel, the Netherlands) for 10 minutes to block endogenous peroxidase activity and washed two times for 5 minutes with PBS + 0.1% Tween (PBST 0.1%). Antigen retrieval was performed with 1 mg/mL pronase (11459643001, Roche Diagnostics, Almere, The Netherlands) and 10 mg/mL hyaluronidase (H3506, Sigma-Aldrich, Saint Louis, USA) in PBS for 30 minutes at 37°C. After washing with PBST 0.1%, the sections were blocked with 5% BSA in PBS for 30 minutes at RT to reduce background interference. Thereafter, the samples were incubated overnight at 4 °C with collagen type II mouse monoclonal antibody (0.4 µg/mL for human and bovine samples and 0.021 µg/mL for canine samples; II-II6B3, DSHB, Iowa City, IA) in 5% BSA in PBS. For the negative control staining, the first antibody was substituted with normal mouse IgG_1 (0.4 µg/mL for human and bovine samples and 0.021 µg/mL for canine samples; 3877, Santa Cruz Biotechnology, Heidelberg, Germany). The next day, the sections were washed with PBST0.1% before the secondary antibody (EnVision + System-HRP Goat Anti-Mouse, K4001, Dako, Glostrup, Denmark) was applied for 60 minutes at room temperature. After washing with PBS, the sections were incubated with the liquid DAB substrate chromogen system (K3468, Dako, Glostrup, Denmark) for 1 minute and counterstained with hematoxylin QS solution (H3404, Vector Laboratories, Burlingame, USA) for 10 seconds. Thereafter, they were washed with tap water for 10 minutes and dehydrated with graded ethanol (70%, 80%, 96% and 100%) and xylene (two times 5 minutes) and mounted. For collagen type I immunohistochemistry, the same procedure was followed with the collagen type I mouse monoclonal antibody (0.1 µg/mL for human and bovine samples and 0.067 µg/mL for canine samples; ab6308, Abcam, Cambridge, UK).

STATISTICAL ANALYSIS

For the statistical analysis, IBM SPSS statistics 22 was used. To check whether the data were normally distributed, a Shapiro Wilks test was performed. General linear regression models based on ANOVAs were used for normally distributed data, whereas a Kruskal Wallis and Mann-Whitney U test were performed on non-normally distributed data. To correct for multiple comparisons, all tests were followed by a Benjamini & Hochberg False Discovery Rate *post-hoc* test. *P*-values < 0.05 were considered significant.

RESULTS

HUMAN NPCS

EFFECTS OF (S)LINK-N ON THE NPC MIRO-AGGREGATES

The micro-aggregates containing NPCs from degenerated human IVDs which were treated with Link-N in high concentration had a significantly increased GAG content compared with the untreated controls (Nx) ($p < 0.05$, Fig. 7a). However, TGF-β_1 treatment induced the highest GAG content (Nx) ($p < 0.05$, Fig. 7a). Furthermore, Link-N treatment in high concentration resulted in a significantly higher GAG content compared with Link-N in low concentration (Nx) ($p < 0.05$; Fig. 7a). No significant effects of (s)Link-N on the DNA content of the micro-aggregates were encountered (Nx and Hx) (Fig. 7b). When the GAG content was corrected for the DNA content (an indication of the GAG production per cell), no significant effects of (s)Link-N were detected (Nx and Hx) (Fig. 7c). The micro-aggregates treated with human (s)Link-N in high concentration released significantly more GAGs in the medium over a time of 28 days compared with the untreated controls, both corrected and not-corrected for the DNA content (Nx) ($p < 0.05$; Fig. 7d). GAG release of the micro-aggregates treated with (s)Link-N in high concentration was significantly higher than of those treated with (s)Link-N in low concentration (Nx) ($p < 0.05$; Fig. 7d). The total GAG production of the micro-aggregates at day 28 was determined by adding together the GAG content of the micro-aggregates and the release of GAGs in the medium during the whole culture period. The micro-aggregates treated with (s)Link-N in high concentration showed a significantly higher total GAG production compared with the untreated controls (Nx) ($p < 0.05$; Fig. 7f). This was again a significant concentration-dependent effect (1 µg/mL > 10 ng/mL and 5 µg/mL > 5 ng/mL) (Nx) ($p < 0.05$; Fig. 7f). In line with the results of the DMMB-assay, Safranin O/Fast Green staining indicated that GAGs were present in the micro-aggregates of all conditions, but most prominently in the TGF-β_1 condition (Fig. 8). Furthermore, the staining showed more GAG deposition in the micro-aggregates treated with (s)Link-N in high concentration compared with those treated with (s)Link-N in low concentration (Fig. 8).

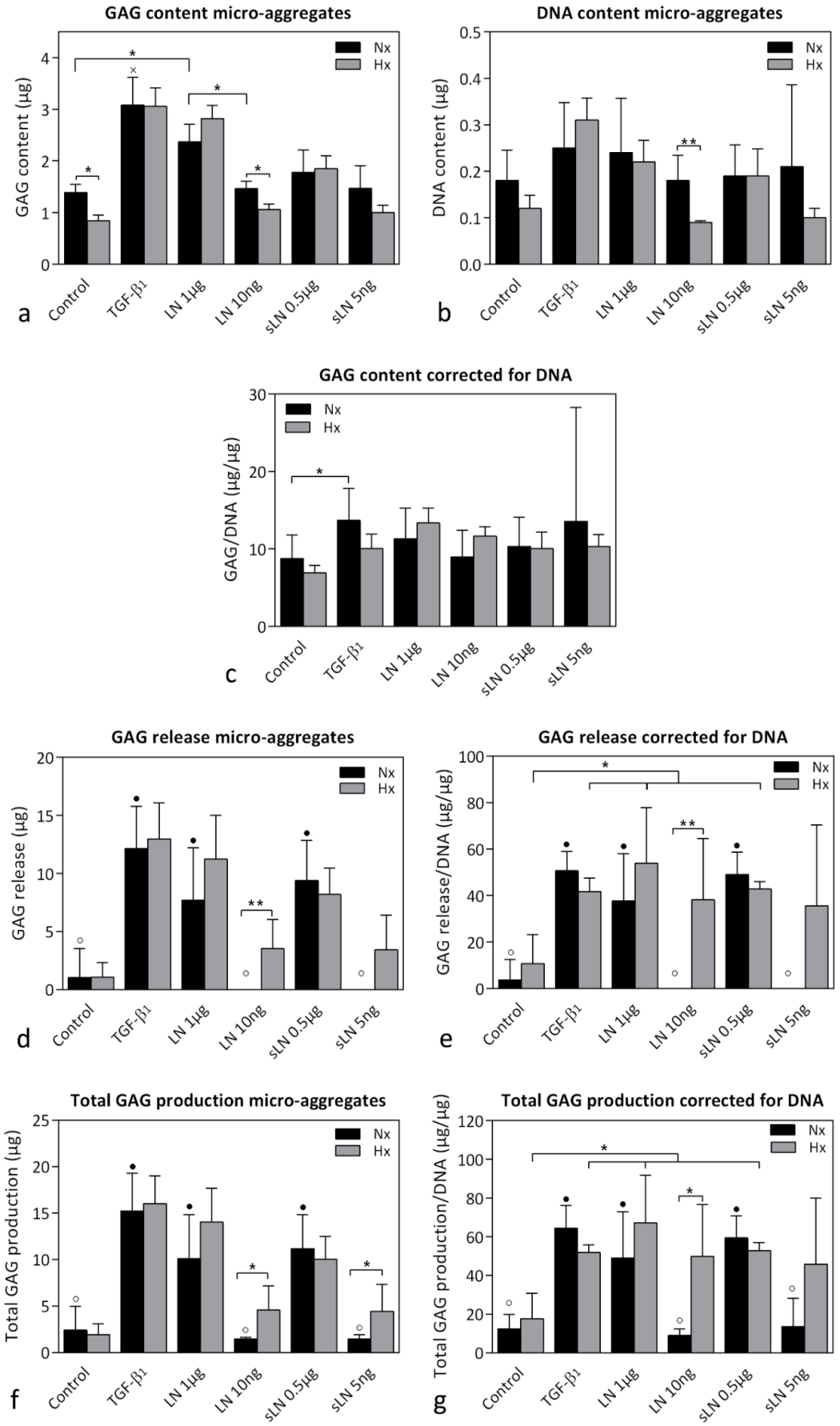

Fig. 7: (s)Link-N exerted concentration dependent regenerative effects on human nucleus pulposus cells (NPCs) when cultured in normoxic conditions. GAG and DNA content (mean + SD) of micro-aggregates derived of human NPCs from degenerated intervertebral discs cultured in micro-aggregates for 28 days in normoxia (Nx, 21% O_2) and hypoxia (Hx, 5% O_2) in different conditions. (**a**) GAG content of the micro-aggregates. (**b**) DNA content of the micro-aggregates. (**c**) GAG content corrected for the DNA content. (**d**) Total amount of GAGs released in the culture medium by the micro-aggregates. (**e**) Total GAG release corrected for the DNA content. (**f**) Total GAG production (GAG content micro-aggregates and amount of GAGs released taken together). (**g**) Total GAG production corrected for the DNA content. Nx: $n = 3$ *per* condition, Hx: $n = 2$ *per* condition. ∗ $p < 0.05$, ∗∗ $p < 0.01$. ● and ○ : significantly different ($p < 0.05$) from all the other conditions in Nx or Hx but the bars with the same symbol. ×: significantly different ($p < 0.05$) from all other conditions (growth factor treatment) in the same group (Hx or Nx).

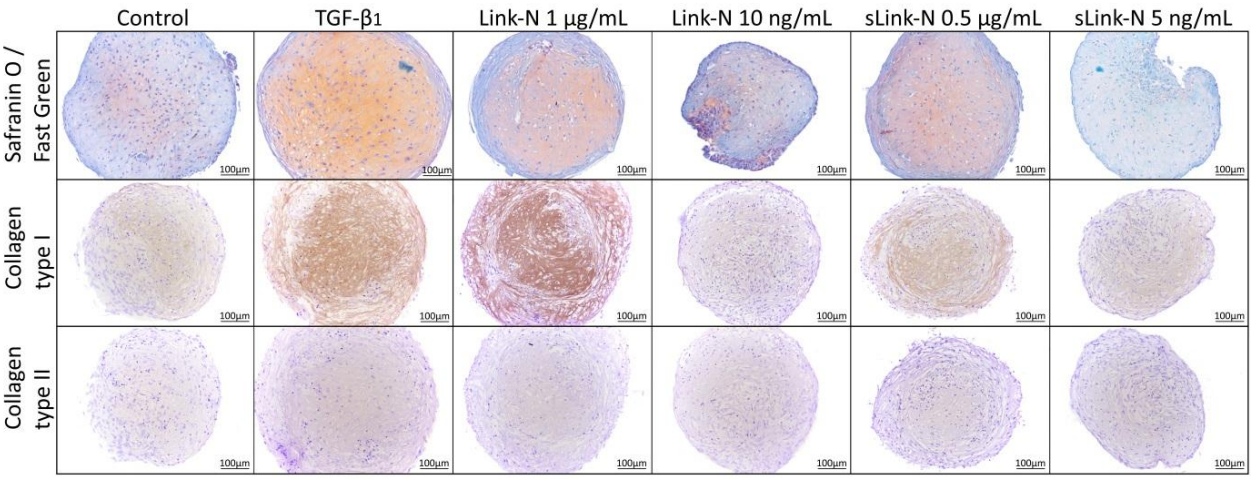

Fig. 8: Representative histological images of human nucleus pulposus cells (NPCs) cultured in micro-aggregates in normoxia (21% O_2) for 28 days stained with Safranin O/Fast green and immunohistochemical staining for collagen type I and II. The Safranin O/Fast Green staining indicated that GAGs were present in the micro-aggregates of all conditions, but most prominently in the ones treated with TGF-β_1 and (s)Link-N in high concentration. Collagen type I protein deposition was increased in the micro-aggregates treated with TGF-β_1 and (s)Link-N in high concentration compared the untreated controls. Collagen type II protein deposition was not affected by treatment with TGF-β_1 or (s)Link-N.

A fibrotic rim was visible around the micro-aggregates treated with TGF-β_1 and (s)Link-N in high concentration, but was less prominent in the micro-aggregates treated with (s)Link-N in low concentration (Fig. 8). In line with these results, IHC showed that collagen type I protein was abundantly present in the micro-aggregates treated with TGF-β_1 and (s)Link-N in high concentration, whereas less collagen type I protein was detected in the micro-aggregates treated with (s)Link-N in low concentration (Fig. 8). However, no significant effects were found on *COL1A1* gene expression determined at day 7 (Fig. 9). IHC indicated that collagen type II protein deposition was not affected by treatment with TGF-β_1 or (s)Link-N (Fig. 8) and the non-upregulated *COL2A1* gene expression at day 7 confirmed these findings (Fig. 9). Although the micro-aggregates treated with Link-N in high concentration demonstrated to have a significantly increased GAG content, *ACAN* gene expression was not significantly upregulated in this condition compared with the untreated controls at day 7 (Fig. 9). Furthermore, no significant differences were encountered for gene expression of *MMP13* and *ADAMTS5* (ECM degradation markers) (Fig. 9). Gene expression of *ALK5* (TGF-β_1 receptor type I), *PAI1* (read-out TGF-β_1 pathway[47]) and *ID1* (read-out BMP pathway[48]) did also not significantly differ between treatment groups (Fig. 9). Also no significant effect of (s)Link-N on the gene expression of *CASP3* and *BAX* (apoptosis markers) and *CCND1* (proliferation marker) was detected (Fig. 9), confirming the non-significant results of the DNA measurement at day 28 (Fig. 7b). Gene expression of *ALK1* (BMP receptor type I) could not be detected.

There were no significant differences encountered between the effects exerted by Link-N and sLink-N in comparable concentration, indicating that sLink-N has a comparable potent effect as Link-N on human NPCs.

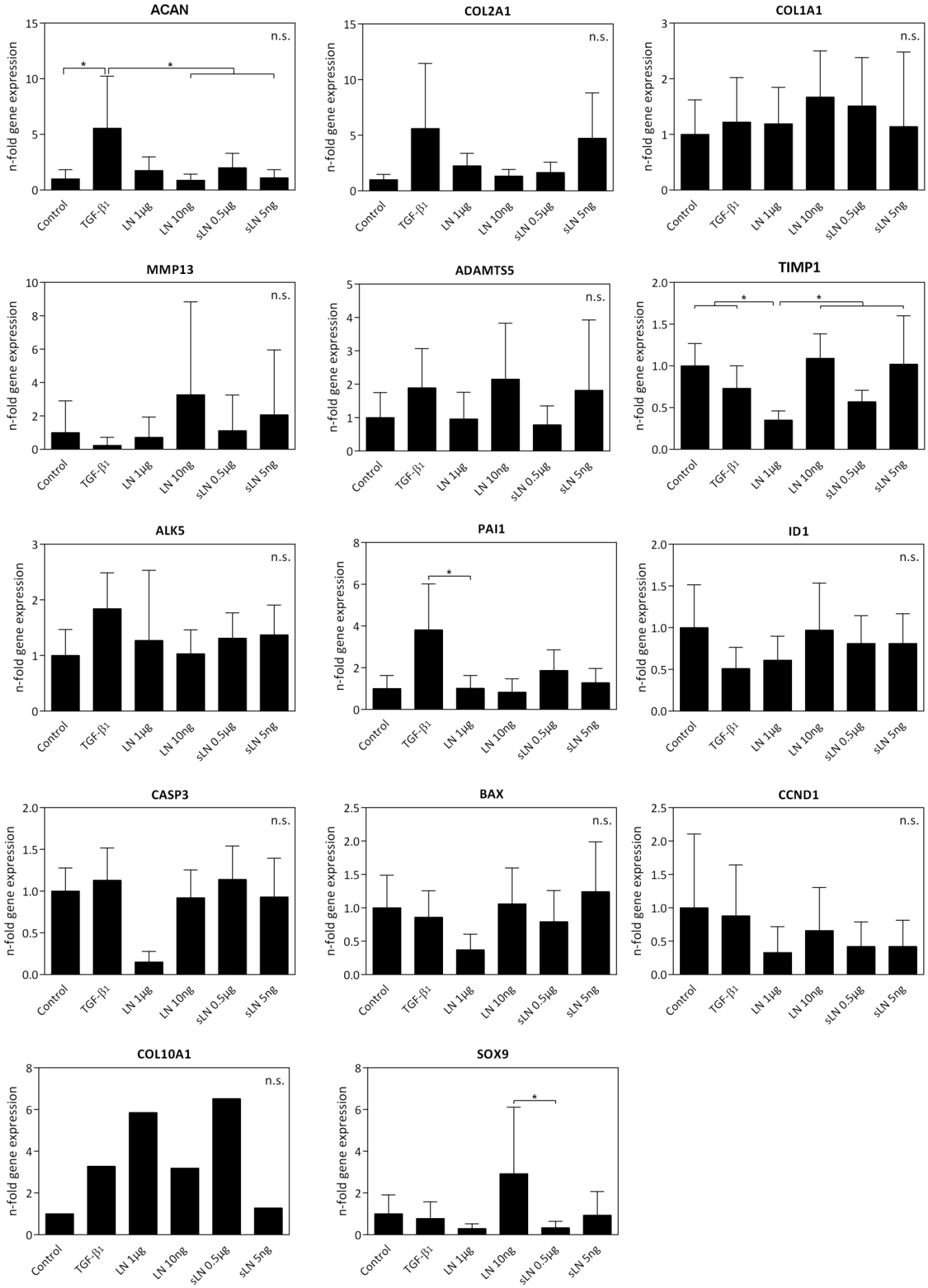

Fig. 9: No regenerative effects of (s)Link-N treatment on gene expression level were detected. Relative *ACAN, COL2A1, COL1A1, MMP13, ADAMTS5, TIMP1, ALK5, PAI1, ID1, CASP3, BAX, CCND1, COL10A1, SOX9* gene expression (mean + SD) of the micro-aggregates derived of human nucleus pulposus cells from degenerated intervertebral discs at day 7 of culture under normoxic conditions (21% O_2). The control micro-aggregates were set at 1. $n = 3$ *per* condition. ∗ $p < 0.05$.

EFFECT OF NX VS. HX CULTURE CONDITIONS

The O_2-saturation only affected the micro-aggregates that contained a low GAG and DNA content, like the untreated controls and the micro-aggregates treated with Link-N in low concentration. The micro-aggregates in those conditions showed a significantly higher GAG and DNA content when cultured in normoxia compared with the micro-aggregates in the same conditions cultured in hypoxia ($p < 0.05$; Fig. 7a, b). This was in contrast with the GAG release and total GAG production which were significantly higher in hypoxic culture conditions compared with normoxic culture conditions when treated with Link in low concentration ($p < 0.05$; Fig. 7d, f). In all the other conditions, no significant differences were detected between the GAG and DNA measurements of the micro-aggregates cultured in hypoxia vs. normoxia. Safranin O/Fast Green staining, IHC and RT-qPCR were only performed on micro-aggregates cultured in normoxic conditions, so the effect of the O_2-saturation on those results could not be determined.

BOVINE NPCS

EFFECTS OF (S)LINK-N ON THE NPC MIRO-AGGREGATES

Only the bovine NPC micro-aggregates treated with human (s)Link-N in high concentration showed a significantly increased DNA and GAG content compared with the untreated controls in both normoxic and hypoxic culture conditions ($p < 0.05$; Fig. 10a, b). The GAG and DNA content of these micro-aggregates were significantly higher than that of the ones treated with (s)Link in low concentration ($p < 0.05$; Fig. 10a, b). When the GAG content was corrected for the DNA content, the micro-aggregates treated with (s)Link-N in high concentration still demonstrated a significantly increased GAG content when cultured in normoxia ($p < 0.05$; Fig. 10d). In hypoxic conditions, all (s)Link-N treatments resulted in a significantly higher GAG content corrected for the DNA content compared with the untreated controls ($p < 0.05$; Fig. 10d). Treatment with (s)Link-N in high concentration resulted in a significantly higher total release of GAGs in the culture medium than the untreated control in both normoxia and hypoxia ($p < 0.05$; Fig. 10d). Furthermore, the micro-aggregates treated with (s)Link-N in high concentration had a significantly higher release of GAGs than when treated with (s)Link-N in low concentration (Nx) ($p < 0.05$; Fig. 10d). When the GAG release was corrected for the DNA content of the micro-aggregates, no significant effects of (s)Link-N were detected (Fig. 10e). The GAG production of the micro-aggregates and the GAG release in the medium taken together demonstrated that treatment with (s)Link-N in high concentration resulted in a higher total GAG production of the micro-aggregates compared with the untreated controls (Nx) ($p < 0.05$; Fig. 10f). The micro-aggregates treated with (s)Link-N in low concentration had a significantly lower total GAG production than the micro-aggregates treated with (s)Link-N in high concentration (Nx and Hx) ($p < 0.05$; Fig. 10f). However, when cultured under hypoxic conditions, all (s)Link-N conditions showed an increased total GAG production compared with the untreated controls ($p < 0.05$; Fig. 10f). When the total GAG production was corrected for the DNA content, no significant differences in the total GAG production between the different treatment groups were found (Fig. 10g).

In contrast with the results of the DMMB-assay, the samples stained with the Safranin O/Fast Green did not show a distinct difference in the amount of GAG deposition between the micro-aggregates in

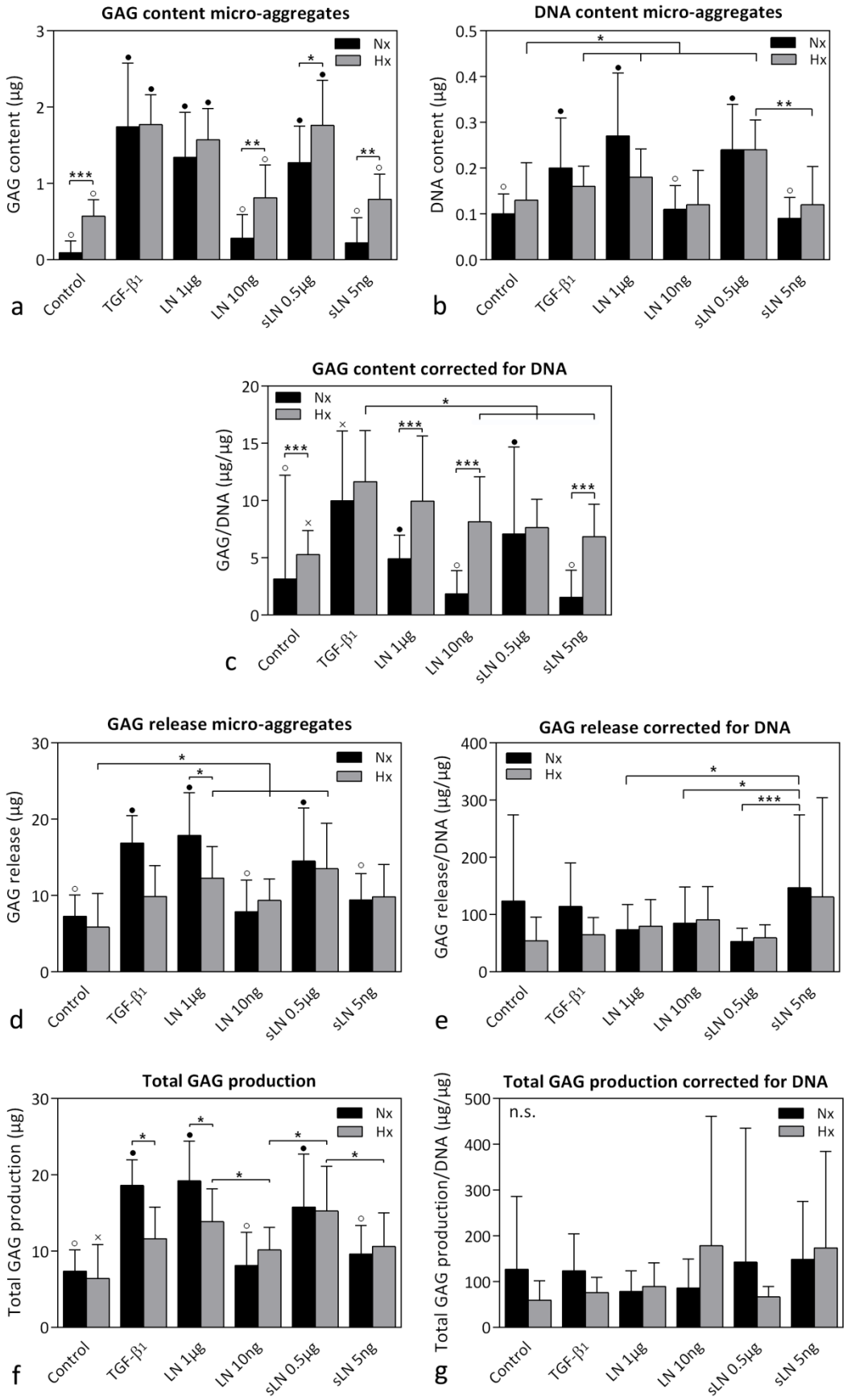

Fig. 10: (s)Link-N exerted concentration dependent regenerative effects on bovine nucleus pulposus cells (NPCs) when cultured in both normoxic and hypoxic conditions. GAG and DNA content (mean + SD) of micro-aggregates derived from bovine NPCs cultured in micro-aggregates for 28 days in normoxia (Nx, 21% O_2) and hypoxia (Hx, 5% O_2) in different conditions. (**a**) GAG content of the micro-aggregates. (**b**) DNA content of the micro-aggregates. (**c**) GAG content corrected for the DNA content. (**d**) Total amount of GAGs released in the culture medium by the micro-aggregates. (**e**) Total GAG release corrected for the DNA content. (**f**) Total GAG production (GAG content micro-aggregates and amount of GAGs released taken together). (**g**) Total GAG production corrected for the DNA content. n = 6 *per* condition. ∗p < 0.05, ∗∗ p < 0.01, ∗∗∗ p < 0.001. ● and ○ : significantly different (p < 0.05) from the other conditions in Nx or Hx but the bars with the same symbol. ×: significantly different (p < 0.05) from all other conditions (growth factor treatment) in the same group (Hx or Nx).

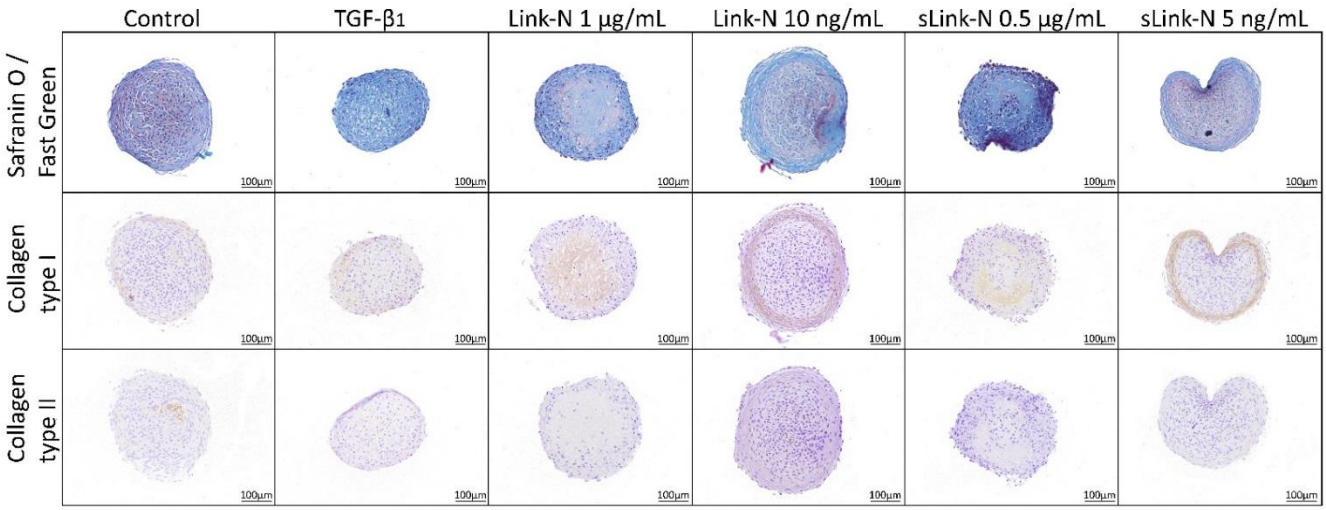

Fig. 11: Representative histological images of bovine nucleus pulposus cells (NPCs) cultured in micro-aggregates in normoxia (21% O_2) for 28 days stained with Safranin O/Fast green and immunohistochemical staining for collagen type I and II. The Safranin O/Fast Green staining showed no clear difference in GAG deposition between the micro-aggregates in the different conditions. Treatment with (s)Link-N in high concentration increased collagen type I protein deposition in the middle of the micro-aggregates, whereas the treatment with (s)Link-N in low concentration increased the collagen type I protein at the rim. Collagen type II protein expression was not affected by treatment with TGF-β_1 or (s)Link-N.

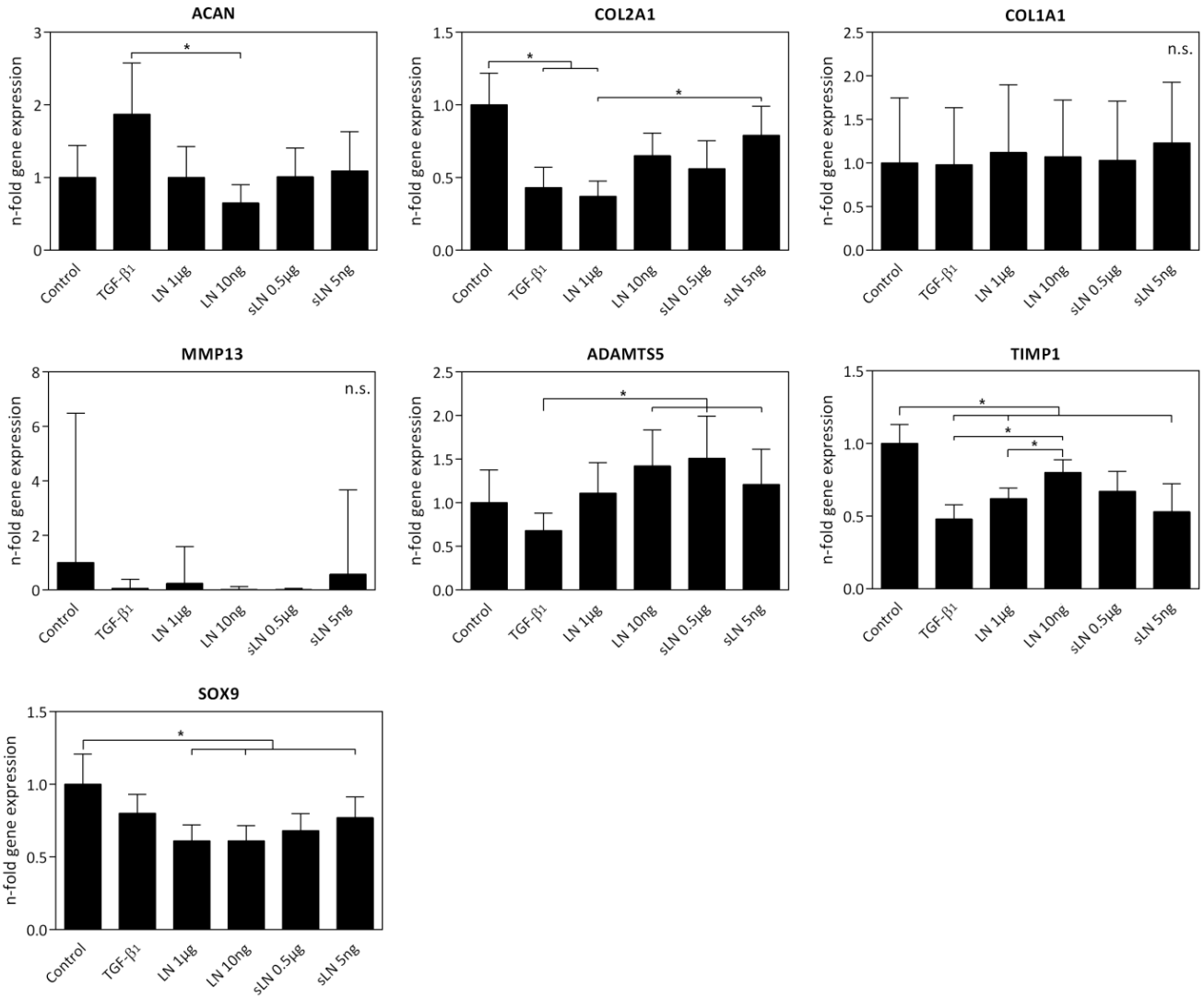

Fig. 12: No regenerative effects of (s)Link-N on gene expression level were detected. Relative *ACAN, COL2A1, COL1A1, MMP13, ADAMTS5, TIMP1, SOX9* gene expression (mean + SD) of the micro-aggregates derived of bovine nucleus pulposus cells at day 7 of culture under normoxic conditions (21% O_2). The control micro-aggregates were set at 1. $n = 6$ *per* condition. ∗ $p < 0.05$.

the different treatment groups (Fig. 11). Slightly increased GAG staining was present in the micro-aggregates of the untreated control group and the ones treated with (s)Link-N in low concentration compared with the micro-aggregates in the other conditions (Fig. 11). A fibrotic rim was detected around the micro-aggregates treated with (s)Link-N in low concentration and in line with these results, IHC showed a rim of collagen type I in these micro-aggregates (Fig. 11). The micro-aggregates treated with (s)Link-N in high concentration showed some collagen type I protein deposition in the middle (Fig. 11). Overall, the deposition of collagen type I protein was increased by the treatment with (s)Link-N compared with the untreated controls, whereas the production of collagen type II was not affected (Fig. 11). Non-upregulated expression of *COL2A1* by (s)Link-N treatment at day 7 confirmed the results of the IHC, whereas the non-significant upregulation of *COL1A1* was in contrast with the results of the IHC (Fig. 12). Contradictory with the results of the DMMB-assay and Safranin O/Fast Green staining, *ACAN* gene expression at day 7 was not significantly up-regulated by (s)Link-N treatment compared with the untreated controls (Fig. 12). Furthermore, no significant differences were encountered for gene expression of *MMP13* and *ADAMTS5* (ECM degradation markers) and neither for the expression of *TIMP1* (anti-catabolic gene) and *SOX9* (marker of chondrogenesis) (Fig. 12). Expression of *COL10A1* (marker of hypertrophic differentiation) was below the detection limit.

There were no significant differences encountered between the effects exerted by Link-N and sLink-N in comparable concentration, indicating that they both have a comparable potent effect on bovine NPCs.

EFFECT OF NX VS HX. CULTURE CONDITIONS

The O_2-saturation only affected the GAG content of the untreated micro-aggregates and the ones treated with (s)Link-N in low concentration. The micro-aggregates in those conditions demonstrated a significantly higher GAG content, both corrected and not corrected for the DNA content, when cultured under hypoxic conditions compared with that of the micro-aggregates cultured under normoxic conditions ($p < 0.01$; Fig. 10a, c). The DNA content, GAG release and total GAG production showed to be not affected by the different O_2-saturation. Safranin O/Fast Green staining, IHC and RT-qPCR were only performed on micro-aggregates cultured in normoxic conditions, so the effect of the O_2-saturation on those results could not be determined.

CANINE NPCS

EFFECTS OF (S)LINK-N ON THE NPC MICRO-AGGREGATES

Under normoxic conditions, the DNA content of the canine NPC micro-aggregates treated with human (s)Link-N was significantly higher compared with the untreated controls after 28 days ($p < 0.05$; Fig. 13b). TGF-β_1 treatment resulted in a significantly higher DNA content compared with all the other conditions ($p < 0.01$; Fig. 13b). When cultured under hypoxic conditions, no proliferative effect of (s)Link-N was found, whereas TGF-β_1 again significantly increased the DNA content compared with all the other conditions ($p < 0.001$; Fig. 13b). The GAG content of the micro-aggregates treated with (s)Link-N in both concentration was significantly higher after 28 days of treatment compared with that of the untreated controls cultured, both corrected and not corrected for the DNA content (Nx) ($p < 0.01$; Fig. 13a, c). There were no significant differences between the effect of the high compared to low concentration of (s)Link-N (Fig. 13). The addition of TGF-β_1 resulted in the highest GAG

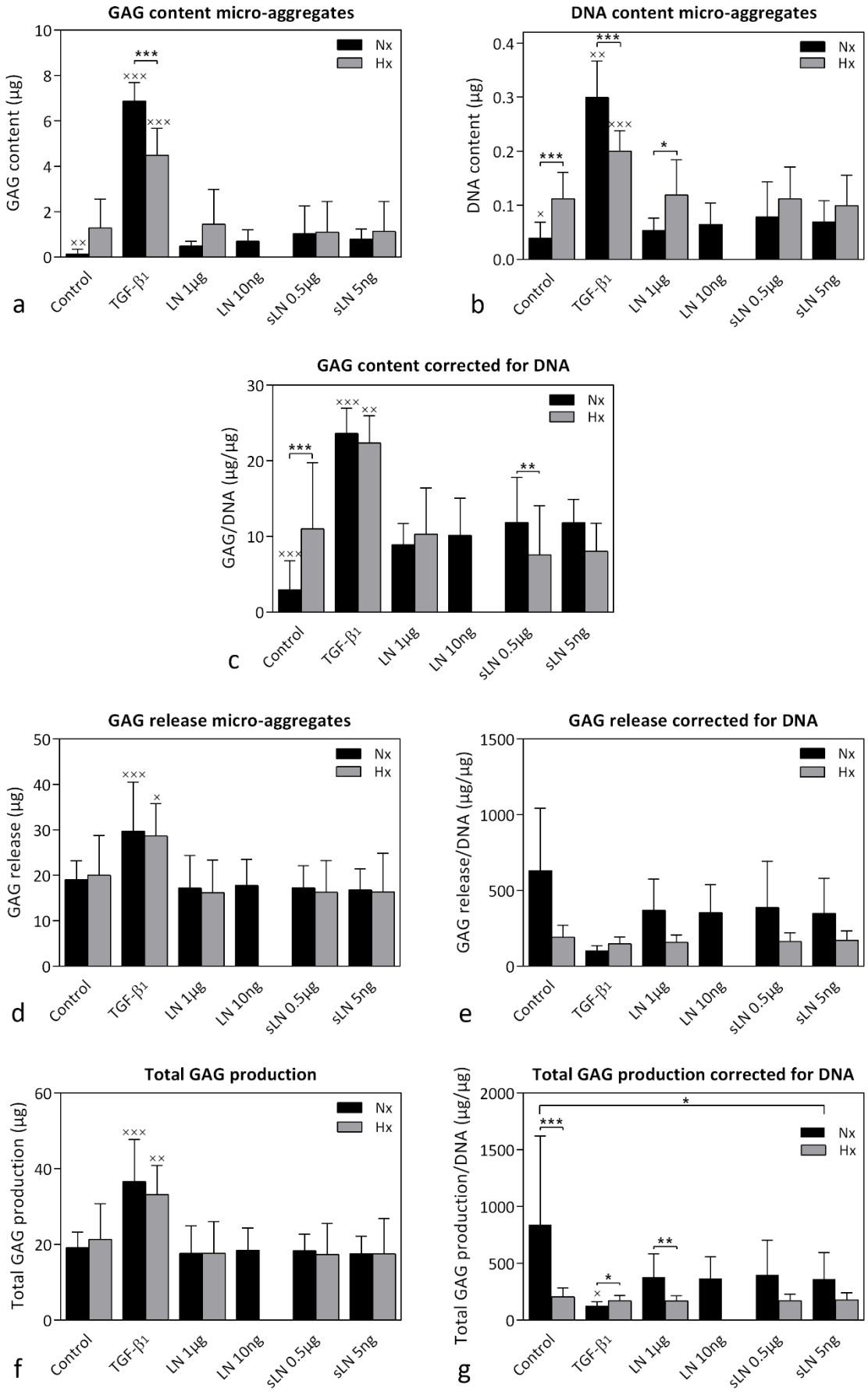

Fig. 13: (s)Link-N exerted a mild, concentration independent, regenerative effects on canine nucleus pulposus cells (NPCs) when cultured under normoxic conditions. GAG and DNA content (mean + SD) of micro-aggregates derived from canine NPCs derived from degenerated intervertebral discs cultured in micro-aggregates for 28 days in normoxia (Nx, 21% O_2) and hypoxia (Hx, 5% O_2) in different conditions. (**a**) GAG content of the micro-aggregates. (**b**) DNA content of the micro-aggregates. (**c**) GAG content corrected for the DNA content. (**d**) Total amount of GAGs released in the culture medium by the micro-aggregates. (**e**) Total GAG release corrected for the DNA content. (**f**) Total GAG production (GAG content micro-aggregates and amount of GAGs released taken together). (**g**) Total GAG production corrected for the DNA content. $n = 6$ *per* condition. ∗ $p < 0.05$, ∗∗ $p < 0.01$, ∗∗∗ $p < 0.001$. ×, ××, ×××: significantly different (respectively $p < 0.05$, $p < 0.01$, $p < 0.001$) from all other conditions (growth factor treatment) in the same group (Hx or Nx).

content compared with all the other conditions, both corrected ($p < 0.001$) and not corrected ($p < 0.01$) for the DNA content (Fig. 13a, c). No significant effect of (s)Link-N treatment on the GAG content was found when the cells were cultured under hypoxic conditions, whereas TGF-β_1 treatment significantly increased the GAG content compared with all the other conditions both corrected and not corrected for the DNA content ($p < 0.01$; Fig. 13a, c). The release of GAGs in the medium and total GAG production of the micro-aggregates were not influenced by the supplementation of (s)Link-N in both normoxic or hypoxic conditions (Fig. 13d, e, f, g).

In line with the results of the DMMB-assay, the Safranin O/Fast Green staining of the micro-aggregates cultured in normoxia treated with (s)Link-N in both concentrations showed to have more GAGs deposited than the untreated controls (Fig. 13a, Fig. 14). Under hypoxic cultured conditions, no increased GAG deposition by (s)Link-N was detected with the Safranin O/Fast Green staining, which was also in line with the results of the DMMB-assay. Again we encountered that addition of TGF-β_1 resulted in the most GAG deposition and the formation of a fibrotic rim around the micro-aggregates (Nx and Hx) (Fig. 14). No clear differences in the deposition of collagen type I and II protein deposition were found between all the conditions (Nx and Hx) (Fig. 14). Since no significant regenerative effects of the treatment with (s)Link-N on gene expression level of all the tested genes were detected in hypoxic conditions (Fig. 16), only the results found in normoxia will be elaborated further (Fig. 15). In contrast with the results of the DMMB-assay and Safranin O/Fast Green staining

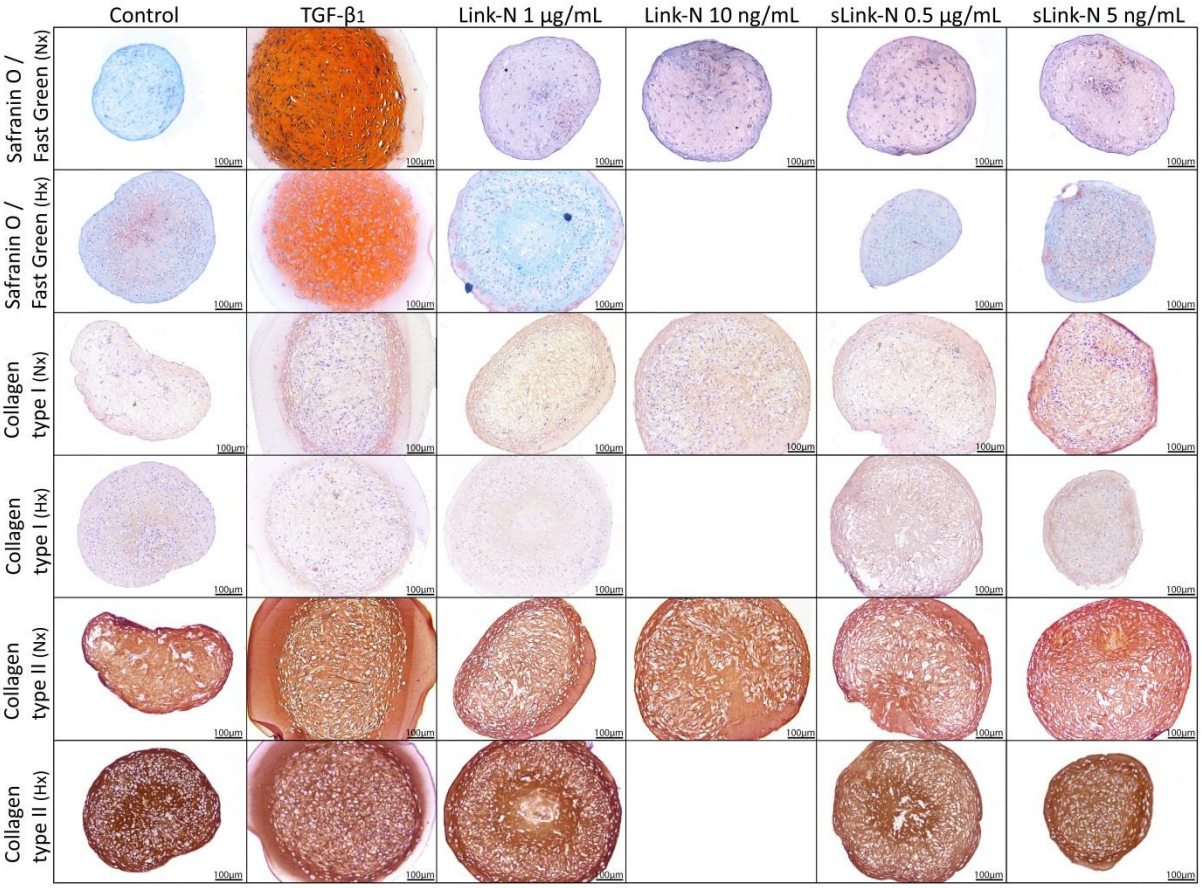

Fig. 14: Representative histological images of canine nucleus pulposus cells (NPCs) cultured in micro-aggregates in normoxia (Nx, 21% O_2) and hypoxia (Hx, 5% O_2) for 28 days stained with Safranin O/Fast green and immunohistochemical staining for collagen type I and II. The Safranin O/Fast Green staining showed an increased GAG deposition in the NPC micro-aggregates treated with (s)Link-N compared with the untreated controls in Nx. The untreated control cultured in hypoxia deposited more GAGs compared with the untreated control micro-aggregates in Nx. TGF-β_1 showed to have the most potent effect on GAG deposition in both Nx and Hx. No clear difference in the expression of collagen type I and II protein between the different conditions was detected.

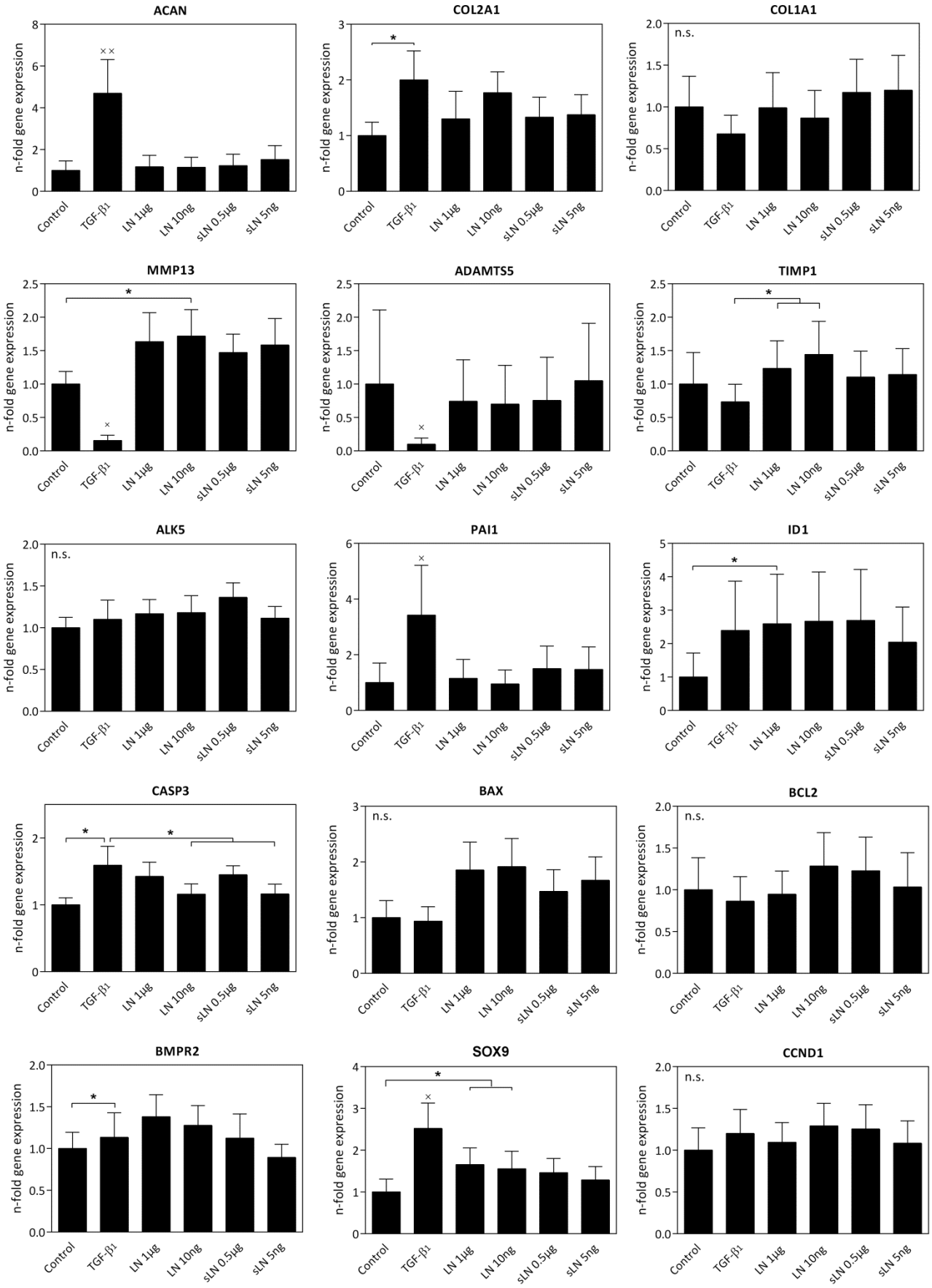

Fig. 15: No effects of (s)Link-N on gene expression level were detected, except from the increased *ID* and *SOX9* expression by Link-N compared with the untreated controls. Relative *ACAN, COL2A1, COL1A1, MMP13, ADAMTS5, TIMP1, ALK5, PAI1, ID1, CASP3, BAX, BCL2, BMPR2, SOX9, CCND1* gene expression (mean + SD) of the micro-aggregates derived of canine nucleus pulposus cells derived from degenerated intervertebral discs at day 7 of culture under normoxic conditions (21% O_2). The control micro-aggregates were set at 1. $n = 6$ *per* condition. ∗ $p < 0.05$. ×, ×× : significantly different (respectively $p < 0.05$ and $p < 0.01$) from all other conditions.

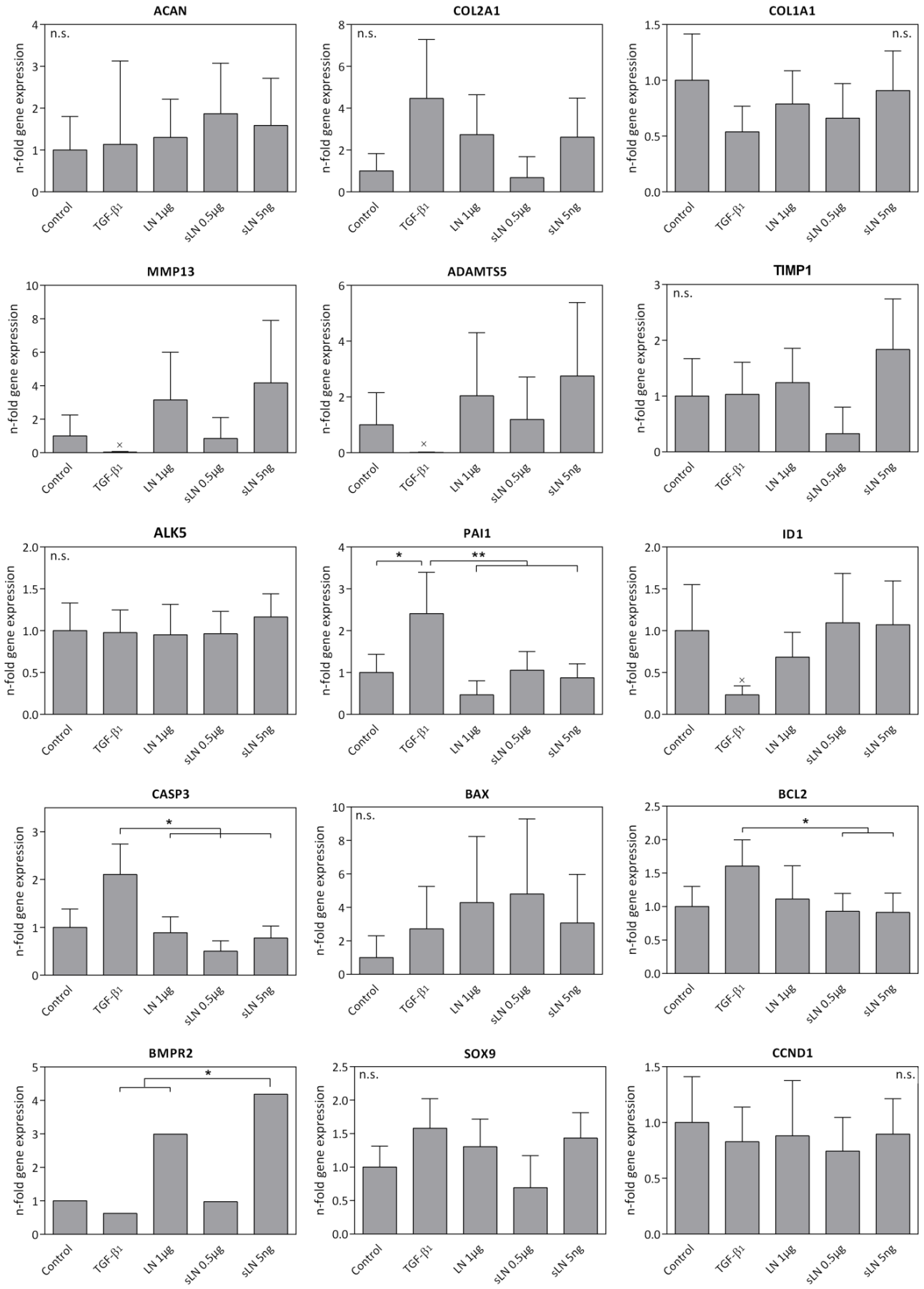

Fig. 16: No regenerative effects of (s)Link-N on gene expression level were detected on canine nucleus pulposus cells (NPCs) cultured under hypoxic conditions. Relative *ACAN, COL2A1, COL1A1, MMP13, ADAMTS5, TIMP1, ALK5, PAI1, ID1, CASP3, BAX, BCL2, BMPR2, SOX9, CCND1* gene expression (mean + SD) of the micro-aggregates derived of canine NPCs derived from degenerated intervertebral discs at day 7 of culture under hypoxic conditions (5% O_2). The control micro-aggregates were set at 1. $n = 6$ *per* condition. ∗ $p < 0.05$, ∗∗ $p < 0.01$. × : significantly different ($p < 0.05$) from all other conditions.

at day 28, *ACAN* gene expression was not upregulated at day 7 (Fig. 13, Fig. 15). In line with the immunohistochemical staining for collagen type I and II protein, there were no differences detected in *COL1A1* and *COL2A1* gene expression at day 7 (Fig. 14, Fig. 15). No significant downregulation in the expression of the catabolic genes *ADAMTS5* and *MMP13* or upregulation of anti-catabolic gene *TIMP1* by (s)Link-N was found (Fig. 15). Additionally, treatment with (s)Link-N did not result in a decreased expression of apoptosis markers *CASP3* and *BAX* or increased expression of anti-apoptosis marker *BCL2* and proliferation marker *CCND1* (Fig. 15). *SOX9* gene expression was significantly higher in the micro-aggregates treated with Link-N in both concentrations, whereas no significant upregulation of the *BMPR2* gene in the micro-aggregates was encountered (Fig. 15). *ALK5* and *PAI1* gene expression were not increased by (s)Link-N treatment, which indicates no increased activation of TGF-β / SMAD2/3 signaling at least at gene expression level (Fig. 15). The expression level of *ID1* was significantly increased when the micro-aggregates were treated with Link-N in high concentration, which indicates activated BMP / SMAD1/5/8 signaling (Fig. 15). Gene expression of *ALK1* (BMP receptor type I) and *COL10A1* (marker of hypertrophy) were both below the detection level.

There were no significant differences encountered between the effects exerted by Link-N and sLink-N in comparable concentration, indicating that they both had a comparable potent effect on canine NPCs.

EFFECT OF NX VS. HX CULTURE CONDITIONS

The untreated controls had a higher DNA and GAG content (corrected for the DNA content), when cultured under hypoxic conditions compared with the micro-aggregates cultured under normoxic conditions ($p < 0.001$; Fig. 13b, c). This was in contrast with the DNA and GAG content of the micro-aggregates treated with TGF-β_1, which demonstrated to be significantly higher when cultured in normoxia compared with hypoxia ($p < 0.001$; Fig. 13a, b). In line with these results, the Safranin O/Fast Green staining showed that the untreated controls deposited more GAGs in hypoxic conditions compared with the ones cultured in normoxia. And furthermore, this staining showed and a more intense GAG staining in the micro-aggregates treated with TGF-β_1 in Nx than Hx. The different O_2-saturation had no effect on the GAG release and total GAG production of the micro-aggregates in all the conditions. IHC showed slightly more collagen type II deposition by the NPC micro-aggregates of all conditions cultured in hypoxia compared with the ones in normoxia.

DISCUSSION

Human Link-N has already shown promising potential for inducing IVD regeneration in different species, including human and bovine IVDs.[38, 41, 46] Firstly, this study sought to determine whether the regenerative effects of Link-N on human and bovine NPCs were reproducible and thus whether our batch of Link-N was effective in the 3D NPC micro-aggregate culture system used in the current study. Since the effect of human Link-N on canine NPCs had never been investigated, the present study was the first to test the regenerative potential of Link-N and sLink-N on NPCs derived from degenerated canine IVDs. The ultimate goal was to determine the potential of (s)Link-N to be used as a future treatment for IVD degeneration in canine patients, but also to determine whether the dog could serve as a suitable large animal model to translate this treatment into human patients.

NPCS PERSEVERE BETTER IN HYPOXIC CULTURE CONDITIONS

Although it has been demonstrated that human degenerative NPCs isolated, expanded, and cultivated in hypoxic conditions could better preserve the cells' regenerative potential[49], in the current study the regenerative response of the human, bovine and canine NPCs to (s)Link-N treatment appeared to be not more potent when cultured under hypoxic compared with normoxic conditions. However, in contrast with human NPCs, the untreated bovine and canine NPCs and the ones treated with (s)Link-N in low concentration had a significantly higher DNA and GAG content when cultured in hypoxia compared with normoxia. This may imply that bovine and canine NPCs persevere better in hypoxic culture conditions, but that the O_2-saturation does not influence their potency to react to (s)Link-N treatment. Since the IVD is an avascular hypoxic structure[50], *in vitro* experiments performed in hypoxic conditions mimic the *in vivo* situation more closely and will therefore facilitate translation into clinical practice.

LINK-N AND SLINK-N SHOW A COMPARABLE REGENERATIVE POTENCY

No significant differences were detected between the effect of Link-N compared with the treatment of sLink-N, indicating that the 8 amino acid structure has an as potent effect as the 16 amino acid structure. This is in line with the results of the study of Gawri *et al.* (2014) which revealed that sLink-N significantly induced proteoglycan production by NP and AF cells to a similar extent as full length Link-N in the presence of the cytokine interleukin-1β. This implied that the biological effect is maintained in the first 8 amino acids of the peptide and indicates that the effect is sustained in an inflammatory environment.[45] Production costs of sLink-N will be even lower than full length Link-N, but of more importance: this smaller structure is beneficial for medicinal chemists, since it is more amenable for optimizing the biological activity and stability.[45]

(S)LINK-N EXERTS REGENERATIVE EFFECTS ON HUMAN AND BOVINE NPCS

The results of the present study reveal that the GAG deposition of the micro-aggregates derived from both human and bovine NPCs was stimulated by the addition of 1 µg/mL Link-N and 0.5 µg/mL sLink-N. This effect was not detected when Link-N and sLink-N were added in the lower concentration, so this indicated a concentration-dependent effect. This confirms the already determined optimal concentration of Link-N by Mwale *et al.* (2014) for isolated bovine disc cells.[38] Gwari *et al.* (2014) also used Link-N and sLink-N in a concentration of 1 µg/mL and 0.5 µg/mL respectively to determine the effect of Link-N, reverse Link-N and sLink-N on human disc cells.[45] However, in a study performed by Petit *et al.* (2011), a concentration of 100 ng/mL Link-N was used and resulted in significant increased collagen type I and II expression of human NP and AF cells.[46] Since a concentration of 100 ng/mL was not used in this study, we cannot exclude that this concentration would also have resulted in a potent regenerative effect. Altogether, the results of the present study showed comparable regenerative effects of (s)Link-N on GAG deposition of human and bovine NPCs as the aforementioned studies, which confirms the validity of the used Link-N batch and 3D micro-aggregate culture system.

In contrast with the quantitative GAG measurements determined using the DMMB assay, GAG deposition imaged by the Safranin O/Fast Green staining of the bovine NPC micro-aggregates showed no distinct differences between the treatment groups. An explanation for this discrepancy is

that the Safranin O/Fast Green staining is a semi-quantitative staining and is considered to be not very sensitive.[51] The GAG deposition induced by (s)Link-N treatment was lower in bovine (± 1.3 µg; Fig. 7a) than in human NPCs (± 2.0 µg; Fig. 10a), thus it could be that the Safranin O/Fast Green staining was not sensitive enough to detect this. Although GAG production of the human and bovine NPCs was significantly increased at day 28 by treatment with (s)Link-N in high concentration, *ACAN* expression at day 7 was not increased. This, together with the other non-significant effects of Link-N at gene expression level, indicates that changes in gene expression were presumably missed at day 7. So future studies should take more time points into account to determine the optimal timing for gene expression profiling.

Collagen type I, but not type II, deposition by the human NPC micro-aggregates was prominently increased by the addition of TGF-β_1 and (s)Link-N in high concentration. This is an already known effect of TGF-β_1 on NPCs[52], and the study by Petit *et al.* (2011) also showed that Link-N increased the collagen type I expression in human NPCs.[46, 53] In contrast, TGF-β_1 treatment did not induce collagen type I and II deposition of bovine NPCs. The study of Zimber *et al*. (1995) showed that TGF-β treatment of bovine chondrocytes led to a substantial increase in GAG synthesis and cell proliferation, but exerted no detectable effects on collagen type I and II as well.[54] The non-increased collagen type II deposition by (s)Link-N treatment was unexpected and in contrast with other studies, which did report increased collagen type II expression in both human and bovine NPCs treated with Link-N.[43, 46] These studies, however, determined collagen type II expression of human and bovine NPCs when cultured under hypoxic conditions in contrast with the current study which determined collagen type II expression in normoxic culture conditions. So future studies, comparing the collagen type II deposition in both normoxic and hypoxic culture conditions, should determine the influence of the O_2 level on collagen deposition by human and bovine NPCs. Another study in which bovine NPCs were cultured in hydrogel, treatment with Link-N also induced prominent collagen type II deposition, which implies that bovine NPCs reside better in hydrogels (unpublished *in vitro* data by the Orthopedics research group, Faculty of Veterinary Medicine, Utrecht University). Hydrogel culture of the same bovine NPC donors in comparable culture conditions should confirm/reject this hypothesis.

There is presumptive evidence that Link-N exerts its effect on rabbit IVD cells by binding to the BMP receptor type II, thereby increasing the activating of SMAD1/5/8, promoting the expression of *SOX9*, and inducing downstream production of both GAGs and collagen type II.[40] The results of the present study show that (s)Link-N treatment increased GAG and collagen type I deposition of human and bovine NPCs, but no evident collagen type II deposition was present. Interestingly, the (s)Link-N effects on human and bovine NPCs closely resembled the effects of TGF-β_1 treatment. Thus, whether (s)Link-N exerts its effect by binding to the BMP receptor type II and activates SMAD1/5/8 signaling of human and bovine NPCs as well, or that it has an effect on the TGF-β_1 pathway (SMAD2/3 signaling) as well remains to be elucidated.

(S)LINK-N EXTERTS NO RELEVANT REGENERATIVE EFFECTS ON CANINE NPCS

Treatment with (s)Link-N resulted in a mild, but significant, concentration independent increase in GAG and DNA content of the canine NPC micro-aggregates, when cultured under normoxic conditions. However, the release of GAGs and total GAG production was not affected by the treatment with (s)Link-N. It is debatable whether this mild regenerative effect exerted by (s)Link-N is

biologically relevant, since it does by far not reach the anabolic effect level of TGF-β_1 treatment. However, it must be mentioned that canine NPCs, for yet unknown reasons, respond more strongly to treatment with TGF-β_1 compared with human and bovine NPCs. When the canine NPCs were cultured under hypoxic conditions, no regenerative effects of (s)Link-N were encountered anymore, mainly due to better performing untreated controls that produced more GAGs. The reason for this mild/absent response of the canine NPCs to treatment with (s)Link-N remains to be elucidated . As stated above, the detected regenerative effects of (s)Link-N on the human and bovine NPCs, cultured in the same culture system, confirm the validity of the Link-N batch and 3D micro-aggregate culture system used in the current study. In addition, the canine NPC micro-aggregates did respond to TGF-β_1 treatment. Thus, the used culture system could likely be excluded as reason for the moderate effect of (s)Link-N on the canine NPCs. In normoxic conditions, an increased expression of *ID1* and *SOX9* at day 7 was detected after Link-N treatment. This implies that Link-N activated the BMP signaling pathway in the canine NPCs which resulted in increased *SOX9* expression. This enhanced expression detected at day 7, however, resulted in only a moderate effect on GAG and collagen type II protein levels at day 28. A possible explanation is that (s)Link-N, beside the BMP receptor, interacts with other receptors that are not elucidated yet, and that those receptors are not present on canine NPCs. Another theory could be that the optimal concentration of (s)Link-N was not reached for the canine NPCs. This latter, however, is not very likely since a preliminary study of Mwale *et al.* demonstrated that 10 Link-N ng/mL was the optimal concentration for the stimulation of ECM production in canine NPCs cultured in 2D monolayers for 7 days (Unpublished *in vitro* data Orthopedics research group, Faculty of Medicine, McGill University). Additionally, no concentration-dependent effect of (s)Link-N treatment on canine NPCs was detected in the current study. Another possibility is that (s)Link-N is degraded more rapidly in canine NPCs compared with human and bovine NPCs. Since there is not much know about the activity of MMPs in canine IVDs, it is possible that canine NPCs produce MMPs that cleave (s)Link-N in biologically inactive fragments.[2]

Altogether, optimization of (s)Link-N as treatment strategy for canine patients with back pain due to IVD degeneration is necessary. It is reported that mesenchymal stromal cells (MSCs) exert regenerative effects in co-culture with NPCs.[55] The MSCs presumably secrete trophic factors and can function as cell-supplement.[38] Furthermore, Link-N showed to enhance chondrogenic differentiation of human MSCs with enhanced ECM production and inhibited hypertrophy.[56] Although it remains to be elucidated whether human Link-N exerts these effects on canine MSCs as well, the combined treatment of human Link-N and canine MSCs seems like a promising approach to increase the regenerative potency of (s)Link-N on the canine NPCs.

LIMITATIONS

The first limitation of this study is that different passages of NPCs were used, varying from passage 1 to 4, which could hamper comparison of results. Cell expansion for one or more passages is likely to induce dedifferentiation of the NPCs, a well-known result of expanding articular chondrocytes.[57] Since dedifferentiated NPCs could respond differently from recently isolated NPCs, future studies should determine the effect of (s)Link-N on NPCs of passage 0 as well. Additionally, in this study the effect of Link-N was tested on NPCs of just one kind of dog breed, namely the Beagle (a chondrodystrophic breed). To successfully develop this new treatment strategy for canine patients, the effect of Link-N on non-chondrodystrophic dogs should also be delineated. It remains to be

elucidated whether (s)Link-N treatment also affects other (symptom modifying) factors than the ones measured in the present study, *e.g.* inflammatory, neurogenic or angiogenic markers. Lastly, in future studies it should be determined whether (s)Link-N treatment results in better preservation of the NPC phenotype than control or TGF-β_1 treatment, *e.g.* by determining species-specific NPC markers.[58]

CONCLUSIONS

In conclusion, the present study showed that both human Link-N and sLink-N stimulated ECM production of human and bovine NPCs, indicating that (s)Link-N exerted regenerative effects on the NPCs in our micro-aggregate culture model. This implies that the results of other studies were reproducible and that our batch of (s)Link-N was functional. Human (s)Link-N exerted a statistically significant, but not biologically relevant, regenerative effect on canine NPCs, suggesting that there are species differences in the response of NPCs to (s)Link-N treatment. The potency of human (s)Link-N as treatment for IVD degeneration in canine patients is debatable, and so is the use of the dog as large animal model for this treatment. Therefore, further research focusing on optimizing this treatment strategy for canine patients is necessary. The addition of canine MSCs to the treatment with (s)Link-N, functioning as cell supplement and additional trophic factor, seems to be a promising approach.

CHAPTER 2: THE REGENERATIVE EFFECTS OF HUMAN LINK-N AND CANINE MESENCHYMAL STEM CELLS ON CANINE NUCLEUS PULPOSUS CELLS

SUMMARY

Background. Link-N and its shorter variant sLink-N have been shown to stimulate regeneration of degenerated intervertebral disc (IVD) cells of different species. However, our previous research showed that (s)Link-N alone exerted only a mild regenerative effect on canine NPCs. IVD repair can also potentially be enhanced by mesenchymal stromal cell (MSC) supplementation to maximize extracellular matrix (ECM) production. Link-N has been shown to inhibit osteogenesis and increase the chondrogenesis of human MSCs *in vitro*. So the combined treatment of MSCs, as cell supplement and as an extra source of trophic factor, and (s)Link-N could be considered as a promising new regenerative treatment strategy for IVD degeneration in canine patients.

Objectives. The focus of this study lays on delineating whether the combined treatment of human (s)Link-N and canine MSCs results in an optimal regenerative effect on canine nucleus pulposus cells (NPCs). Furthermore we aimed at determining the survival rate of MSCs in a micro-aggregate co-culture system with NPCs.

Methods. NPCs and MSCs from Beagle donors were cultured in both NPC and NPC:MSC (50:50) micro-aggregates (35,000 cells) for 28 days with and without human Link-N (1 µg/mL) or sLink-N (5 ng/mL and 0.5 µg/mL). ECM production, cell proliferation, and MSC survival were determined by gene expression profiling, histological evaluation and measuring DNA and GAG content.

Results. Human (s)Link-N treatment alone resulted in no increased GAG content, GAG release and total GAG production of the canine NPC and NPC:MSC micro-aggregates, nor an increase in collagen type I and II protein deposition compared with untreated controls. Addition of the MSCs to the treatment with (s)Link-N did not result in an increased GAG and DNA content, GAG release or total GAG production. Collagen type I deposition was prominently increased by the addition of MSCs regardless the treatment, whereas collagen type II protein deposition was not affected. After 28 days, the amount of (un)differentiated MSCs in all the conditions was around 11%, regardless the treatment.

Conclusions. The addition of canine MSCs to the treatment with human (s)Link-N resulted in no additive regenerative effects on canine NPCs *in vitro*: cell proliferation and ECM production were not increased by the treatment. The addition of MSCs resulted even in an undesired increased deposition of collagen type I. So human (s)Link-N treatment alone or in combination with canine MSCs exerted no regenerative effects on canine NPCs, implying that these treatment strategies are not suitable for dogs that suffer back pain due to from IVD degeneration. Species differences in the amino acid sequence of (s)Link-N could play a role is this, and therefore it would be interesting to investigate the regenerative effects of the canine variant of (s)Link-N on canine NPCs.

INTRODUCTION

During IVD (intervertebral disc) degeneration, increased matrix catabolism and a decreased synthesis of aggrecan and collagen type II occurs.[2] Additionally, this process is associated with cell loss through apoptosis.[2] Thus, to accomplish functional IVD restoration, it is necessary to increase extracellular matrix (ECM) production and cell proliferation. This can be stimulated using exogenous growth factors, such as transforming growth factor beta (TGF-β) and bone morphogenetic proteins (BMPs).[33, 34] Since growth factors are expensive to produce and potentially cause side-affects[35, 36], Link-N seems like a suitable alternative.[45] Growth factor-like stimulation alone may, however, be insufficient for IVD repair since cell viability is impaired and cell numbers are decreased in degenerated IVDs.[31] Therefore, a combined cell and growth factor-based therapy could provide a suitable approach to maximize ECM production by degenerated IVDs. However, which cells are suitable for this approach is not obvious, because autologous IVD cells cannot be harvested and the use of allogeneic disc cells is not favorable.[56] The use of mesenchymal stromal cells (MSCs) could provide the solution. These cells are multipotent stem cells and have the ability of self-renewal and differentiation into a variety of cells, including osteoblasts, chondrocytes, and adipocytes.[59] With their immunosuppressive properties and secretion of trophic factors, MSCs have profound potential to support IVD regeneration.[59]

The study of Antoniou *et al.* (2012) revealed that the human Link-N variant promoted the chondrogenic differentiation of human MSCs and retarded their hypertrophic and osteogenic differentiation.[56] The study, in which Link-N was used as a molecular agent and human MSCs as a cell supplement to treat bovine IVDs, demonstrated that Link-N and MSCs have the ability to restore the proteoglycan content separately but that no additional effect was detected by a combination of the two.[38]

Since our previous study revealed that human Link-N and its shorter variant sLink-N exerted no/mild regenerative effects on canine nucleus pulposus cells (NPCs), optimization of the treatment is needed. The combined treatment of (s)Link-N and MSC seems like a promising approach to accomplish this, but has only been tested on human and bovine NPCs. Therefore, this study aimed to determine the trophic effect of canine bone marrow derived MSCs in co-culture with NPCs and the additional regenerative effects of MSCs when combined with (s)Link-N treatment.

MATERIALS AND METHODS

HARVEST, ISOLATION AND CULTURE OF THE NPCS AND MSCS

Bone marrow derived MSCs from a female canine donor (Table 5) were readily available and were harvested, isolated and expanded as described by Malagola *et al.* (2016).[60] The harvest and isolation of the NPCs was performed following the same procedure as described in chapter 1.

NPCs from degenerated IVDs of 6 male canine donors (Table 5) were expanded in the same manner as described in chapter 1. After expansion, micro-aggregates of 35,000 cells were formed with NPCs and MSCs (ratio 50:50) and only NPCs using the same protocol as in chapter 1. The next day (day 0), chondrogenic culture medium was replaced with either basal culture medium for the negative control micro-aggregates, basal culture medium supplemented with 10 ng/mL human recombinant

TGF-β_1 (240-B, R&D Systems, Inc., Minneapolis, USA) for the positive control micro-aggregates, basal culture medium supplemented with 1 µg/mL human Link-N (DHLSDNYT-LDHDRAIH; CanPeptide, Montreal, Canada), or 0.5 µg/mL or 5 ng/mL human sLink-N (DHLSDNYT; CanPeptide, Montreal, Canada). The micro-aggregates were cultured for 28 days in hypoxic conditions (5% O_2, 5% CO_2, 37°C). Media were changed two times a week.

Table 5: Overview of the used donors in the study and their characteristics

Number	Gender	Cell type	Species	Thompson score	Age (years)
1399617	Male	NPC	Canine (Beagle)	III	4.5
7140151	Male	NPC	Canine (Beagle)	III	4.5
7283679	Male	NPC	Canine (Beagle)	III	2.5
7207859	Male	NPC	Canine (Beagle)	III	5.0
7200013	Male	NPC	Canine (Beagle)	III	5.0
1208156	Male	NPC	Canine (Beagle)	III	1.5
4317	Female	MSC	Canine (Beagle)	N.A.	2

GENE EXPRESSION PROFILING

RNA isolation, cDNA synthesis and the quantitative RT-PCR, determining the relative gene expression of the same genes, were performed following the same protocols as in chapter 1.

GAG AND DNA MEASUREMENTS

The GAG and DNA content were determined following similar protocols as described in chapter 1.

HISTOLOGY

Safranin O/Fast Green staining and immunohistochemistry for collagen type I and II protein was performed according to the same protocol as describe in chapter 1.

QUANTITATIVE *SRY/GAPDH* RT-PCR

To determine the fate of the MSCs after 28 days, the NPC (male):MSC (female) ratio was determined by *SRY:GAPDH* PCR. The genomic (g)DNA was isolated from the papain digested samples using the DNEasy Blood and tissue kit (69581, Qiagen). Next, the DNA was diluted 10 times and used for *SRY* or *GAPDH* qPCR (Table 6). The male DNA percentage of the samples was determined using a standard series with known female:male gDNA amounts. Finally, the amount of male DNA was interpolated from that of the known standard series using the quantitation cycle *SRY*/*GAPDH*.

Table 6: Overview of the primers used to determine gene expression of *GAPDH* and *SRY*

Genes	Forward sequence 5' → 3'	Reverse sequence 5' → 3'	Amplicon size	Annealing temp (°C)
Reference gene				
GAPDH	TGTCCCCACCCCCAATGTATC	CTCCGATGCCTGCTTCACTACCTT	100	58
Target gene				

SRY	TGAAAGCGGAGGAAACGGTA	TGCTGATCTCTGAGTTTTGCATTT	130	55.5

STATISTICAL ANALYSIS

For the statistical analysis, IBM SPSS statistics 22 was used. To check whether the data were normally distributed, a Shapiro Wilks test was performed. General linear regression models based on ANOVAs were used for normally distributed data, whereas a Kruskal Wallis and Mann-Whitney U test were performed on non-normally distributed data. To correct for multiple comparisons, all tests were followed by a Benjamini & Hochberg False Discovery Rate *post-hoc* test. *P*-values < 0.05 were considered significant.

RESULTS

EFFECTS OF (S)LINK-N TREATMENT ON NPCS

As already described in the previous study (chapter 1), human (s)Link-N alone exerted no regenerative effects on the canine NPC micro-aggregates when cultured under hypoxic conditions: no increased GAG and DNA content compared with the untreated NPC micro-aggregates were detected (Fig. 17a, b). As positive control, TGF-β_1 treatment resulted in a significantly increased DNA and GAG content (both corrected and not corrected for the DNA content) ($p < 0.01$; Fig. 17a, b, c). Furthermore, no effect of (s)Link-N on the GAG release and total GAG production of the NPC micro-aggregates was found, whereas treatment with TGF-β_1 again resulted in a significant increase compared with the untreated NPC control micro-aggregates ($p < 0.05$; Fig. 17d, f). In line with these results, the Safranin O/Fast Green staining showed no increased GAG deposition of the NPC micro-aggregates treated with (s)Link-N compared with the untreated NPC controls (Fig. 18). This staining also indicated that GAG deposition was most prominently present in the NPC micro-aggregates treated with TGF-β_1. IHC showed no clear effect of (s)Link-N treatment on collagen type I expression of the NPC micro-aggregates (Fig. 18). Additionally, no significant regenerative effects at gene expression level were detected of (s)Link-N treatment on the NPC micro-aggregates (Fig. 19).

EFFECTS OF (S)LINK-N TREATMENT ON CANINE MSCS

Treatment with human (s)Link-N did not exert positive effects on the NPC:MSC micro-aggregates: the NPC:MSC micro-aggregates treated with (s)Link-N did not have an increased DNA content compared with the untreated NPC:MCS micro-aggregates and the GAG content of the NPC:MSC micro-aggregates treated with (s)Link-N was even significantly lower than that of the untreated NPC:MSC micro-aggregates ($p < 0.05$; Fig. 17a). Furthermore, (s)Link-N treatment did not result in an increased GAG release or total GAG production of the NPC:MSC micro-aggregates compared with the untreated NPC:MSC micro-aggregates (Fig. 17d, e, f, g). Histological evaluation showed no increased GAG, collagen type I or II deposition by the addition of (s)Link-N to the NPC:MSC micro-aggregates as well (Fig. 18). Furthermore, (s)Link-N treatment did not result in increased *SOX9* (marker of chondrogenic differentiation) expression of the NPC:MSC micro-aggregates at day 7 (Fig. 19). In line with the aforementioned results, no beneficial effects on the expression of the other tested genes exerted by (s)Link-N treatment on the NPC:MSC micro-aggregates were detected (Fig. 19). In contrast, the addition of TGF-β_1 did result in a significant increase in GAG content, DNA

content (both corrected and not corrected for the DNA content), GAG release and total GAG production of the NPC:MSC micro-aggregates compared to the NPC:MSC micro-aggregates in all the other conditions ($p < 0.01$; Fig. 17a, b, c, d, f), which was confirmed by the Safranin O/Fast Green staining (Fig. 18). Additionally, TGF-β_1 treatment downregulated gene expression of matrix metalloproteinase *MMP13* expression of the NPC:MSC micro-aggregates compared with the untreated NPC:MSC controls ($p < 0.01$; Fig. 19).

EFECTS OF CANINE MSCS ON UNTREATED NPCS

No regenerative effects of the potential trophic factors secreted by the MSCs were detected since the DNA and GAG content, GAG release and total GAG production were significantly higher in the untreated NPC micro-aggregates compared with the untreated NPC:MSC micro-aggregates ($p < 0.05$; Fig. 17a, b, d, f). These findings were confirmed by the histological evaluation, which showed no increased GAG and/or collagen type II deposition in the untreated NPC:MSC micro-aggregates compared with the untreated NPC micro-aggregates (Fig. 18). Additionally, the size of the untreated NPC:MSC micro-aggregates appeared to be smaller than that of the untreated NPC micro-aggregates (Fig. 18). On gene expression level, only an undesired significant increase in expression of *COL1A1* and *MMP9* by the addition of MSCs to the untreated NPCs was encountered ($p < 0.001$ and $p < 0.05$ respectively; Fig. 19).

ADDITIONAL EFECTS OF MSCS ON (S)LINK-N TREATMENT

No additive regenerative effects of the addition of MSCs to the treatment with human (s)Link-N were detected: the GAG and DNA content of the NPC:MSC micro-aggregates treated with sLink-N were even significantly lower compared with those of the NPC micro-aggregates treated with (s)Link-N ($p < 0.05$; Fig. 17a, b). No additive effects of the MSCs were detected on the release of GAGs in the medium and total GAG production, since those were significantly higher in the NPC micro-aggregates compared with the NPC:MSC micro-aggregates treated with (s)Link-N ($p < 0.01$; Fig. 17d, f). The results of the Safranin O/Fast Green staining confirmed these findings, since the NPC micro-aggregates treated with sLink-N deposited more GAGs and had a bigger size compared with the NPC:MSC micro-aggregates in the same conditions (Fig. 18). Collagen type I deposition was prominently increased by the addition of MSCs regardless the condition (Fig. 18). The deposition of collagen type II appeared not very different between conditions (Fig. 18). In line with the results of the DMMB-assay, *ACAN* gene expression was significantly higher in the NPC micro-aggregates treated with sLink-N compared with the NPC:MSC micro-aggregates at day 7 ($p < 0.05$; Fig. 19). Confirming the IHC for collagen type I, gene expression of *COL1A1* was significantly higher in the NPC:MSC micro-aggregates compared with the NPC micro-aggregates in all conditions ($p < 0.01$; Fig. 19). No significant downregulation of the expression of catabolic genes *ADAMTS5*, *MMP9* and *MMP13* or upregulation of anti-catabolic gene *TIMP1* by the addition of MSCs to (s)Link-N treatment were found (Fig. 19). Although the histological images showed a center of apoptotic cells in some (s)Link-N treated NPC and NPC:MSC micro-aggregates (Fig. 18), no significant differences were encountered for the expression of apoptosis markers *CASP3* and *BAX* or anti-apoptosis marker *BCL2* and proliferation marker *CCND1* (Fig. 19). *SOX9* gene expression, which is a marker of chondrogenesis, was not upregulated by the addition of MSCs to (s)Link-N treatment, and neither was the expression of the gene coding for the BMP receptor type II: *BMPR2* (Fig. 19). *ALK5* and *PAI1*

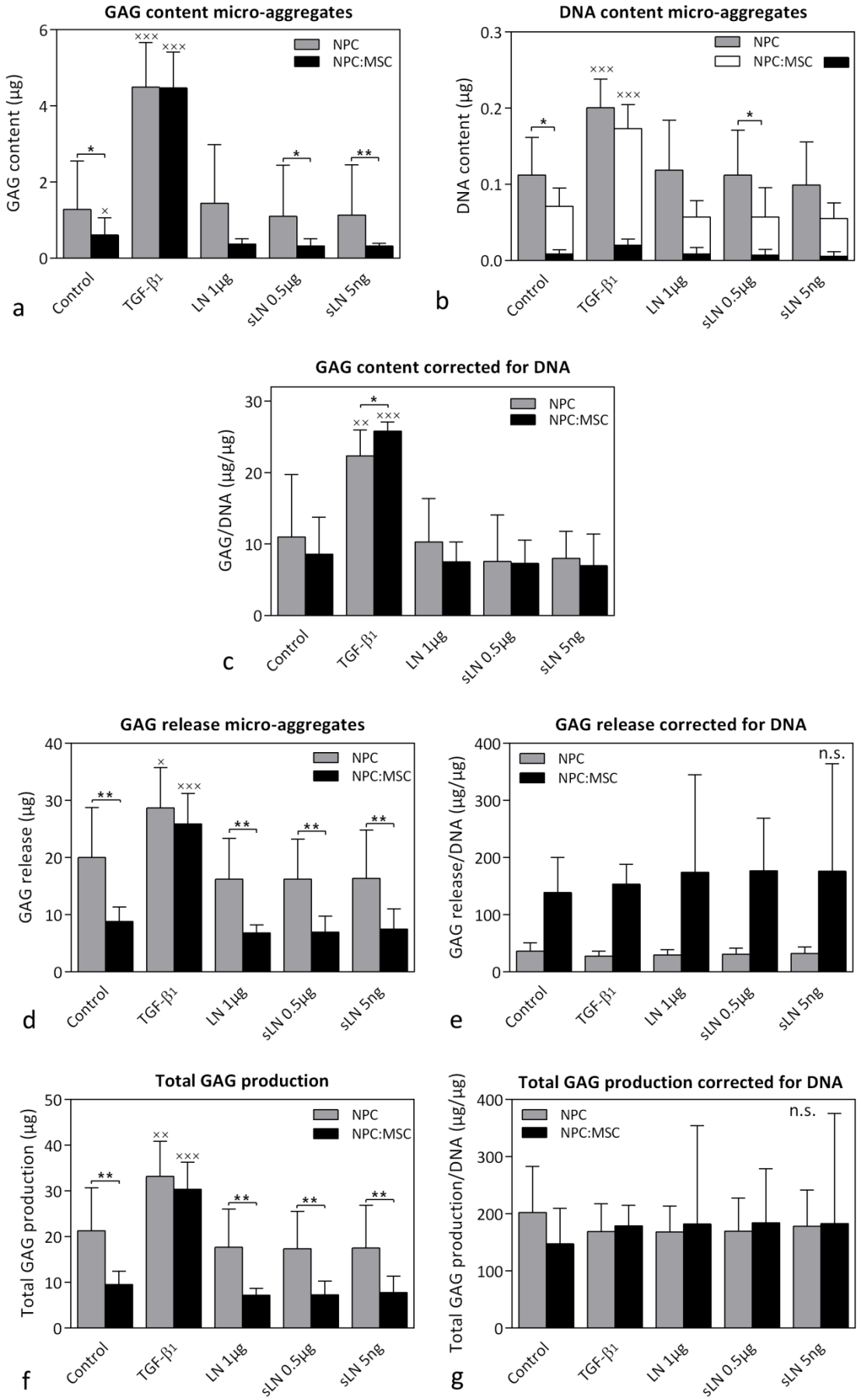

Fig. 17: (s)Link-N exerted no regenerative effect on the canine nucleus pulposus cells (NPCs) and no additive regenerative effects of the mesenchymal stromal cells (MSCs)were detected. GAG and DNA content (mean + SD) of canine NPC and NPC:MSC micro-aggregates cultured for 28 days in hypoxia (5% O_2) in different conditions. (**a**) GAG content of the micro-aggregates. (**b**) DNA content of the micro-aggregates. (**c**) GAG content corrected for the DNA content. (**d**) Total amount of GAGs released in the culture medium by the micro-aggregates. (**e**) Total GAG release corrected for the DNA content. (**f**) Total GAG production (GAG content micro-aggregates and amount of GAGs released taken together). (**g**) Total GAG production corrected for the DNA content. *n* = 6 *per* condition. ∗*p* < 0.05, ∗∗*p* < 0.01. ● and ○ : significantly different (p < 0.05) from the other conditions in the same group but the bars with the same symbol. ×, ××, ×××: significantly different (respectively *p* < 0.05, *p* < 0.01, *p* < 0.001) from all other conditions (growth factor treatment) in the same group (NPC or NPC: MSC).

expression was not increased by supplementation of MSCs to (s)Link-N treatment, which indicates no increased activation of TGF-β / SMAD2/3 signaling at least at gene expression level (Fig. 19). The expression level of *ID1* was not significantly increased when the micro-aggregates were treated with (s)Link-N combined with MSCs compared with (s)Link-N treatment alone, which implied no activated BMP / SMAD1/5 signaling (Fig. 19). Additionally, gene expression of *ALK1* (BMP receptor type I) was beneath the detection level. *COL10A1* gene expression was also not detected, indicating that the cells did not undergo hypertrophic differentiation. No significantly differences in the expression of *CCND1* (marker of cell proliferation) between the different treatment groups were found (data not shown).

TGF-β_1 treatment of the NPC:MSC micro-aggregates significantly increased the GAG deposition per DNA compared with the NPC micro-aggregates treated with TGF-β_1 ($p < 0.05$; Fig. 17c). Confirming this result, the NPC:MSC Safranin O/Fast Green staining of the micro-aggregates treated with TGF-β_1 showed a more intense GAG deposition compared with the NPC micro-aggregates treated with TGF-β_1 (Fig. 18).

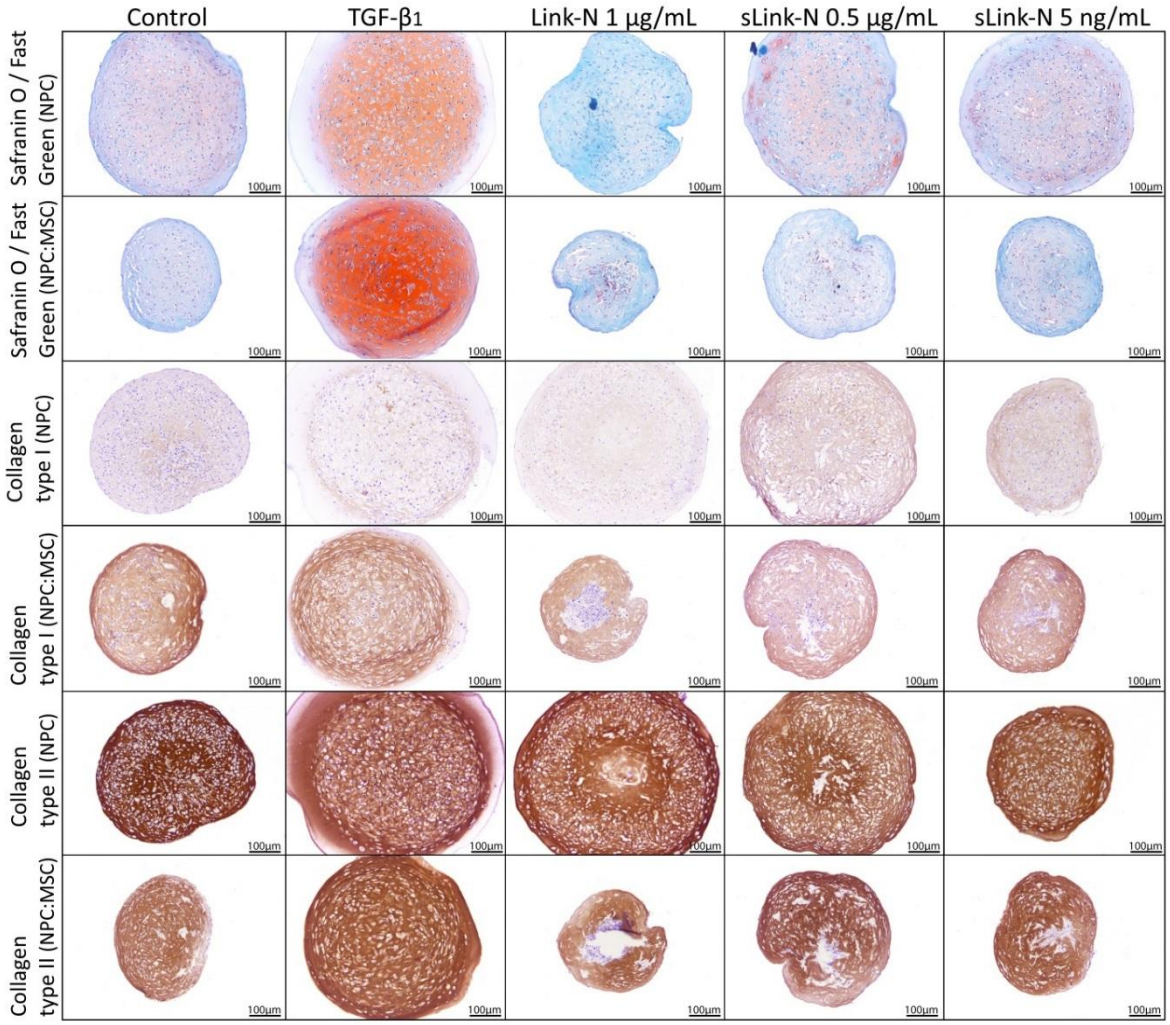

Fig. 18: Representative histological images of canine nucleus pulposus cell (NPC) and NPC: mesenchymal stromal cell (MSC) micro-aggregates cultured in hypoxic conditions (5% O_2) for 28 days stained with Safranin O/Fast Green and analyzed with immunohistochemistry for collagen type I and II. Safranin O/Fast Green staining showed no increased GAG deposition by the addition of (s)Link-N nor an additive effect of the MSCs. Collagen type I protein deposition was slightly increased by the treatment with sLink-N in the NPC micro-aggregates compared with the untreated controls and was prominently increased by the addition of MSCs in all the conditions. No clear differences were detected in collagen type II deposition.

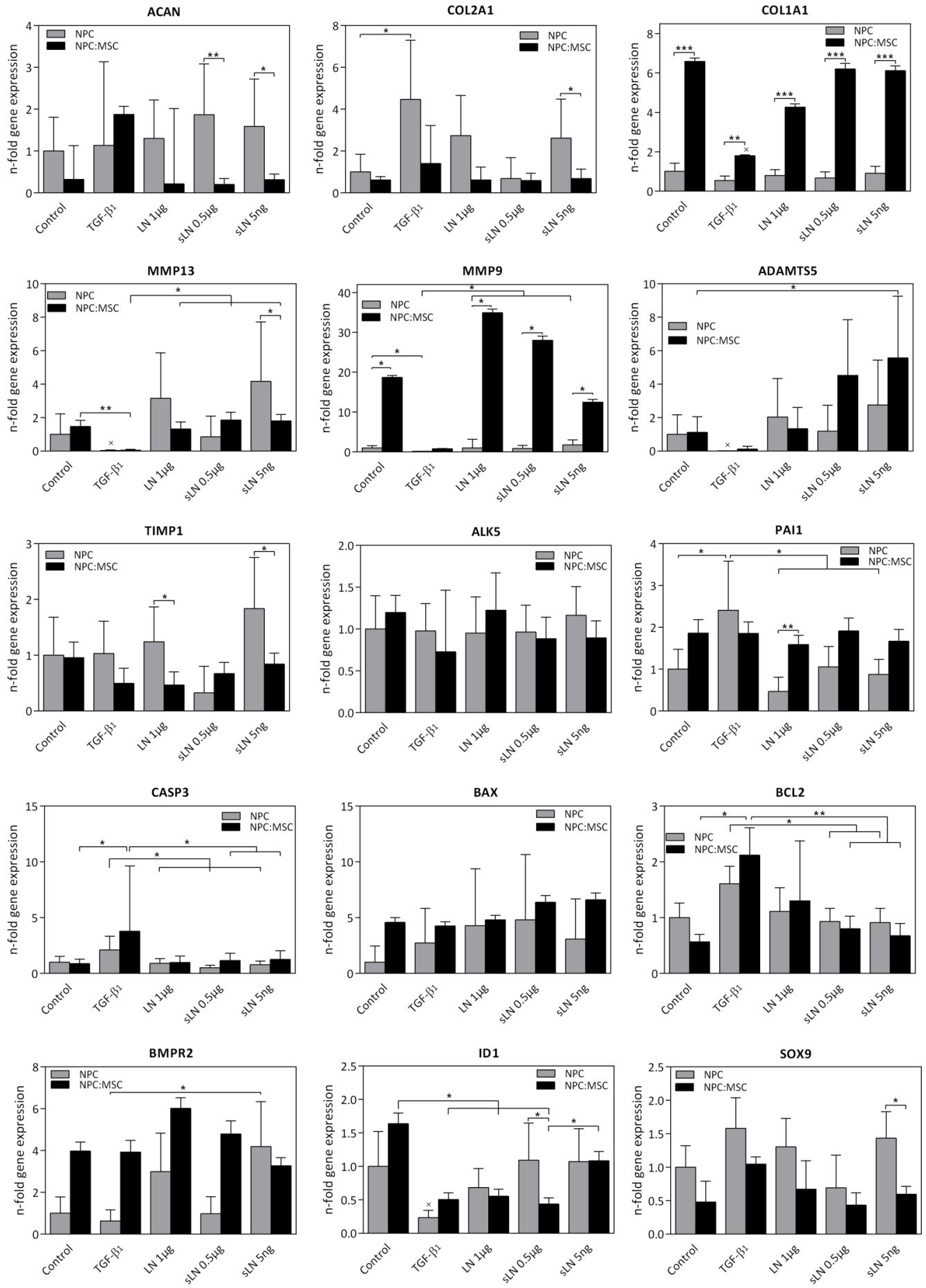

Fig. 19: No regenerative effects of (s)Link-N on gene expression level and also no additive regenerative effects of the addition of mesenchymal stromal cells (MSCs) were detected. Relative *ACAN, COL2A1, COL1A1, MMP13, MMP9, ADAMTS5, TIMP1, ALK5, PAI1, CASP3, BAX, BCL2, BMPR2, ID1, SOX9* gene expression (mean + SD) of the canine nucleus pulposus cell (NPC) and NPC:MSC micro-aggregates at day 7 of culture under hypoxic conditions (5% O_2). The control NPC micro-aggregates were set at 1. $n = 6$ *per* condition. ∗: $p < 0.05$, ∗∗: $p < 0.01$, ∗∗∗: $p < 0.001$. × : significantly different ($p < 0.05$) from all other conditions (growth factor treatment) in the same group (NPC or NPC:MSC micro-aggregates).

SURVIVAL OF THE MSCS IN CULTURE

At day 0, the NPC:MSC micro-aggregates were formed with 50% NPCs of male canine donors and 50% MSCs from a female canine donor (Fig. 20). After 3 days, 83% of the untreated control micro-aggregates contained the *SRY* gene and therefore appeared to be NPC-derived, which indicates that only 17% of the MSCs had survived (Fig. 20). After 28 days, the amount of (un)differentiated MSCs in all the conditions was around 11%, regardless the treatment (Fig. 20). Thus, the percentage MSCs present within the micro-aggregates was significantly decreased at day 3 and 28 of culture compared with day 0 ($p < 0.01$; Fig. 20).

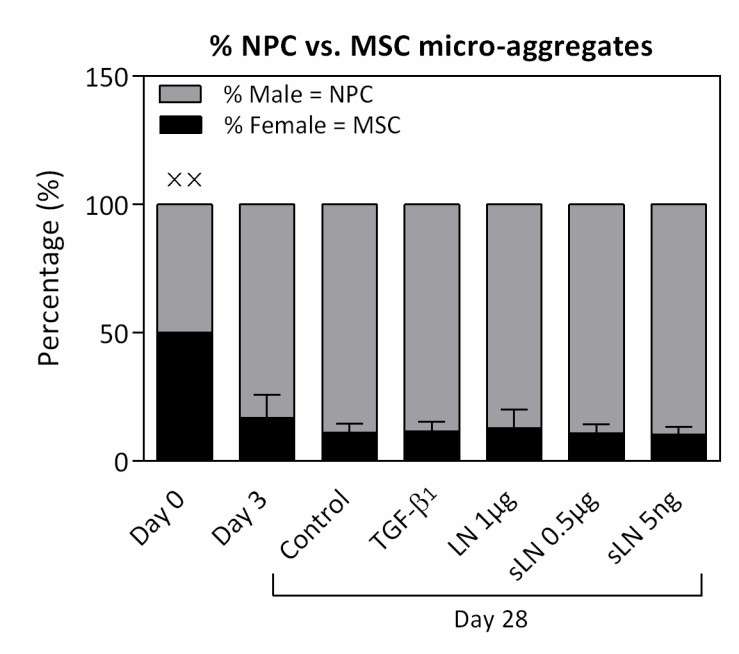

Fig. 20: Survival of the mesenchymal stromal cells (MSCs) in micro-aggregate co-culture with nucleus pulposus cells (NPCs) under hypoxic conditions (5% O_2). At day 0, the NPC:MSC micro-aggregates were seeded in a 50:50 ratio. After 3 days, only 17% of the untreated control micro-aggregates was composed of (un)differentiated MSCs. The last 5 bars show that after 28 days, the amount of (un)differentiated MSCs was approximately 11% regardless the treatment. $n = 6$ *per* condition. ××: significantly different ($p < 0.01$) from all other % MSC bars.

DISCUSSION

Our previous research revealed that human (s)Link-N alone exerted only very mild/no regenerative effects on canine NPCs. Therefore, in this study, the trophic effects of canine bone marrow derived MSCs on canine NPCs were delineated and the additional regenerative effects of the MSCs when combined with human (s)Link-N treatment.

MSCS DO NOT EXERT REGENERATIVE EFFECTS ON UNTREATED NPCS

Although the study of Wu *et al.* (2012) showed that MSCs stimulated chondrocyte proliferation and ECM production, the present study detected no trophic effects of the MSCs in co-culture with canine NPCs.[55] This could be due to the fact that only 11% of the NPC:MSC micro-aggregates was composed of MSCs after 28 days of culture, indicating that only a small amount of the MSCs survived. The IVD is a hypoxic tissue and it is reported that this low O_2-saturation stimulates cell viability, NP-like differentiation and ECM production of the MSCs.[2,61] Because of the aforementioned reasons, the MSCs and NPCs were cultured under hypoxic conditions in the present study. In contrast, the study of Rodrigues *et al.* (2010) reported that hypoxia increased MSC stress and could thus be considered as a reason for the low survival rate of the MSCs in the current study.[62] Other factors that potentially contribute to poor viability of MSCs *in vivo* are anoikis (disruption of cell-matrix interactions), pH

changes, and inflammatory factors.[62] Since the ultimate goal is to use MSC-based treatments *in vivo*, it is important that the cells have the ability to adapt to the low oxygen concentration and low pH microenvironment present in the IVD.[62] To improve the survival rate, growth factors could be used since it is reported that they possess the potential to stimulate human MSC proliferation, expansion, and survival *in vivo.*[63] However, in the current study, there was no significant difference detected in the survival rate of the MSCs between the different conditions, which indicates that the addition of TGF-β_1, Link-N or sLink-N had no positive effect on the survival of the canine MSCs *in vitro*.

The decreased *SOX9* expression of the untreated NPC:MSC control micro-aggregates compared with the untreated control micro-aggregates only containing NPCs indicates that NPC:MSC co-culture did not stimulate the differentiation of the MSCs to a NP-like phenotype. There is presumptive evidence that undifferentiated MSCs have a high basal production of collagen type I (unpublished *in vitro* data by the Orthopedics research group, Faculty of Veterinary Medicine, Utrecht University). Therefore, the increased collagen type I deposition of the untreated NPC:MSC micro-aggregates compared with the untreated NPC micro-aggregates is another indication that the MSCs did not differentiate. This could be due to a non-optimal NPC:MSC ratio used in the current study. The used 50:50 ratio was based upon the study of Sobajima *et al.* (2008)[64] which revealed that the greatest increase in ECM production was yielded when the human NPCs and MSCs were culture in 75:25 and 50:50 ratio.[64] The experiments of Vadala *et al.* (2008), in which NPCs and MSCs were also cultured in 50:50 ratio in an alginate hydrogel suggested that human MSCs acquire a more chondrogenic gene expression profile after being co-cultured with NPCs, but a trophic effect on the NPCs was not observed as well.[65] The study from Richardson *et al.* (2006) showed that co-culture of NPCs and MSCs causes MSC differentiation to an NP-like phenotype only if cells have cell-cell contact and that the optimal NPC:MSC ratio for differentiation is 75:25.[63] Furthermore, the use of a hydrogel could facilitate the differentiation of MSCs to a NPC-like phenotype and has the potential to deliver MSCs *in vivo* via minimally invasive injection.[66] Taken together, the current study shows that canine MSCs did not exert regenerative effects on canine NPCs under hypoxic conditions when they were seeded in a 50:50 ratio in micro-aggregates. Future studies should focus on optimizing the co-culture conditions for canine NPC and MSCs so that the potential beneficial effects of MSCs can be enhanced, *e.g.* by testing different MCS:NPC ratio's, comparing normoxic with hypoxic conditions and/or by using hydrogels.

MSCS DO NOT EXERT AN ADDITIVE REGENERATIVE EFFECT ON GROWTH FACTOR TREATMENT OF NPCS

Our results reveal that the addition of canine MSCs to the treatment with (s)Link-N resulted in an even decreased GAG and DNA content. The study of Mwale *et al.* (2014) demonstrated regenerative effects of the addition of MSCs and Link-N separately on bovine NPCs but did not show additional regenerative effects of the combined treatment as well.[38] Furthermore, in the current study, no indications of increased chondrogenic differentiation of the MSCs by (s)Link-N was detected, since the expression of the chondrogenic differentiation marker *SOX9* was not increased. Additionally, the abundant presence of collagen type I deposition (both on gene and protein level) in NPC:MSC micro-aggregates treated with (s)Link-N could, as stated before, be an indication of the presence of undifferentiated MSCs. These results were in contrast with a study of Antoniou *et al.* (2012), which showed that human Link-N promoted the chondrogenic differentiation of MSCs and retarded their

hypertrophic and osteogenic differentiation.[56] Whether (s)Link-N retarded hypertrophic differentiation of the NPCs and MSCs could in the current study could not be determined, since the absent *COL10A1* expression of the micro-aggregates at day 7 in all conditions implied that no hypertrophic differentiation occurred at all. To determine hypertrophic differentiation at day 28 at protein level, future experiments should also include IHC for collagen type X.

When TGF-β_1 was added to the NPC:MSC micro-aggregates, the GAG per DNA content was significantly increased compared with the NPC micro-aggregates. It is unlikely that this increased GAG/DNA production was caused by chondrogenic differentiation of the MSCs, since *SOX9* expression was not significantly increased and abundant collagen type I deposition was present. It could be that TGF-β_1 stimulates the MSCs to secrete trophic factors that induced GAG deposition of the NPCs, but since most of the MSCs died within the first 3 days of culture, this seems unlikely as well. Another option would be that the increased GAG/DNA content is due to decreased cell numbers in the NPC:MSC micro-aggregates (since almost all MCSs died within 3 days). This would indicate that the NPCs were able to react more potently to the TGF-β_1-treatment at lower cell density. Cells grown in 3D micro-aggregate culture systems receive nutrients/growth factors and exchange gasses only by diffusion.[67] Typical zones of cell proliferation may arise in the layers of the 3D culture system because of the different grade of diffusion of oxygen, CO_2 and nutrition (Fig. 21).[68] Although the IVD is considered an avascular tissue[2], it could be that diffusion is less sufficient *in vitro* compared with the *in vivo* situation. Therefore, the size of the micro-aggregates could be of influence on the performance of the cells, since the cells in larger micro-aggregates or pellets may not all have adequate exposure to nutrients/growth factors. The current study thus stresses the importance of using proper controls to compare MSC co-culture results with. To account for possible differences in available nutrition and factors that inhibit cell proliferation and ECM production, control samples should be seeded at comparable density as the MSC co-culture, but also at lower density.

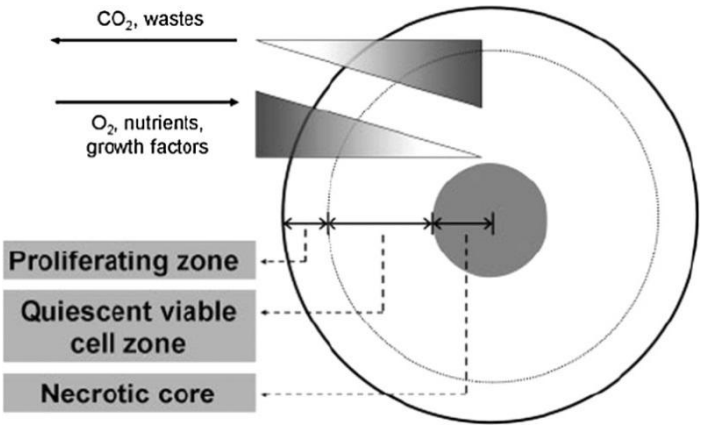

Fig. 21: The schematic diagram of typical zones of cell proliferation in a 3D spheroid, with the models of oxygenation, nutrition, and CO_2 removal. Lin *et al.* 2008.[68]

THE COMBINED TREATMENT OF HUMAN LINK-N AND MSCS DOES NOT EXERT REGENERATIVE EFFECTS ON CANINE NPCS

In the current study, the combined treatment of Link-N and MSCs did not increase the GAG and/or DNA content compared with the NPC control micro-aggregates, indicating that no regenerative effect was exerted by this treatment. This was surprising, because although there were no additive effects of the MSCs detected in the study of Mwale *et al.* (2014), combined treatment with human MSCs and Link-N still exerted regenerative effects compared with untreated controls.[38] An explanation for this discrepancy could be that there are interspecies differences in MSC characteristics. The study of Bertolo *et al.* (2015) reported that compared to human MSCs, canine

MSCs have significantly shorter life *in vitro* reflected by a lower number of cell doublings and higher proportion of senescent cells in the population.[69] Furthermore, their results indicated that the potential of canine MSCs to undergo chondrogenic differentiation *in vitro* is limited not only in time but also in scale.[69]

According to the results of the present study, the combined treatment of canine MSCs and human (s)Link-N is no suitable treatment strategy for IVD degeneration in canine patients. Although human Link-N has already been shown to have regenerative potential on NPCs of several species, it could be that canine NPCs are not able to react to the human variant of Link-N. The amino acid sequence of the canine variant of Link-N differs four and the bovine variant differs three amino acids from human Link-N (Unpublished data Orthopedics research group, Faculty of Medicine, McGill University). The amino acid sequence of canine and bovine sLink-N is similar and differs only one amino-acid from human sLink-N (Fig. 22).

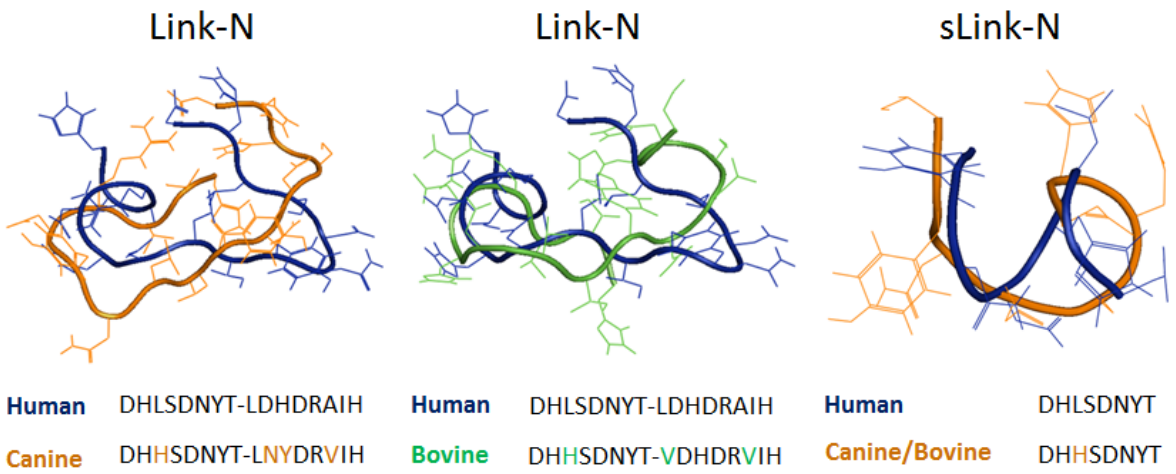

Fig. 22: Alignment of human, canine and bovine (s)Link-N performed using Pymol and 3D structures were generated with PEP-FOLD by Michael Grant (McGill University, Montreal, Canada). Amino-acids that differ from human (s)Link-N are indicated in color.

It depends on the properties of the amino acids whether those differences have an influence on the 3D structure of the peptide and thereby potentially affect protein function.[70] The properties of the amino acids of (s)Link-N that differ between species are shown in Table 7. Particularly the substitution of the third amino acid of human (s)Link-N (histidine) by leucine (bovine/canine (s)Link-N) is likely to influence the 3D structure due to the difference in polarity. This is confirmed by the 3D illustrations of human and canine/bovine sLink-N, which show the result of this substitution (Fig. 22). Additionally, substitutions in the active part of the peptide are more likely to affect their function.[71]

Table 7: Overview of the Link-N amino acids that differ between species and their characteristics

Amino acid	Abbreviation	Side-chain polarity	Side-chain charge (pH 7.4)	Side-chain
Histidine	H	Polar	Positive (10%) Neutral (90%)	$-CH_2-C_3H_3N_2$
Leucine	L	Nonpolar	Neutral	$-CH_2CH(CH_3)_2$
Asparagine	N	Polar	Neutral	$-CH_2CONH_2$
Aspartic acid	D	Acidic polar	Negative	$-CH_2COOH$
Tyrosine	Y	Polar	Neutral	$-CH_2-C_6H_4OH$

Valine	V	Nonpolar	Neutral	$-CH(CH_3)_2$
Alanine	A	Nonpolar	Neutral	$-CH_3$

It is reported that (s)Link-N exerts it effect by direct binding to the BMP receptor type II, at least in rabbit IVD cells.[40] It is possible that the species differences in amino acid sequence, resulting in an altered 3D structure, affected the docking of the human variant of (s)Link-N on canine NPCs. Consequently, the activation of the BMP signaling pathway could be impaired. Therefore, follow up studies will determine the influence of the these species differences in amino-acid sequence on the regenerative potency of the (s)Link-N peptide. In this way, we aim at delineating whether canine (s)Link-N exerts more potent regenerative effects on canine NPCs, and thus has therapeutic potential to be used as treatment for IVD degeneration in canine patients. Even more so, given that it is unclear how the 3D conformation of Link-N influences its function and we cannot exclude that the canine variant of Link-N may also be as active as human Link-N on human NPCs, the effect of canine (s)link-N will also be tested on human NPCs.

LIMITATIONS

In this study, the effects of Link-N and MSCs were tested on NPCs of just one kind of dog breed, namely the Beagle. This a chondrodystrophic breed so to successfully develop this new treatment strategy for canine patients, the effect of Link-N on non-chondrodystrophic dogs should also be delineated. Furthermore, it remains to be elucidated if other factors were affected by treatment with Link-N and MSCs than the ones measured in the present study, *e.g.* inflammatory, neurogenic or angiogenic markers. In the current study, only one NPC:MSC ratio was tested in hypoxic culture conditions and no hydrogel was used. Since the optimal co-culture conditions of canine NPCs and MSCs are not determined yet, future studies should test more different NPC:MSC ratio's, the effect of normoxic compared with hypoxic conditions, and/or the use of a hydrogel.

CONCLUSIONS

Human (s)Link-N treatment alone yielded no regenerative effects on canine NPCs derived from degenerated IVDs. MSCs exerted no trophic effects on untreated as well as growth factor (TGF-β_1 and (s)Link-N)-treated canine NPCs derived from degenerated IVDs. Moreover, they even increased undesired collagen type I deposition. These results imply that the treatment with human (s)Link-N and canine MSCs alone or combined has no potential to treat IVD degeneration in canine patients. Since the amino acid sequence of (s)Link-N differs between species, follow up studies will focus on testing the regenerative effects of the canine variant of (s)Link-N on human, bovine and especially canine NPCs derived from degenerated IVDs.

CHAPTER 3: THE REGENERATIVE EFFECTS OF THE CANINE VARIANT OF LINK-N ON HUMAN, BOVINE AND CANINE NUCLEUS PULPOSUS CELLS

SUMMARY

Background. The human variant of Link-N (DHLSDNYT-LDHDRAIH) has already proven to be a promising new regenerative treatment for intervertebral disc (IVD) repair in different species. Our previous studies, however, showed that canine nucleus pulposus cells (NPCs) only responded moderately to treatment with human Link-N. The optimization of this treatment using canine bone marrow derived mesenchymal stromal cells did not result in an (additive) regenerative effect. These results could be due to species differences in the amino acid sequence of (s)Link-N. Canine Link-N (DHHSDNYT-LNYDVIH) differs four and bovine Link-N (DHHSDNYT-VDHDRVIH) three amino-acids from human Link-N. The canine and bovine short variants of Link-N (sLink-N) are similar and differ only one amino-acid from human sLink-N.

Objective. Investigating the regenerative effects of the canine variant of (s)Link-N on human, bovine, and especially canine NPCs derived from degenerated IVDs.

Methods. NPCs from degenerated human, bovine and canine IVDs were cultured in 3D micro-aggregates (35,000 cells) for 28 days in basal culture medium with and without supplementation of canine Link-N (1 µg/mL and 10 ng/mL) and sLink-N (0.5 µg/mL and 5 ng/mL). Extracellular matrix production and cell proliferation was determined by gene expression profiling, histological evaluation and measuring the DNA and GAG content.

Results. Treatment with canine (s)Link-N did not increase the GAG and/or DNA content of human NPCs and had no influence on the collagen type I and II deposition. The GAG, but not the DNA content of the bovine NPC micro-aggregates was slightly increased by canine (s)Link-N treatment in high concentration, whereas collagen type II deposition was prominently stimulated. A small, significant, concentration-independent increase in GAG and DNA content of the canine micro-aggregates was induced by treatment with canine (s)Link-N, but the biological relevance was negligible. Collagen type I and II deposition were not affected by canine (s)Link-N treatment.

Conclusions. Canine (s)Link exerted no regenerative effects on human NPCs and stimulated prominent production of collagen type II instead of GAGs in bovine NPCs, presumably due to the different amino acid sequence of the human compared with the canine variant of (s)Link-N. Canine (s)Link-N treatment resulted in no biologically relevant effects on canine NPCs. The results of the present study indicate that canine (s)Link-N is no potential treatment for IVD degeneration in canine or human patients. Species differences in amino-acid sequence of (s)Link-N influence its effect, but treatment with the species own sequence does not intrinsically result in the most potent effect. To better understand the effects of human and canine sLink-N on NPCs of different species, follow up studies will focus on delineating which intracellular signaling pathways they transduce.

INTRODUCTION

The human variant of Link-N has already proven to be a promising new regenerative treatment for intervertebral disc (IVD) repair in different species.[38, 41, 42] Our previous studies, however, showed that canine NPCs only responded moderately to treatment with human Link-N and its shorter variant sLink-N. The optimization of this treatment using canine bone marrow derived mesenchymal stromal cells, as cell supplement and extra trophic factor, did not result in an (additive) regenerative effect. These results could be due to species differences in the amino acid sequence of (s)Link-N. The amino acid sequence of canine Link-N differs four amino acids from human Link-N and bovine Link-N differs three amino acids from human Link-N (Unpublished data Orthopedics research group, Faculty of Medicine, McGill University). The amino acid sequence of canine and bovine sLink-N is similar and differs only one amino-acid from human sLink-N (Table 8).

Table 8: Species differences in amino acid sequence of (s)Link-N

	Link-N	**sLink-N**
Human	DHLSDNYT-LDHDRAIH	DHLSDNYT
Bovine	DHHSDNYT-VDHDRVIH	DHHSDNYT
Canine	DHHSDNYT-LNYDRVIH	DHHSDNYT

Amino-acids that differ from the human variant are underlined.

Particularly the substitution of the third amino acid of human (s)Link-N (histidine) by leucine (bovine/canine (s)Link-N) is likely to influence the 3D structure due to the difference in polarity. Additionally, substitutions in the active part of the peptide are more likely to affect the function.[71] Studies on rabbit IVD cells implicated that Link-N exerts its effect by direct interaction with the bone morphogenetic protein (BMP) receptor type II.[40] The different 3D structure of canine sLink-N compared with human sLink-N likely affects the docking of the peptide on the BMP receptor type II (Fig. 23), and potentially on non-elucidated receptors that may be involved in the working mechanism of (s)Link-N as well.

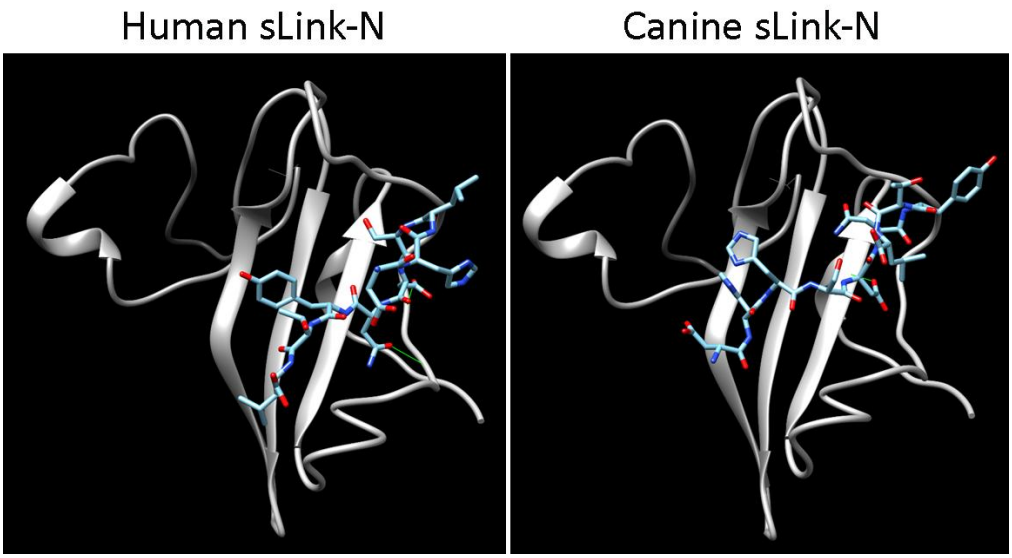

Fig. 23: Human and canine sLink-N docked on a modeled structure of canine bone morphogenetic protein receptor type II (extracellular domain) using SwissDock. The most energetically favorable fit was used for imaging and images were created with UCSF Chimera by Michael Grant (McGill University, Montreal, Canada).

To investigate whether the species differences in amino-acid sequence influence the effect of the (s)Link-N peptide on NPCs of different species, the aim of the present study was to delineate whether the canine variant of (s)Link-N exerts regenerative effects on human and bovine, but especially canine NPCs. The ultimate goal of the current study was to determine the potential of canine (s)Link-N as a treatment for IVD denegation in canine patients, and furthermore to contribute to the development of a suitable large animal model to facilitate translation of Link-N treatment into human patients. The human and bovine NPCs were taken along to determine whether species own variant of (s)Link-N exerts more potent effects. Since the canine variant of (s)Link-N differs in polarity from the human variant it is hypothesized that it exerts less potent/no regenerative effects on human NPCs. The bovine and canine variant of Link-N do not differ in polarity and sLink-N is actually similar between the two species, so canine (s)Link-N treatment will presumably result into a more potent effect on bovine NPCs. Finally it is hypothesized that the canine variant of (s)Link-N will exert potent regenerative effects on canine NPCs since it is species own variant of the peptide.

MATERIALS AND METHODS

HARVEST AND ISOLATION OF THE NPCS

The harvest and isolation of the canine NPCs was performed following the same protocol as described in chapter 1. Human and bovine NPCs were supplied by other research institutes, but were harvested in a similar manner as the canine NPCs.

MICRO-AGGREGATE CULTURE OF THE NPCS

NPCs from degenerated IVDs of 6 canine, 6 bovine, and 2 human donors (Table 9) were expanded using the same protocol as described in chapter 1. After expansion, micro-aggregates of 35,000 were formed following the same protocol as in chapter 1 as well. The next day (day 0), basal culture medium was replaced with either basal culture medium for the negative control micro-aggregates, basal culture medium supplemented with 10 ng/mL human recombinant TGF-β_1 (240-B, R&D Systems, Inc., Minneapolis, USA) for the positive control micro-aggregates, basal culture medium supplemented with 1 µg/mL or 10 ng/mL canine Link-N (DHHSDNYT-LNYDRVIH; CanPeptide, Montreal, Canada), or 0.5 µg/mL or 5 ng/mL canine sLink-N (DHHSDNYT; CanPeptide, Montreal, Canada). or basal culture medium supplemented with canine Link-N (1 µg/mL and 10ng/mL) or canine sLink-N (0.5 µg/mL and 5 ng/mL). The micro-aggregates were cultured for 28 days under hypoxic conditions (5% O_2, 5% CO_2, 37°C). Medium was changed two times a week.

Table 9: Overview of the used donors in the study and their characteristics

Number	Gender	Species	Thompson score	Age (years)
S14-80	Female	Human	III	63
S13-142	Female	Human	III	47
B1	Unknown	Bovine	I/II	± 2
B2	Unknown	Bovine	I/II	± 2

B3	Unknown	Bovine	I/II	± 2
B4	Unknown	Bovine	I/II	± 2
B5	Unknown	Bovine	I/II	± 2
B6	Unknown	Bovine	I/II	± 2
1399617	Male	Canine (Beagle)	III	5
7140151	Male	Canine (Beagle)	III	5.5
7283679	Male	Canine (Beagle)	III	6
7207859	Male	Canine (Beagle)	III	5
0906743	Female	Canine (Beagle)	III	6.5
7164092	Female	Canine (Beagle)	III	5.5

GENE EXPRESSION PROFILING

RNA isolation, cDNA synthesis and the quantitative RT-PCR determining the relative gene expression of the below mentioned genes were performed following the same protocols as described in chapter 1.

QUANTITATIVE RT-PCR

HUMAN NPCS

Four reference genes (TATAA-box binding protein (*TBP*), hypoxanthine-guanine phosphoribosyltransferase (*HPRT*), succinate dehydrogenase subunit A (*SDHA*), and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide *(YWHAZ*)) were used to normalize gene expression of the target genes (inhibitor of DNA binding-1 (*ID1*), plasminogen activator inhibitor-1 (*PAI1*), activin receptor-like kinase 1 (*ALK1*), TGF-β receptor type I (*ALK5*), bone morphogenetic protein 4 (*BMP4*), and SRY-box 9 (*SOX9*)) (Table 10).

Table 10: Overview of the primers used to asses gene expression of the human NPCs

Genes	Forward sequence 5' → 3'	Reverse sequence 5' → 3'	Amplicon size	Annealing temp (°C)
Reference genes				
HPRT	TATTGTAATGACCAGTCAACAG	GGTCCTTTTCACCAGCAAG	192	60
SDHA	TGGGAACAAGAGGGCATCTG	CCACCACTGCATCAAATTCATG	86	58
TBP	TGCACAGGAGCCAAGAGTGAA	CACATCACAGCTCCCCACCA	132	63.5
YWHAZ	ACTTTTGGTACATTGTGGCTTCAA	CCGCCAGGACAAACCAGTAT	94	64
Target genes				
ALK1	GCAACCTGCAGTGTTGCATC	CGGATCTGCTCGTCCAGCAC	139	62.5
ALK5	GCCGTTTGACTGAAGGCTG	GGGCATCCCAAGCCTCATC	146	61
ID1	CTCTACGACATGAACGGCTGT	TGCTCACCTTGCGGTTCTG	76	65
PAI1	GCTGGTGAATGCCCTCTAC	GGCAGCCTGGTCATGTTG	318	65
SOX9	CCCAACGCCATCTTCAAGG	CTGCTCAGCTCGCCGATGT	242	65.5
BMP4	CCGCAGCCTAGCAAGAGTG	GCTCAGGATACTCAAGACCAGTG	115	58

CANINE NPCS

Four reference genes (Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), Hypoxanthine-guanine phosphoribosyltransferase (*HPRT*), Ribosomal protein S19 (*RPS19*) and succinate dehydrogenase complex subunit A (*SDHA*)) were used to normalize gene expression of the target genes (inhibitor of DNA binding-1 (*ID1*), plasminogen activator inhibitor-1 (*PAI1)*, activin receptor-like kinase 1 (*ALK1*), TGF-β receptor type I (*ALK5*), SRY-box 9 (*SOX9*), bone morphogenetic protein receptor type II (*BMPR2*), bone morphogenetic protein receptor type IA (*ALK3*), bone morphogenetic protein receptor type IB (*ALK6*), bone morphogenetic protein 7 (*BMP7*), Noggin (*NOG*)) (Table 11).

Table 11: Overview of the primers used to asses gene expression of the canine NPCs

Genes	Forward sequence 5' → 3'	Reverse sequence 5' → 3'	Amplicon size	Annealing temp (°C)
Reference genes				
GAPDH	TGTCCCCACCCCCAATGTATC	CTCCGATGCCTGCTTCACTACCTT	100	58
HPRT	AGCTTGCTGGTGAAAAGGAC	TTATAGTCAAGGGCATATCC	104	58
RPS19	CCTTCCTCAAAAAGTCTGGG	GTTCTCATCGTAGGGAGCAAG	95	61
SDHA	GCCTTGGATCTCTTGATGGA	TTCTTGGCTCTTATGCGATG	92	56.5
Target genes				
ALK1	CCTTTGGTCTGGTGCTGTG	CGAAGCTGGGATCATTGGG	107	61
ALK5	GAGGCAGAGATTTATCAGACC	ATGATAATCTGACACCAACCAG	116	59.5
BMP2R	GTCTTCACAGTATGAACATGATGG	AACACTTTCACAGCAACTGG	150	65
ID1	CTCAACGGCGAGATCAG	GAGCACGGGTTCTTCTC	135	59.5
PAI1	AAACCTGGCGGACTTCTC	ACTGTGCCACTCTCATTCAC	98	61.5
SOX9	CGCTCGCAGTACGACTACAC	GGGGTTCATGTAGGTGAAGG	105	62
NOG	TGCCGAGCGAGATCAAAGGG	AGCCACATCTGTAACTTCCTCCG	99	63
ALK3	TTTGGGAAATGGCTCGTC	CGTATGATGGATCGTTGGG	N.A.	60
ALK6	CCCTATCATGACCTAGTGCC	TGCCTCAGACACTCATCAC	N.A.	63
BMP7	CAACGCCATCTCTGTCCTCTACTTC	AGGCTCGGACGACCATGTTTCTGT	N.A.	59

GAG AND DNA MEASUREMENTS

The GAG and DNA content were determined following the same protocol described in the chapter 1.

HISTOLOGY

Safranin O/Fast Green staining and immunohistochemistry for collagen type I and II protein were performed according to the same protocol as described in chapter 1.

STATISTICAL ANALYSIS

For the statistical analysis, IBM SPSS statistics 22 was used. To check whether the data were normally distributed, a Shapiro Wilks test was performed. General linear regression models based on ANOVAs were used for normally distributed data, whereas a Kruskal Wallis and Mann-Whitney U test were performed on non-normally distributed data. To correct for multiple comparisons, all tests were followed by a Benjamini & Hochberg False Discovery Rate *post-hoc* test. *P*-values < 0.05 were considered significant.

HUMAN NPCS

Canine (s)Link-N treatment in both concentrations did not increase the GAG content of the human NPC micro-aggregates compared with the untreated controls, whereas TGF-β_1 treatment did ($p < 0.05$; Fig. 24a). In agreement, the Safranin O/Fast Green staining showed the most prominent presence of GAGs in the micro-aggregates treated with TGF-β_1 compared with all the other conditions (Fig. 25). No significant differences between the DNA content of the micro-aggregates cultured in the different conditions were encountered (Fig. 24b). When the GAG content was corrected for the DNA content (an indication of the GAG production per cell), no significant increase by treatment with canine (s)Link-N or TGF-β_1 was detected. However, the GAG/DNA content of the micro-aggregates treated with 5 ng/mL sLink-N was significantly higher compared with the ones treated with 10 ng/mL Link-N ($p < 0.05$; Fig. 24c), indicating a more potent effect of this shorter variant of the peptide. The significantly higher *ALK5* (TGF receptor type I) expression of the micro-aggregates treated with sLink-N compared with the ones treated with Link-N both in low concentration at day 7 ($p < 0.05$; Fig. 26) confirms this higher GAG/DNA content by more activation of the TGF-β pathway. In contrast, the Safranin O/Fast Green staining indicated slightly increased GAG deposition by Link-N treatment in low concentration compared with the all other (s)Link-N treatments (Fig. 25). The GAG release and total GAG production were not significantly different between the micro-aggregates cultured in the different conditions, corrected and not corrected for the DNA content (Fig. 24d, e, f, g). The deposition of collagen type I protein was prominently increased by treatment with TGF-β_1 and slightly increased by treatment with (s)Link-N in low concentration compared with the untreated controls (Fig. 25). Collagen type II protein deposition was prominently increased in the samples treated with TGF-β_1 compared with the untreated controls, whereas no effect of treatment with (s)Link-N was detected (Fig. 25). No significant differences between the effects of the applied treatments on *PAI1* (read-out of the TGF pathway[47]), *ID1* (read-out of the BMP pathway[48]) and *SOX9* (marker of chondrogenic differentiation) gene expression were encountered (Fig. 26). *ALK1* (BMP receptor type I) and *BMP4* expression were too low to be detected.

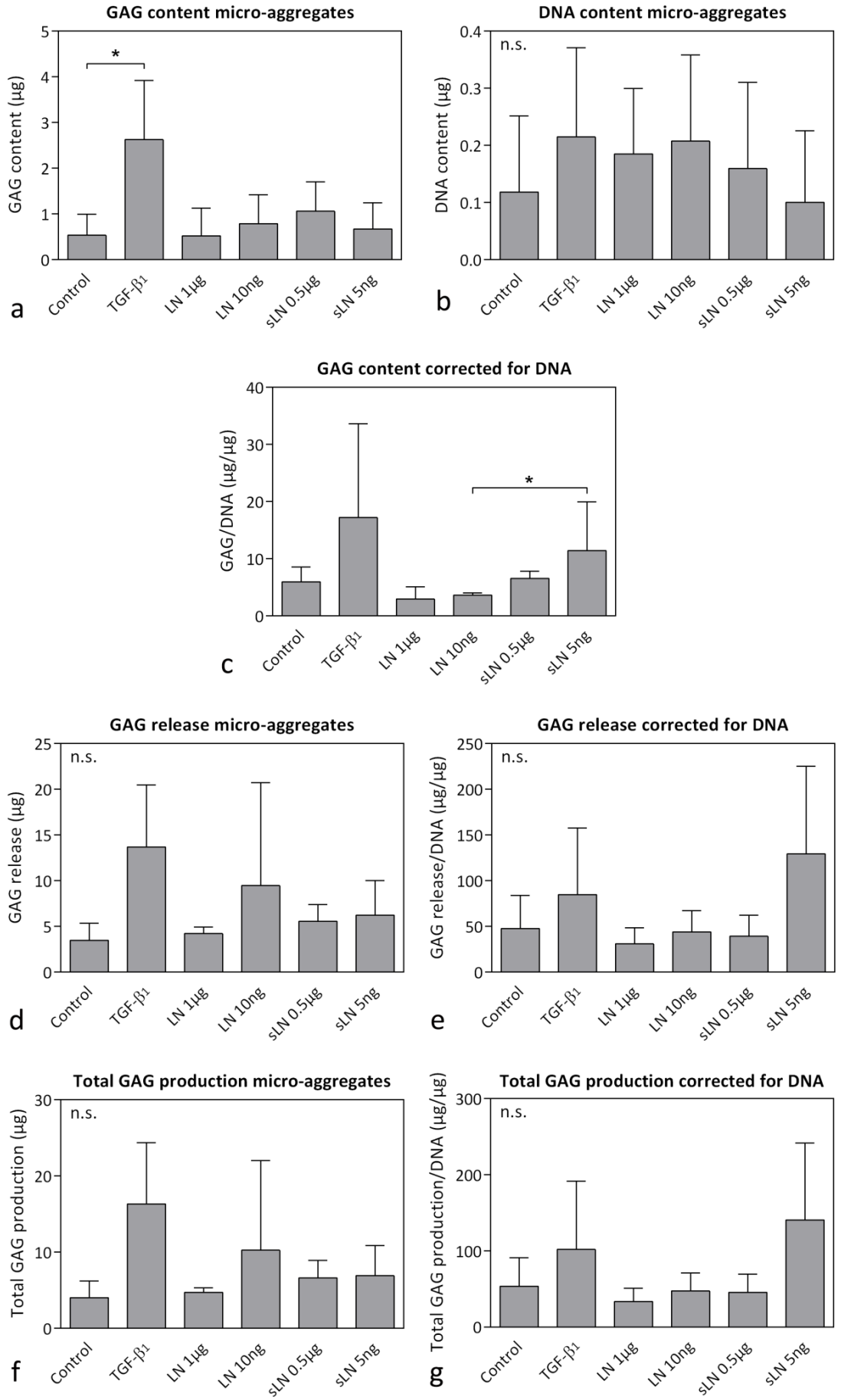

Fig. 24: Canine (s)Link-N exerted no regenerative effect on the human nucleus pulposus cells (NPCs). GAG and DNA content (mean + SD) of human NPC micro-aggregates cultured for 28 days in hypoxia (5%O_2) in different conditions. (**a**) GAG content of the micro-aggregates. (**b**) DNA content of the micro-aggregates. (**c**) GAG content corrected for the DNA content. (**d**) Total amount of GAGs released in the culture medium by the micro-aggregates. (**e**) Total GAG release corrected for the DNA content. (**f**) Total GAG production (GAG content micro-aggregates and amount of GAGs released taken together). (**g**) Total GAG production corrected for the DNA content. $n = 3$ *per* condition. * $p < 0.05$.

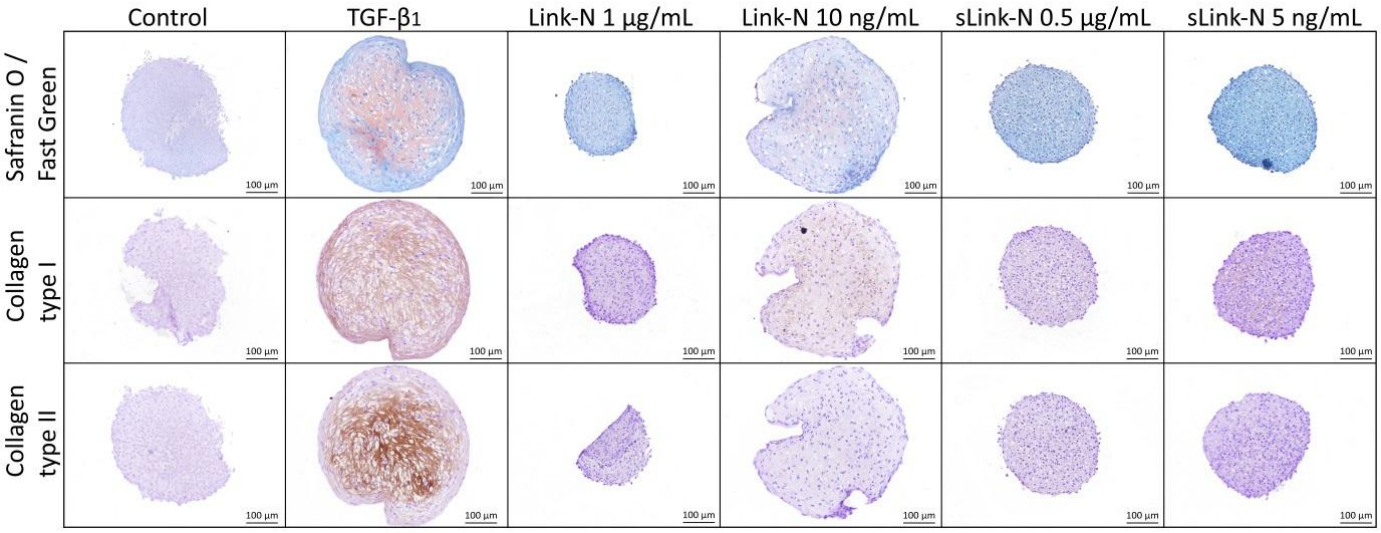

Fig. 25: Representative histological images of human nucleus pulposus cell (NPC) micro-aggregates cultured in hypoxic conditions (5% O_2) for 28 days stained with Safranin O/Fast green and analyzed with immunohistochemistry for collagen type I and II. Canine Link-N in a concentration of 10 ng/mL showed to slightly increase GAG and collagen type I protein deposition. No effect of the other (s)Link-N conditions was detected, whereas treatment with TGF-β_1 showed a prominent increase in GAG, collagen type I and II protein deposition.

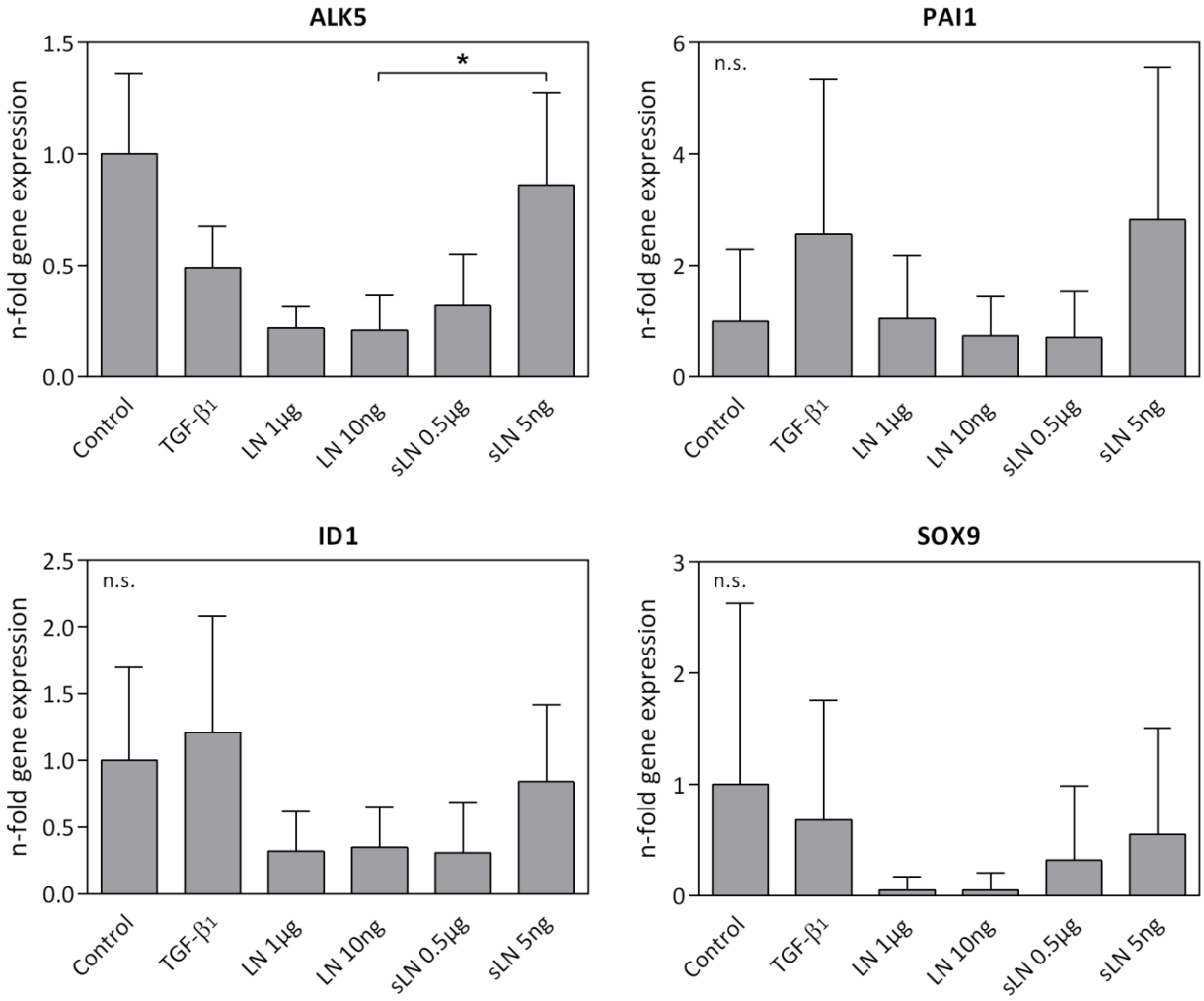

Fig. 26: No regenerative effects of (s)Link-N on gene expression level were detected. Relative *ALK5, PAI1, ID1, SOX9* gene expression of the micro-aggregates derived of human nucleus pulposus cells derived from degenerated intervertebral discs treated with canine (s)Link-N at day 7 of culture under hypoxic conditions (5%, O_2). The control micro-aggregates were set at 1. $n = 2$ *per* condition. $* \; p < 0.05$.

BOVINE NPCS

A slight, but significant, increase in GAG content was detected when the micro-aggregates derived of NPCs from bovine spines were treated with canine (s)Link-N in high concentration compared with the untreated controls ($p < 0.05$; Fig. 27a). However, TGF-β_1 treatment resulted in the most potent increase in GAG content ($p < 0.01$; Fig. 27a). The GAG content of the micro-aggregates treated with (s)Link-N in high concentration had a significantly higher GAG content compared with (s)Link-N treatment in low concentration, indicating a concentration-dependent effect ($p < 0.05$; Fig. 27a). Treatment with (s)Link-N did not significantly increase the DNA content of the micro-aggregates, whereas TGF-β_1 treatment resulted in an increased DNA content compared with the micro-aggregates in all the other conditions ($p < 0.01$; Fig. 27b). When the GAG content was corrected for the DNA content, no significant differences were encountered between the different conditions (Fig. 27c). In contrast with the GAG measurements, Safranin O/Fast Green staining showed that treatment with (s)Link-N in both concentrations induced GAG deposition at least as potent as treatment with TGF-β_1 (Fig. 28). The Safranin O/Fast Green staining did not explicitly demonstrate the prominently increased GAG deposition by treatment with TGF-β_1 measured with the DMMB-assay. Collagen type I protein was slightly present in the micro-aggregates of all the conditions except the untreated controls, but most prominent in the ones treated with Link-N in low and sLink-N in high concentration (Fig. 28). (s)Link-N in both concentrations prominently increased collagen type II protein deposition compared with the untreated control micro-aggregates in which also a slight deposition was detected (Fig. 28). TGF-β_1 treated micro-aggregated contained less collagen type II protein deposition compared with the untreated control (Fig. 28).

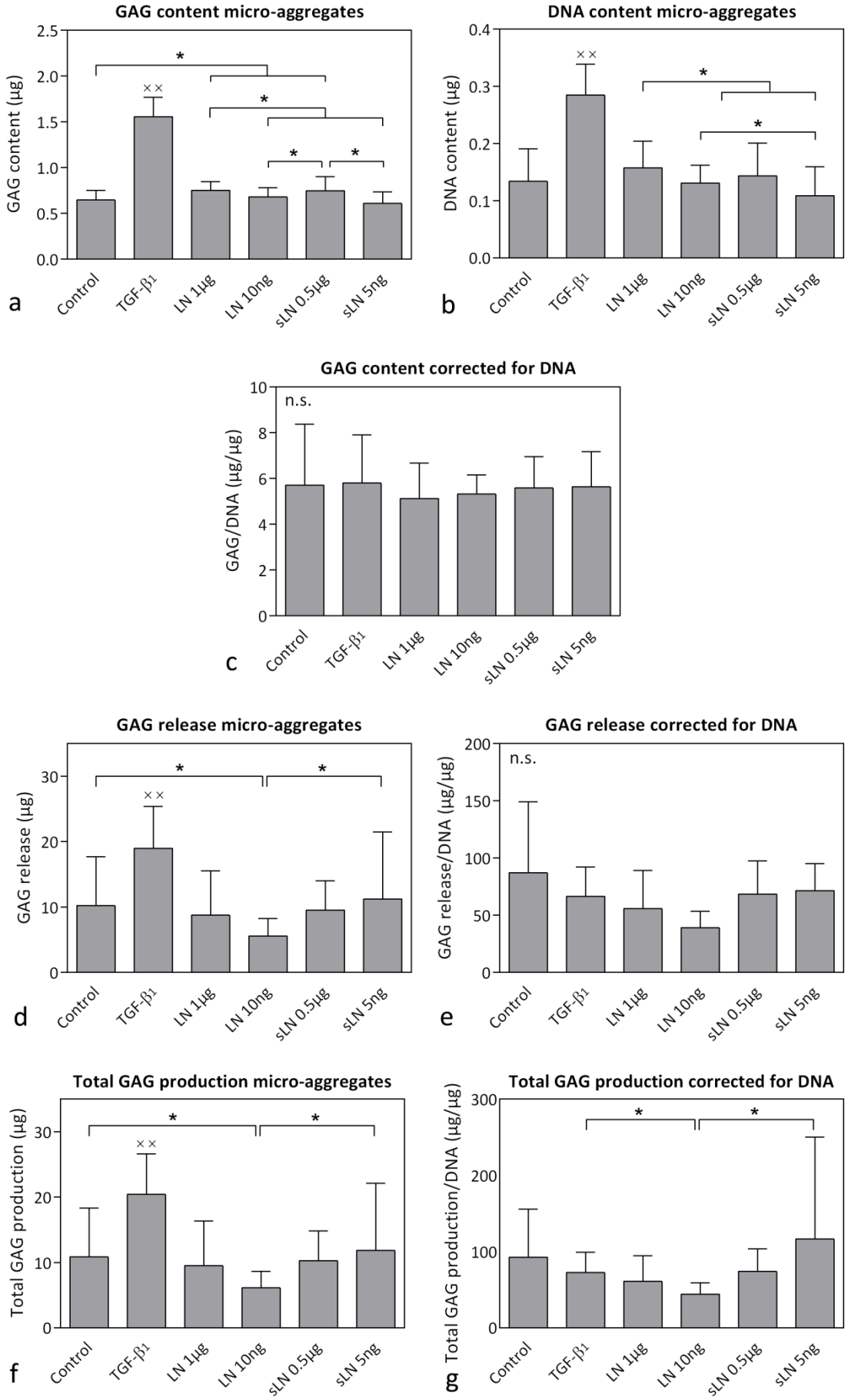

Fig. 27: Canine (s)Link-N exerted no potent regenerative effect on the bovine nucleus pulposus cells (NPCs). GAG and DNA content (mean + SD) of bovine NPC micro-aggregates cultured for 28 days in hypoxia (5%O_2) in different conditions (**a**) GAG content the micro-aggregates. (**b**) DNA content of the micro-aggregates. (**c**) GAG content corrected for the DNA content. (**d**) Total amount of GAGs released in the culture medium by the micro-aggregates. (**e**) Total GAG release corrected for the DNA content. (**f**) Total GAG production (GAG content micro-aggregates and amount of GAGs released taken together). (**g**) Total GAG production corrected for the DNA content. $n = 6$ *per* condition. $*p < 0.05$. ××: significantly different ($p < 0.01$) from all other conditions (growth factor treatment).

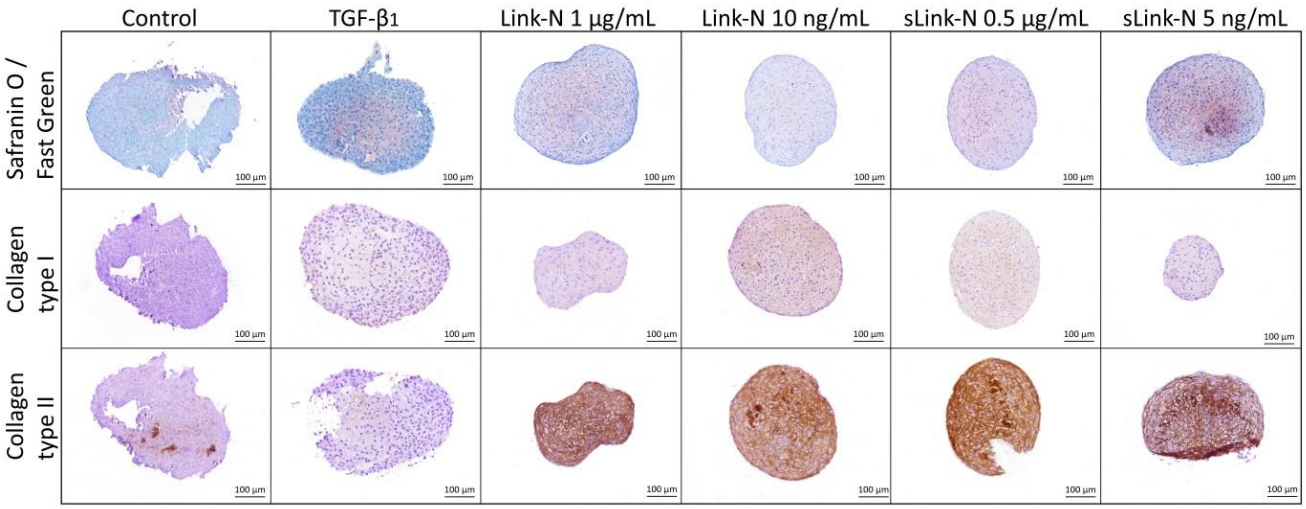

Fig. 28: Representative histological images of bovine nucleus pulposus cell (NPC) micro-aggregates cultured in hypoxic conditions (5% O_2) for 28 days stained with Safranin O/Fast Green and analyzed with immunohistochemistry for collagen type I and II. Canine (s)Link-N in both concentrations slightly increased GAG and collagen type I protein deposition and prominently increased collagen type II protein deposition.

CANINE NPCS

The micro-aggregates containing NPCs from degenerated canine IVDs treated with canine Link-N in high concentration and sLink-N in both concentrations had a slightly, but significantly, higher GAG and DNA content compared with the untreated control micro-aggregates ($p < 0.05$; Fig. 29a, b). Treatment with TGF-β_1 induced the highest GAG and DNA content ($p < 0.001$; Fig. 29a, b). The GAG/DNA content was also significantly increased by treatment with Link-N in high concentration and sLink-N in both concentrations, but the effect of TGF-β_1 was again more potent ($p < 0.05$; Fig. 29c). Treatment with (s)Link-N in both concentrations did not result in increased GAG release or total GAG production compared with the untreated controls, whereas TGF-β_1 did induce a prominent increase ($p < 0.001$; Fig. 29d, e, f, g). Safranin O/Fast Green staining showed a slight increase in GAG deposition by (s)Link-N in high concentration compared with the untreated controls, whereas the low concentration of (s)Link-N did not affect this. The effect by (s)Link-N on GAG deposition, however, was far not as potent as treatment with TGF-β_1, which resulted in an abundant presence of GAGs (Fig. 31). Although DNA measurements showed a slight increase in DNA content by treatment with (s)Link-N, histological images show no increase and even a decrease in size (Fig. 31). In line with the DNA measurements, treatment with TGF-β_1 induced an increased micro-aggregates size (Fig. 31). Collagen type I and II deposition were not influenced by treatment with (s)Link-N, whereas treatment with TGF-β_1 resulted in a fibrotic rim of collagen type I and prominent increase of collagen type II (Fig. 31). Confirming these results, *SOX9* expression at day 7 was the highest in the micro-aggregates treated with TGF-β_1, but only significantly higher than sLink-N treatment in high concentration (p <0.05; Fig. 30). No significant differences in *ALK5* (TGF receptor type I), *PAI1* (read-out parameter of the TGF pathway), *ALK3* (BMP receptor type IA), *ID1* (read-out parameter of the BMP pathway) and *BMP2R* expression were encountered between the different treatment groups (Fig. 30). Gene expression of *ALK6* (BMP receptor type IB), *BMP7* and *NOG* (antagonist of BMP signaling) were too low to detect.

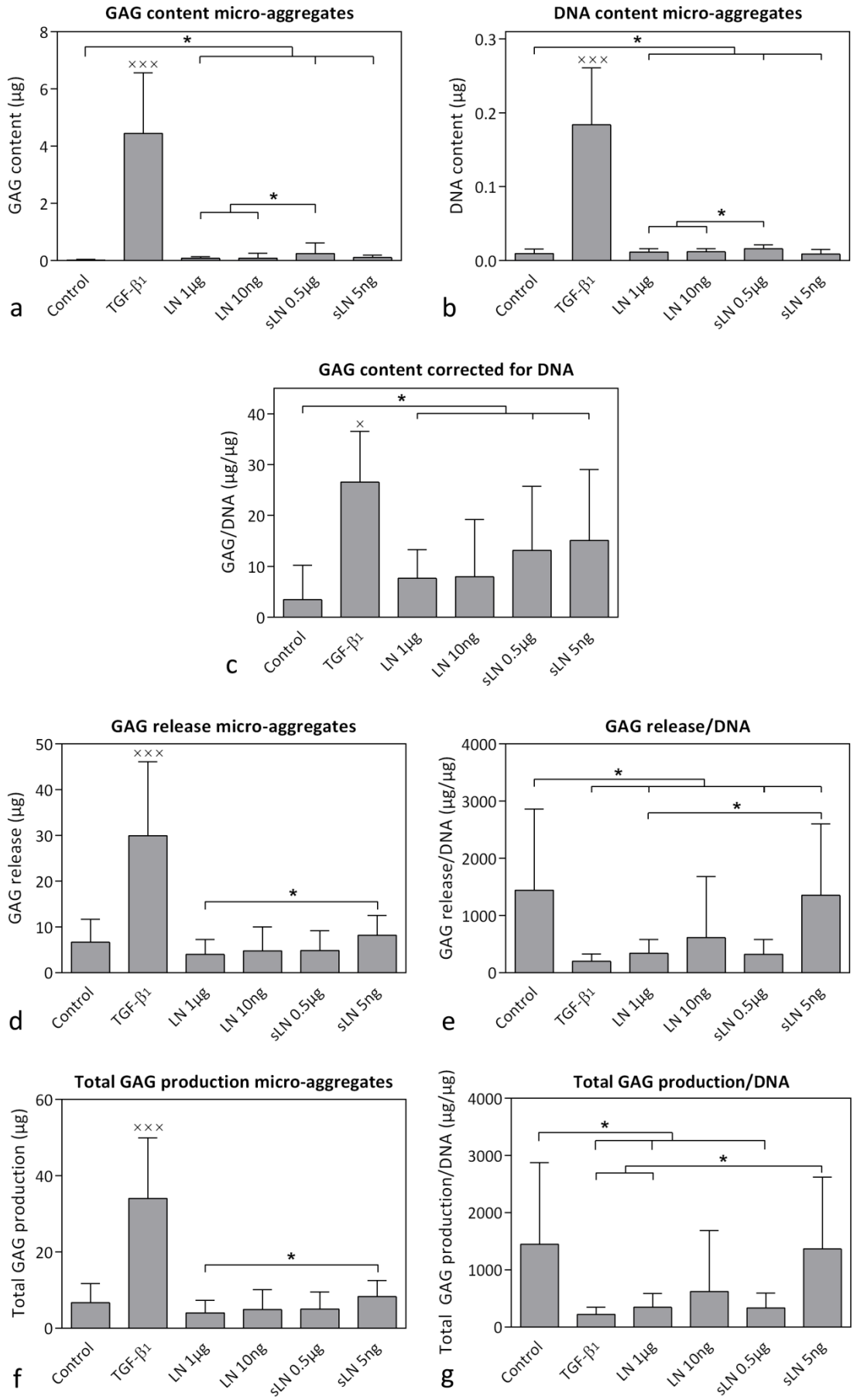

Fig. 29: Canine (s)Link-N slightly increased the DNA and GAG content of the canine nucleus pulposus cells (NPCs). GAG and DNA content (mean + SD) of canine NPC micro-aggregates cultured for 28 days in hypoxia (5%O_2) in different conditions (**a**) GAG content of the micro-aggregates. (**b**) DNA content of the micro-aggregates. (**c**) GAG content corrected for the DNA content. (**d**) Total amount of GAGs released in the culture medium by the micro-aggregates. (**e**) Total GAG release corrected for the DNA content. (**f**) Total GAG production (GAG content micro-aggregates and amount of GAGs released taken together). (**g**) Total GAG production corrected for the DNA content. n = 6 *per* condition. ∗$p < 0.05$. ×, ×××: significantly different ($p < 0.05$ and $p < 0.001$ respectively) from all other conditions (growth factor treatment).

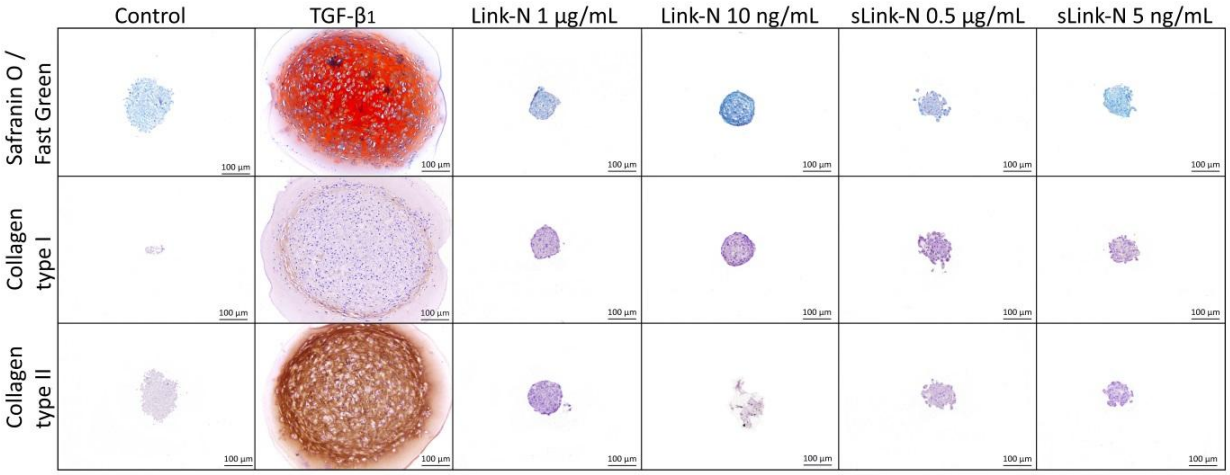

Fig. 31: Representative histological images of canine nucleus pulposus cell (NPC) micro-aggregates cultured in hypoxic conditions (5% O_2) for 28 days stained with Safranin O/Fast green and analyzed with immunohistochemistry for collagen type I and II. No effects of canine (s)Link-N treatment were detected, whereas TGF-β_1 did prominently increase GAG and collagen type II deposition.

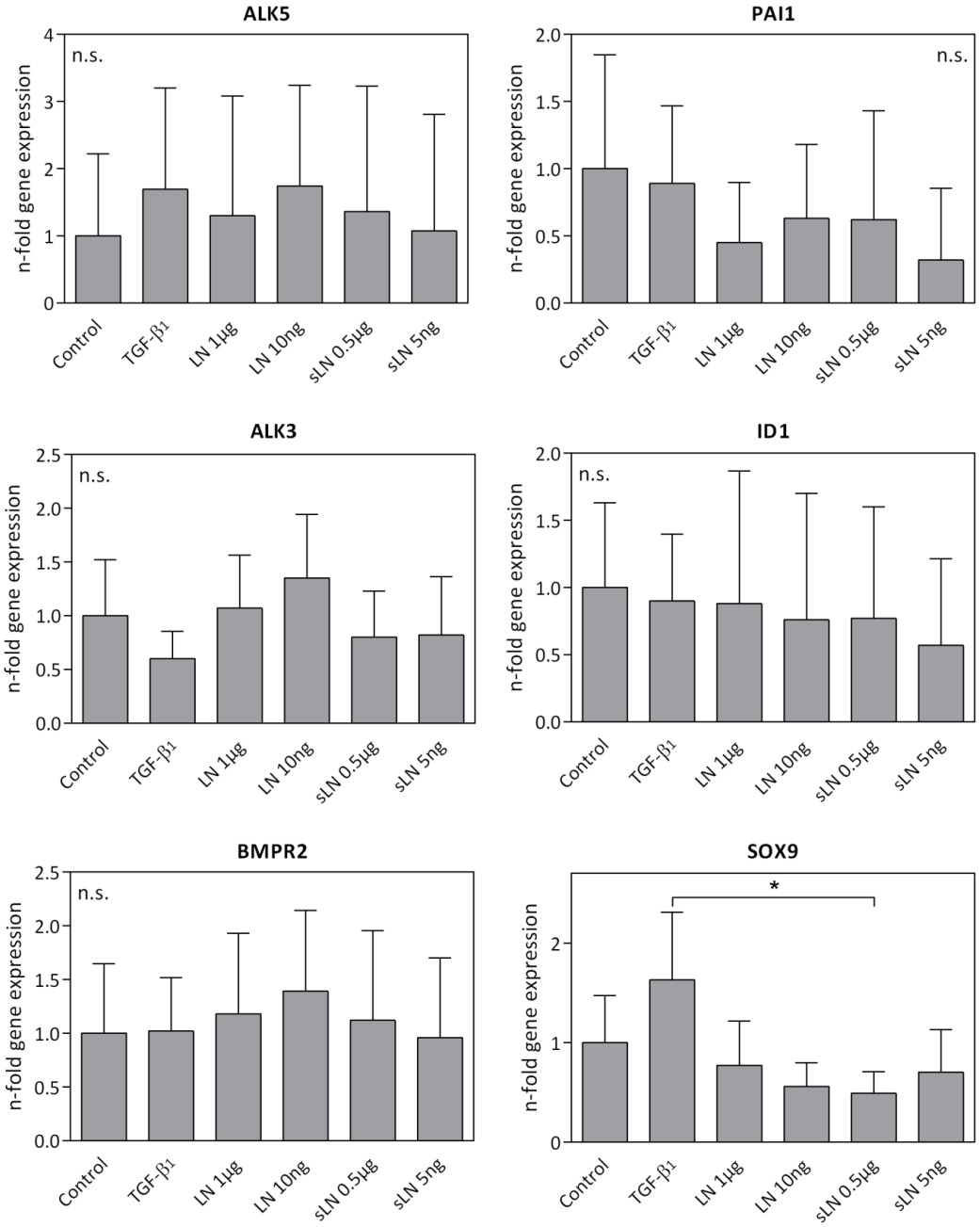

Fig. 30: No regenerative effects of (s)Link-N on gene expression level were detected. Relative *ALK5, ALK3, ID1, BMPR2, SOX9* gene expression of the micro-aggregates derived of canine nucleus pulposus cells derived from degenerated intervertebral discs treated with canine (s)Link-N at day 7 of culture under hypoxic conditions (5%, O_2). The control micro-aggregates were set at 1. $n = 6$ *per* condition. * $p < 0.05$.

DISCUSSION

Our two previous studies showed that human (s)Link did not exert regenerative effects on canine NPCs. The amino acid sequence of (s)Link-N differs between species (Unpublished data Orthopedics research group, Faculty of Medicine, McGill University) and each amino acid substitution potentially affects protein function.[70] Particularly the substitution of the third amino acid of human (s)Link-N (histidine) by leucine (bovine/canine (s)Link-N) is likely to influence the 3D structure and subsequently the workings mechanism of the peptide due to the difference in polarity. Therefore, the aim of the present study was to elucidate the regenerative effects of the canine variant of (s)Link-N on human, bovine, and especially canine NPCs derived from degenerated IVDs.

CANINE (S)LINK-N DOES NOT EXERT REGENERATIVE EFFECTS ON HUMAN NPCS

Our first study as well as the literature shows that human (s)Link-N exerts regenerative effects on NPCs derived of degenerated human IVDs *in vitro*.[41, 46] The results of the present study, however, reveal that treatment with the canine variant of (s)Link-N did not increase ECM production or cell proliferation of the human NPCs. This implies that the substitution of histidine (human variant) by leucine (canine variant) indeed influences the working mechanism of the Link-N peptide. It is likely that the species difference in 3D structure of the peptide interferes with the docking of (s)Link-N to the BMP receptor type II or other non-delineated receptors involved in the working mechanism of (s)Link-N. This confirms the finding of Hermes *et al.* (1990), which demonstrated that substitutions in the active part of a peptide are likely to affect the function of a protein.[71]

CANINE (S)LINK-N STIMULATES COLLAGEN TYPE II, BUT NO RELEVANT GAG DEPOSITION IN BOVINE NPCS

Based upon literature and the results of our first study, it could be concluded that human (s)Link-N exerted regenerative effects on bovine NPCs, in terms of ECM production and cell proliferation.[38, 43] Thus, bovine NPCs are able to react to human (s)Link treatment despite the difference in polarity compared with the bovine variant of (s)Link-N. Since the bovine and canine variant of Link-N do not differ in polarity and sLink-N is actually similar between the two species, it was hypothesized that canine (s)Link-N exerted a more potent effect on bovine NPCs. The present study, however, showed that canine (s)Link-N only slightly increased GAG deposition and had no effect on the DNA content of the bovine NPC micro-aggregates. Safranin O/Fast Green staining indicated that GAG deposition was more prominently increased by (s)Link-N treatment than measured with the DMMB-assay. An explanation for this discrepancy is that the Safranin O/Fast Green staining is a semi-quantitative method and thus provides an indication of the presence of GAGs but not of the exact amount.[51] In contrast to human (s)Link-N, canine (s)Link-N prominently increased collagen type II protein deposition in bovine NPCs. This implies that canine (s)Link-N activates other pathways than human (s)Link-N and that the species difference in amino-acid sequence thus influences the working mechanism of (s)Link-N. TGF-β_1 did not induce collagen type II protein deposition of the bovine NPCs in the first study, and neither in the current study. This indicates that bovine NPCs do not produce collagen type II protein under influence of TGF-β_1 at least when cultured in micro-aggregates. Additionally, the study of Zimber *et al*. (1995) showed that TGF-β treatment of bovine chondrocytes exerted no detectable effects on collagen type I and II as well.[54] The collagen type II deposition in the

present study was determined after culture under hypoxic conditions (5% O_2), while the first study, using human (s)Link-N, was performed under normoxic conditions (21% O_2). It has been demonstrated that human degenerative NPCs isolated, expanded, and cultivated in hypoxic conditions could better preserve the cells' regenerative potential.[49] Although the O_2-saturation did not influence the collagen type II deposition of the bovine NPCs treated with TGF-β_1, further research should determine whether the oxygen level influences the collagen type II deposition of the bovine NPCs when treated with human/canine (s)Link-N treatment.

CANINE (S)LINK-N EXERTS NO BIOLOGICALLY RELEVANT REGENERATIVE EFFECT ON CANINE NPCS

The main aim of the current study was to delineate the regenerative effects of canine (s)Link-N on canine NPCs derived from degenerated IVDs, since our previous studies showed that human (s)Link-N only exerted moderate effects, and the addition of canine MSCs did not result in additive regenerative effects. Optimizing this treatment strategy using canine (s)Link-N aimed at developing a new regenerative treatment for IVD degeneration in canine patients. Furthermore, as a step towards translation into *in vivo* use in human patients, dogs could then serve as suitable large animal model. The results of the present study, however, did not show more potent regenerative effects of the canine variant of (s)Link-N on canine NPCs. Treatment with canine (s)Link-N resulted in a slight, but significant, increase in DNA and GAG content. However, this minimal increase is not of any biological relevance. Thus, the results of this study imply that using the canine variant of (s)Link-N did not optimize the Link-N treatment efficiency. Explanations for the only mild effects of human and canine Link-N treatment on canine NPCs should therefore be investigated. The study from Dean *et al*. (2000) reported that the proteolysis and inactivation of Link-N was entirely carried out by membrane-associated peptidases.[72] Since the exact MMPs produced by canine NPCs are not delineated yet[2], it is possible that the minimal effects of (s)Link-N are due to rapid breakdown of the peptide in the culture medium. The 3D micro-aggregate culture system was validated by testing the effect of human (s)Link-N on human and bovine NPCs as well. The obtained regenerative effects on the human and bovine NPCs comparable with several other studies[38, 41, 43, 46] implied that the used batch of human and canine (s)Link-N was effective in the micro-aggregate culture system. However, it could still be that canine NPCs reside better in a hydrogel culture and require such an environment to be able to react to (s)Link-N more potently. Therefore, future studies should determine the regenerative effects of canine and human (s)Link-N on canine NPCs when cultured in hydrogels which are preferably already used in clinical practice for intradiscal cell transplantation. In the study of Benz *et al.* (2012) they injected *in vitro* expanded IVD cells, which were supported by a polyethylene glycol-crosslinked serum albumin/hyaluronan hydrogel, in ovine discs. The results indicated that this treatment did not compromise endogenous repair and may even be beneficial.[73]

It is reported that Link-N exerts its effect by direct binding to the BMP receptor type II in rabbits NPCs which initiates SMAD1/5/8 signaling and upregulates BMP proteins, including BMP-4 and BMP-7.[40] Through a cell-autonomous loop, BMP-4 and BMP-7 promote expression of *SOX9* and downstream *ACAN* and *COL2A1* genes by binding to the BMP receptor type II, which sustains or amplifies SMAD1/5/8 signal transduction.[40] It could be that this intracellular signaling pathway does not function optimally in canine NPCs, for example due to inhibition of the positive feedback loop by BMP antagonists such as noggin and/or chordin.[74] Although no expression of *NOG* was detected in

this study at day 7, it could be that its expression is upregulated at a different time point. Another possibility is that there are other receptors, beside the BMP receptor type II, involved in the working mechanism of (s)Link-N, and that there are interspecies differences in the expression of those receptors. Possibly, canine NPCs express lower numbers of these yet unknown receptors than human and bovine NPCs. The study of Wang *et al.* (2013) determining the binding of (s)Link-N to the BMP receptor type II used rabbits with an age of 6 months.[40] Rabbits have large numbers of notochordal cells (NCs) at least until 12 months of age.[7] Since humans, cows, and chondrodystrophic dog breeds may have NCs at birth, but the numbers decrease rapidly[7], this hampers the comparison with the NC-rich rabbit IVDs. Therefore, follow up studies will focus on analyzing the potential SMAD pathways which are activated in the NPCs (instead of NCs) of different species treated with the human and canine variant of (s)Link-N.

The results of the present study, together with the results of our two previous studies, imply that human and canine (s)Link-N are no suitable treatment for IVD repair in canine patients. Furthermore, the differences in response of human NPCs compared with canine NPCs to Link-N treatment, indicate that the dog is no suitable large animal model for translation of this treatment into human patients. However, since human (s)Link still has promising potential to be used as treatment for IVD degeneration in human patients, the search for another suitable large model for preclinical investigation should continue. Based upon the comparable potent effects of human (s)Link-N on bovine NPCs, cows could be considered as suitable option.[38] However, considerations of size, housing, and reproduction limit their use as animal model.[75] Since the cell type and biomechanical loading of the sheep spine is comparable to the human spine and ovine models are already extensively used in literature[76-79], sheep are a promising option. However, it first needs to be investigated whether (s)Link-N exerts comparable regenerative effects on ovine NPCs as detected on human and bovine NPCs *in vitro*.

LIMITATIONS

In the present study, the effect of canine (s)Link-N was only tested on NPCs of chondrodystrophic (CD) dogs. Since non-chondrodystrophic dogs (NCD) retain their notochordal cells throughout life, it would be interesting to delineate whether canine (s)Link-N has a more potent effect on NPCs derived of NCD dog spines. Furthermore, it remains to be elucidated whether other factors were affected by canine (s)Link-N treatment and differ from human (s)Link-N treatment than the ones measured in the present study, *e.g.* inflammatory, neurogenic or angiogenic markers. In the current study, only a micro-aggregate culture system was used, so future studies should also take other culture systems into account to investigate the influence of *e.g.* hydrogels on the regenerative potency of the NPCs.

CONCLUSIONS

Canine (s)Link exerted no regenerative effects on human NPCs derived from degenerated IVDs, presumably due to the different amino acid sequence. The increased GAG deposition by bovine NPCs induced by canine (s)Link-N was negligible, while collagen type II production was prominently increased. So the response of the bovine NPCs to canine (s)Link-N treatment differed from human (s)Link-N treatment, which potently increased GAG instead of collagen type II production. Canine

(s)Link-N exerted no biologically relevant regenerative effects on canine NPCs. Together, the results of the present study indicate that canine (s)Link-N is no suitable treatment for IVD degeneration in canine or human patients. Species differences in amino-acid sequence of (s)Link-N influence its effect, but treatment with the species own sequence does not intrinsically result in the most potent effect. To better understand the effect of human and canine sLink-N on NPCs of different species, follow up studies will focus on which intracellular SMAD signaling pathways they transduce.

ACKNOWLEDGEMENTS

We would like to thank Michael Grant (McGill University, Montreal, Canada) for the 3D conformational analysis of Link-N and docking on the canine bone morphogenetic receptor type II.

CHAPTER 4: WORKING MECHANISM OF HUMAN AND CANINE LINK-N ON HUMAN, BOVINE, CANINE NUCLEUS PULPOSUS CELLS

SUMMARY

Background. Human Link-N and its shorter variant sLink-N have shown to possess the potential to stimulate extracellular matrix production of chondrocytes and NPCs of different species and are therefore considered as a promising candidate to be used for intervertebral disc (IVD) repair. However, our previous studies revealed interspecies differences in the response of human, bovine, and canine nucleus pulposus cells (NPCs) to treatment with the human and canine variant of (s)Link-N. It is reported that human Link-N exerts its effect by direct interaction with the BMP receptor type II, followed by SMAD1 signaling, at least in rabbit NPCs The working mechanism by which (s)Link-N exerts its effect on NPCs of other species has not been delineated yet.

Objective. The present study aimed at analyzing if and which SMAD signaling pathways are activated by treatment with the human and canine variant of (s)Link-N in human, bovine, and canine NPCs.

Methods. Human, bovine, and canine NPCs were cultured in monolayers in hypoxic conditions and stimulated for 24 hours with or without TGF-β_1 (10 ng/mL), BMP-2 (250 ng/mL), human or canine Link-N (1 µg/mL) or sLink-N (0.5 µg/mL). Activated signaling pathways were determined by measuring phosphorylated (p)SMAD1 and 2 levels and gene expression profiling.

Results. Although not significant, both pSMAD1 and pSMAD2 levels were increased compared with the untreated controls in human and bovine NPCs by treatment with the human and canine variant of (s)Link-N. Beside gene expression of BMP-2 pathway markers (*ALK1, ID1, BMP4*), also expression of TGF-β_1 pathway markers (*ALK5, PAI1*) was increased in human NPCs by treatment with mainly canine (s)Link-N. Both human and canine Link-N significantly increased pSMAD1 levels in canine NPCs, whereas only canine Link-N treatment significantly increased pSMAD2 levels.

Conclusions. The human and canine variant of (s)Link-N induced a slight increase in both SMAD1 and SMAD2 signaling in human, bovine, and canine NPCs, indicating activation of both the TGF-β_1 and BMP-2 pathway. This does not explain the interspecies differences in the potency of the regenerative effects that human and canine (s)Link-N exert on the NPCs. Further research investigating the receptor interactions and degradation of human and canine (s)Link-N by the NPCs of different species will hopefully provide more decisive answers.

INTRODUCTION

Intervertebral disc (IVD) degeneration is considered as one of the major causes of back pain in both humans and dogs. During IVD degeneration, increased matrix catabolism and a decreased synthesis of aggrecan and collagen type II occurs.[2] Additionally, this process is associated with cell loss through apoptosis.[2] Thus, to accomplish functional IVD restoration, it is necessary to increase extracellular matrix (ECM) production and cell proliferation. This can be stimulated using exogenous growth factors, such as transforming growth factor beta (TGF-β) and bone morphogenetic proteins (BMPs).[33, 34] There are three subtypes of TGF-β, *i.e.* TGF-β_1, -β_2, and -β_3 from with subtype TGF-β_1 is most often used in regenerative cartilage medicine.[80] BMP-2, BMP-4, BMP-6, and BMP-7 are the most commonly used subtypes of the BMP family.[81] All of them show to possess chondrogenic abilities, but it has been reported that BMP-2 is most essentially involved in skeletal development.[82] The specific signaling pathways of TGF-β_1 and BMP-2, which both results in increased ECM production and cell proliferation, are described below.

TGF-B1 SIGNALING PATHWAY

The cascade starts with the binding of TGF-β_1 to the TGF-β_1 receptor type II, which is an active kinase.[83] This receptor then phosphorylates and thereby activates the TGF-β_1 receptor type I, known as the activating receptor-like kinase-5 (ALK5).[83] Next, ALK5 phosphorylates SMAD2 and 3 which allows them to form a complex with SMAD4. This complex is transferred into the nucleus of the cell, where it interacts with several co-receptors, co-activators and transcription factors.[83] One of those transcription factors is SOX9, which is considered as the master regulator of chondrogenic differentiation.[44, 84] *SOX9* expression is essential for ECM specific gene expression of collagen type 2 (*COL2A1*) and aggrecan (*ACAN*).[85] Beside SOX9, other members of the SOX-family are transcribed as well, such as SOX5 and SOX6.[86] By binding to the SMAD-complex, SOX9 is capable of recruiting a co-activator complex known as CREB-binding protein (CBP)/p300.[44] Subsequently, SOX9 associates with the SOX9 binding site and thereby activates the *COL2A1* enhancer, stimulating the transcription of *COL2A1* (Fig. 32).[44] This connection is secured by the binding of SOX5 and 6.[86] SOX9 stimulates the expression of *ACAN* by binding to and activating the *ACAN* enhancer in a similar way as that of *COL2A1*.[86, 87] However, *ACAN* transcription is beside this pathway also regulated by other pathways, such as the mitogen activated protein kinase (MAPK) pathway.[88] This pathway involves extracellular

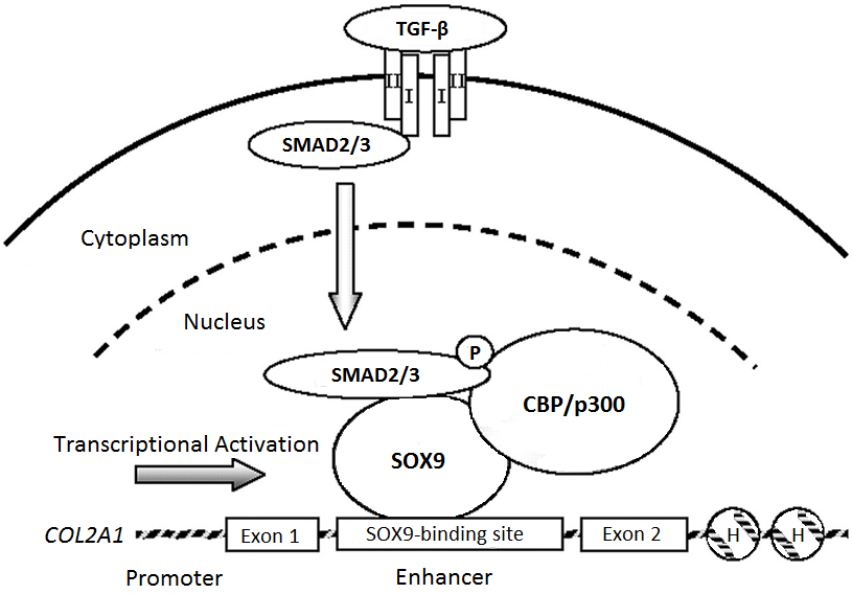

Fig. 32: The TGF-β / SMAD2/3 signaling pathways promote the SOX9-dependent transcriptional activity by facilitating the recruitment of CBP/p300 on the *COL2A1* enhancer. Modified from Furumatsu *et al*. (2005).[44]

signal regulated protein kinase (ERK)1/2 and p38 kinase.[88] The SMAD and MAPK pathways are both activated by TGF-β_1 and regulate *ACAN* expression at the same time. However, it is reported that after prolonged TGF-β_1 stimulation, the regulation of *ACAN* expression is predominantly dependent on the MAPK signaling pathway.[89]

BMP-2 SIGNALING PATHWAY

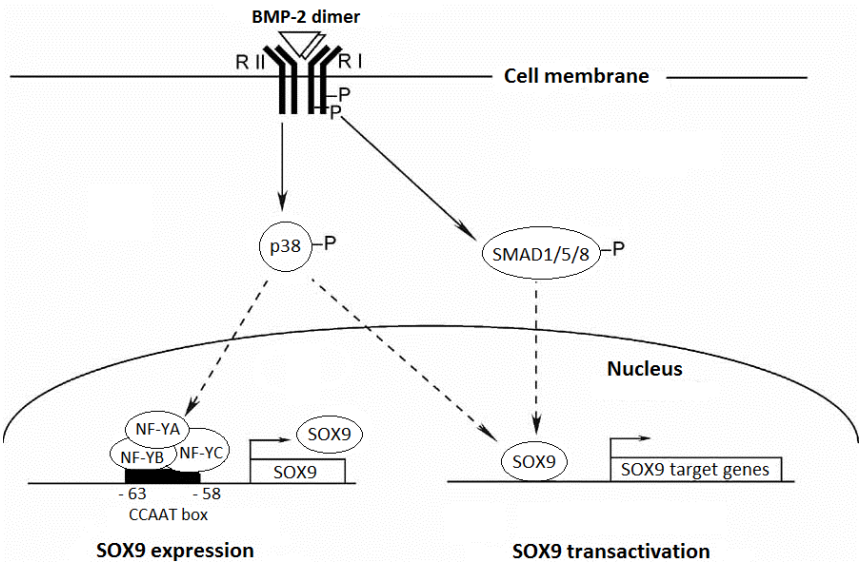

Fig. 33: Schematic model of the pathways by which BMP-2 stimulates SOX9. BMP-2 binds to the BMP receptors and stimulates both p38 and SMAD phosphorylation. Activation of p38 kinase may increase the binding of NF-Y transcription activator to the proximal promoter of SOX9 gene, and enhance SOX9 activity to regulate the expression of it's target genes. Modified from Pan *et al.* (2008).[94]

BMP-2 exerts its signal via the BMP receptor type I and II, which are transmembrane serine/threonine kinase receptors. There are different kinds of type I receptors but BMP-2 preferably binds to BMP receptor type IA (ALK3) and BMP receptor type IB (ALK6).[90-92] Heteromeric complexes of the type I and II receptors are formed prior or after binding of BMP-2.[93] When BMP-2 binds to preformed receptor complexes it induces SMAD1/5/8 signaling, whereas when complexes are formed after BMP-2 binding it results in activation of the MAPK signaling pathway.[93] Thus, when BMP-2 docks on the preformed complex, BMP receptor type II is being activated and phosphorylates ALK3 (BMP receptor type IA) or ALK6 (BMP receptor type IB).[90-92] These type I receptors phosphorylate SMAD1/5/8 which on their turn form a complex with SMAD4. When transferred into the nucleus, this complex modulates gene transcription by interacting with co-activators, -repressors and transcription factors.[91] Similar to the TGF-β_1 signaling pathway, this also results in increased *SOX9* expression (Fig. 33).[94] However, when BMP-2 docks on non-preformed receptor complexes, p38-MAPK gets activated, which results in binding of the transcription activator complex NF-Y/p300 to the promoter of the *SOX9* gene (Fig. 33).[94] This leads to increased *SOX9* expression and subsequently increased *ACAN* and *COL2A1* expression as described above.

LINK-N SIGNALING PATHWAY

However, the use of the aforementioned growth factors is limited because of their high costs and potential side effects.[35, 36] A review about the 'dark sides' of tissue repair reported that TGF-β may be responsible for tissue damage caused by scarring in many diseases.[36] And a review, assessing the effectiveness and harms of BMP-2 in spinal fusion, reported that the use of BMP-2 in anterior cervical spine fusion was associated with increased risk for wound complications and dysphagia.[35] Therefore alternative agents, with growth factor-like properties, are sought. Human Link-N and its

shorter variant sLink-N have shown to possess the potential to stimulate ECM production of chondrocytes and NPCs and are therefore considered a promising candidates.[37, 39, 43, 46] It is reported that Link-N exerts its effect by direct interaction with the BMP receptor type II, at least in rabbit NPCs.[40] This interaction initiates SMAD1/5/8 signaling and upregulates BMP proteins, including BMP-4 and BMP-7. Through a cell-autonomous loop, BMP-4 and BMP-7 promote expression of *SOX9* and downstream *ACAN* and *COL2A1* genes by binding to the BMP receptor type I, thereby sustaining or amplifying SMAD1/5/8 signal transduction (Fig. 6).[40] However, rabbit IVDs contain large numbers of notochordal cells (NCs) at least until 12 months of age.[7] Since human, bovine, and chondrodystrophic dog breed IVDs may contain NCs at birth but numbers decrease rapidly, comparison with the rabbit IVDs is hampered.[7] Therefore, the working mechanism by which (s)Link-N exerts it effect on NPCs of other species should be investigated as well. Our first study (chapter 1) demonstrated that human (s)Link-N exerted regenerative effects on human and bovine NPCs, but that canine NPCs responded only moderately to human (s)Link-N. These results may imply that human (s)Link-N transduces signaling pathways in bovine and human NPCs more potently compared with canine NPCs. Furthermore, interspecies differences in NPC response to canine compared with human (s)Link-N were detected (chapter 3). Since the human and canine variant of (s)Link-N differ in amino-acid sequence and 3D structure (chapter 3), it could be that this influences the docking on receptors and subsequently the activation of signaling pathways. Therefore, the present study aimed at delineating the working mechanism of human and canine (s)Link-N by analyzing whether and to what extent human and canine (s)Link-N activate signaling pathways in human, bovine and canine NPCs. Based upon the study of Wang *et al.* (2014) we mainly focused at investigating the activation of the TGF (SMAD2/3) and BMP (SMAD1/5/8) pathway.[40]

MATERIALS AND METHODS

HARVEST AND ISOLATION OF THE NPCS

The harvest and isolation of the canine NPCs was performed following the same protocol as described in chapter 1. Human and bovine NPCs were supplied by other research institutes, but were harvested in a similar manner as the canine NPCs.

NPC MONOLAYER CULTURE

Since micro-aggregates do not contain enough protein for performing an ELISA, a 2D monolayer culture system was used in the current study. Therefore, NPCs from degenerated IVDs of human, canine and bovine donors (Table 12) were plated in a density of 200,000 cells per well (12-wells plate, 665180, Greiner CELLSTAR®) in expansion medium containing hgDMEM+Glutamax (31966, Invitrogen, Paisley, UK) with 10% FBS (Gibco 16000-044, Life Technologies, Bleiswijk, the Netherlands), 1% P/S (P11-010, GE Healthcare Life Sciences, Eindhoven, the Netherlands), 0.1 mM Ascorbic acid 2-phosphate (A8960, Sigma-Aldrich, Saint Louis, USA), 10^{-9} M dexamethasone (AD1756, Sigma-Aldrich, Saint Louis, USA) and 1 ng/mL bFGF (PHP105, AbD Serotec, Puchheim, Germany) in an incubator under hypoxic conditions (5% O_2, 5% CO_2, 37°C). After 2 days, the expansion medium was replaced by basal culture medium (hgDMEM+Glutamax with 1% P/S, 1% ITS + premix (354352, Corning Life Sciences, Amsterdam, the Netherlands), 0.04 mg/mL L-proline (P5607, Sigma-Aldrich, Saint Louis, USA), 0.1 mM Ascorbic acid 2-phosphate, and 1.25 mg/mL Bovine Serum Albumin (Sigma

Aldrich, A9418)) for the negative control group. Basal culture medium supplemented with 10 ng/mL human recombinant TGF-β_1 (240-B, R&D Systems, Inc., Minneapolis, USA) or 250 ng/mL BMP-2 (generous gift by TETEC AG) was used to create the positive control groups for SMAD2 and SMAD1 signaling levels, respectively. The other treatment groups were created by addition of basal culture medium supplemented with 1 µg/mL human Link-N (DHLSDNYT-LDHDRAIH; CanPeptide, Montreal, Canada), 0.5 µL human sLink-N (DHLSDNYT; CanPeptide, Montreal, Canada), 1 µg/mL canine Link-N DHHSDNYT-LNYDRVIH; CanPeptide, Montreal, Canada) or 0.5 µg/mL canine sLink-N (DHHSDNYT; CanPeptide, Montreal, Canada). The cells were stimulated for 24 hours (based on the study of Wang *et al.*[40]) in an incubator under hypoxic (5% O_2, 5% CO_2, 37°C) conditions.

Table 12: Overview of the used donors in the study and their characteristics

Number	Gender	Species	Thompson score	Age (years)
RT-qPCR				
S14-80	Female	Human	III	63
S13-022	Male	Human	III	68
S13-023	Male	Human	III	66.5
S14-043	Female	Human	III	63
S14-212	Male	Human	III	44.5
S14-219	Male	Human	III	47
ELISA				
S14-80	Female	Human	III	63
S13-142	Female	Human	III	47
S13-023	Male	Human	III	66.5
S14-212	Male	Human	III	44.5
S14-219	Male	Human	III	47
B1	Unknown	Bovine	I/II	± 2
B2	Unknown	Bovine	I/II	± 2
B3	Unknown	Bovine	I/II	± 2
B4	Unknown	Bovine	I/II	± 2
B5	Unknown	Bovine	I/II	± 2
1399617	Male	Canine (Beagle)	III	4.5
7140151	Male	Canine (Beagle)	III	5.5
7283679	Male	Canine (Beagle)	III	6
13055503	Female	Canine (Beagle)	III	2
7207859	Male	Canine (Beagle)	III	5.5

PROTEIN COLLECTION

For protein collection the monolayer samples were homogenized in RIPA buffer containing 0.6 mM phenylmethylsulphonyl fluoride, 17 µg/mL aprotinin and 1 mM sodium orthovanadate (Sigma-Aldrich). 200 µL of this RIPA buffer was added to each well and incubated for 30 minutes on ice, where after the wells were scratched and the cell lysate was collected. After centrifugation (1500 g, 4°C, 10 minutes)the supernatant was pipetted and stored at -20°C until further use.

TOTAL PROTEIN MEASUREMENTS

The total protein of the NPC lysate samples was measured using the Qubit® Protein Assay Kit (Q32851, Invitrogen). Firstly, Qubit® working solution was prepared by diluting the Qubit® Protein Reagent 1:200 in Qubit® Protein Buffer. Thereafter, the standard assay tubes were prepared by adding 10 µL standard from the kit to 190 µL of working solution. The sample assay tubes were prepared by adding 1 µL of sample to 199 µL of working solution. All tubes were vortexed for 2-3 seconds and incubated for 15 minutes at room temperature. After determining the standard line using the standard assay tubes, the assay tubes were inserted in Qubit® 2.0 and readings were taken.

ELISA PSMAD1 AND 2

A sandwich ELISA kit was used to perform an *in vitro* enzyme-linked immunosorbent assay (ELISA) to measure the phosphorylated SMAD1 (SER463/465, PEL-SMAD1-S463, RayBiotech) and SMAD2 levels (S245/250/255, PEL-SMAD2-S245, RayBiotech) of the human, bovine, and canine NPC lysate samples. First, a positive control (standard line) was prepared: 400 µL of Assay Diluent was added to the positive control vial, resulting in a P-1 solution. 300 µL of Assay Diluent was pipetted into 4 different tubes, after which 150 µL of P-1 was added to another tube to produce P-2, 150 µL of P-2 to produce P-3, completing the dilution series until P-5. The NPC lysate samples were not diluted for SMAD2 detection, whereas samples for SMAD1 detection were diluted 5 times. 100 µL of each sample or positive control was pipetted into appropriated wells, which were coated with an anti-pan SMAD1 or anti-pan SMAD2 antibody. The wells were covered with a plate holder and incubated for 2.5 hours at room temperature on a shaker. The present SMAD1 or SMAD2 in the sample was bound to the wells by the immobilized antibody. Next, the dilution was discarded and the wells were washed four times with 1x Wash Solution to wash away the unbound antibody. Washing was performed by adding 300 µL of Wash Buffer to each well, after which the liquid was removed. Next, 100 µL of prepared detection antibody, anti-phosphoSMAD1 (Ser463/465) or anti-phosphoSMAD2 (Ser245/250/255), was added and incubated for 1 hour at room temperature on the shaker. The solution was discarded and again the wells were washed for four times using 1x Wash Solution. Afterwards, 100 µL of prepared HRP-conjugated anti-rabbit IgG against anti-SMAD1 (Ser463/465) or anti-SMAD2 (Ser245/250/255) was added to each well and incubated for 1 hour at room temperature on the shaker. After the solution was discarded, the wash step was repeated four times and each well was filled with 100 µL of TMB One-Step Substrate Reagent. Incubation occurred for 30 minutes at room temperature in the dark with shaking. As final step 50 µL of Stop Solution was added to each well. Immediately after adding the solution, the plate was read at 450 nm using the DTX880 Multimode Detector (Beckman Coulter Nederland B.V., Woerden, The Netherlands). The control samples were set at 1 where after the relative SMAD phosphorylation of the samples in the other treatment groups was calculated.

GENE EXPRESSION PROFILING

Gene expression profiling was performed on the NPC lysate of a pool of human donors (Table 12) cultured in monolayers as described previously. RNA isolation of the samples followed by cDNA

synthesis and RT-qPCR were performed following the same protocols as described in the first chapter.

QUANTITATIVE RT-PCR

HUMAN NPC MONOLAYERS

Four reference genes (TATAA-box binding protein (*TBP*), hypoxanthine-guanine phosphoribosyltransferase (*HPRT*), succinate dehydrogenase subunit A (*SDHA*) and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide *(YWHAZ*)) were used to normalize gene expression of the target genes (inhibitor of DNA binding-1 (*ID1*), plasminogen activator inhibitor-1 (*PAI1*), activin receptor-like kinase 1 (*ALK1*), TGF-β receptor type I (*ALK5*) bone morphogenetic protein 4 (*BMP4*) and SRY-box 9 (*SOX9*)) (Table 13).

Table 13: Overview of the primers used to asses gene expression of the human NPCs

Genes	Forward sequence 5' → 3'	Reverse sequence 5' → 3'	Amplicon size	Annealing temp (°C)
Reference genes				
HPRT	TATTGTAATGACCAGTCAACAG	GGTCCTTTTCACCAGCAAG	192	60
SDHA	TGGGAACAAGAGGGCATCTG	CCACCACTGCATCAAATTCATG	86	58
TBP	TGCACAGGAGCCAAGAGTGAA	CACATCACAGCTCCCCACCA	132	63.5
YWHAZ	ACTTTTGGTACATTGTGGCTTCAA	CCGCCAGGACAAACCAGTAT	94	64
Target genes				
ACAN	CAACTACCCGGCCATCC	GATGGCTCTGTAATGGAACAC	160	63.5
ADAMTS5	GCCAGCGGATGTGCAAGC	ACACTTCCCCCGGACGCAGA	130	62.5
ALK1	GCAACCTGCAGTGTTGCATC	CGGATCTGCTCGTCCAGCAC	139	62.5
ALK5	GCCGTTTGACTGAAGGCTG	GGGCATCCCAAGCCTCATC	146	61
BAX	GGACGAACTGGACAGTAACATGG	GCAAAGTAGAAAAGGGCGACAAC	150	60
BCL2	ATCGCCCTGTGGATGACTGAG	CAGCCAGGAGAAATCAAACAGAGG	125	64
BMP4	CCGCAGCCTAGCAAGAGTG	GCTCAGGATACTCAAGACCAGTG	115	58
CASP3	CAGTGGAGGCCGACTTCTTG	TGGCACAAAGCGACTGGAT	102	58
COL1A1	TCCAACGAGATCGAGATCC	AAGCCGAATTCCTGGTCT	191	61
COL2A1	AGGGCCAGGATGTCCGGCA	GGGTCCCAGGTTCTCCATCT	195	63.5
COL10A1	CACTACCCAACACCAAGACA	CTGGTTTCCCTACAGCTGAT	225	61
CCND1	AGCTCCTGTGCTGCGAAGTGGAAAC	AGTGTTCAATGAAATCGTGCGGGGT	480	65
ID1	CTCTACGACATGAACGGCTGT	TGCTCACCTTGCGGTTCTG	76	65
MMP13	TCCCAGGAATTGGTGATAAAGTAGA	CTGGCATGACGCGAACAATA	123	64
PAI1	GCTGGTGAATGCCCTCTAC	GGCAGCCTGGTCATGTTG	318	65
SOX9	CCCAACGCCATCTTCAAGG	CTGCTCAGCTCGCCGATGT	242	65.5
TIMP1	CTTCTGGCATCCTGTTGTTG	GGTATAAGGTGGTCTGGTTG	153	64

STATISTICAL ANALYSIS

For the statistical analysis, IBM SPSS statistics 22 was used. To check whether the data were normally distributed, a Shapiro Wilks test was performed. General linear regression models based on ANOVAs were used for normally distributed data, whereas a Kruskal Wallis and Mann-Whitney U test were performed on non-normally distributed data. To correct for multiple comparisons, all tests were followed by a Benjamini & Hochberg False Discovery Rate *post-hoc* test. *P*-values < 0.05 were considered significant.

HUMAN NPCS

Although not significant, human and canine (s)Link-N slightly increased pSMAD1 and pSMAD2 levels of the human NPCs after 24 hours o

f stimulation compared with the untreated controls (Fig. 34a, b). As positive control for the BMP-2 signaling pathway, BMP-2 treatment induced the highest pSMAD1 levels ($p < 0.05$; Fig. 34a). Though not significant, an increase in pSMAD2 was detected after BMP-2 stimulation as well (Fig. 34b). Most pSMAD2 was detected after TGF-β_1 treatment, however this increase was not significant due to large donor variations (Fig. 34b). The ratio between pSMAD1/2 was the highest after BMP-2 treatment, but again not significant due to the high standard deviation and small samples size (Fig. 34c). No prominent differences between the potency of the different (s)Link-N variants were detected.

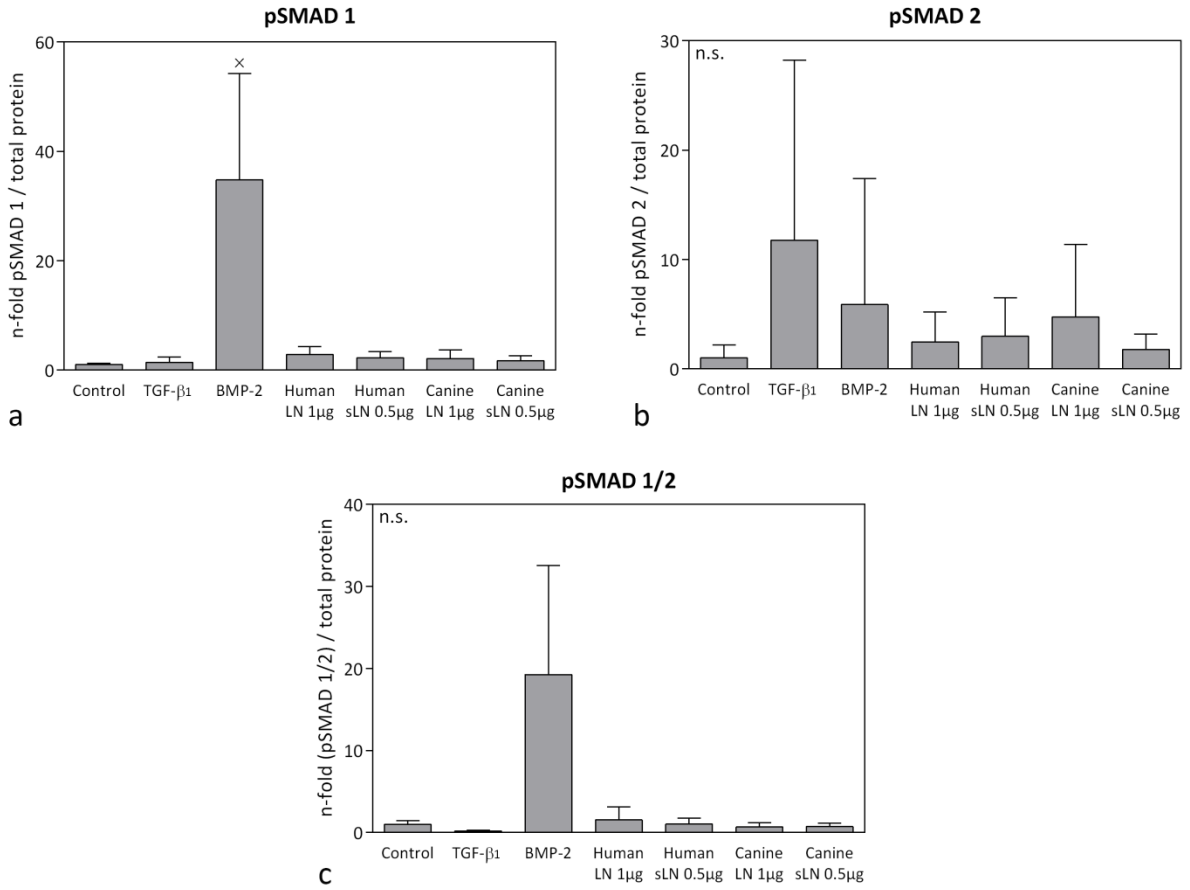

Fig. 34: A slight, not significant, increase in pSMAD1 and 2 was obtained by treatment with human and canine (s)Link-N. Relative pSMAD levels (mean + SD) of human NPC monolayers stimulated for 24 hours in hypoxia (5% O_2) with different treatments. (**a**) Relative pSMAD1 levels. (**b**) Relative pSMAD2 levels. (**c**) Relative pSMAD1/2 levels. TGF-β_1 and BMP-2 treatment were taken along as positive control for SMAD activation. The untreated control micro-aggregates were set at 1. $n = 5$ *per* condition. ×: significantly different ($p < 0.05$) from all other conditions.

At gene expression level, BMP-2 treatment induced the highest *ACAN* expression after 24 hours of stimulation, whereas TGF-β_1 treatment induced the most prominent *COL1A1* expression (Fig. 35). *ACAN* and *COL1A1* expression were also slightly increased by treatment with (s)Link-N compared with the untreated control except for the human variant of Link-N (Fig. 35). In line with the low *ACAN* expression, human Link-N treatment induced the highest *ADMATS5* (protease enzyme) expression (Fig. 35). The expression of *TIMP1*, also a matrix metalloproteinase, was increased by all treatments compared with the untreated controls except for treatment with human Link-N (Fig. 35). Gene expression of the apoptotic markers *BAX* and *CASP3* were most prominently increased by treated with the canine variant of (s)Link compared with the untreated control (Fig. 35). Cell proliferation marker *CCND1* expression was increased by all treatments, except for the human variant of Link-N, compared with the untreated control (Fig. 35). Canine (s)Link-N treatment induced the most *ALK1* (BMP receptor type I) expression whereas, in line with the pSMAD1 results, BMP-2 treatment induced the most *ID1* (read-out of the BMP pathway) (Fig. 35). *BMP4* and *SOX9* expression were the highest after treatment with the canine variant of (s)Link-N. As expected TGF-β_1 treatment increased *PAI1* (read-out of the TGF-β_1 pathway) most prominently (Fig. 35). Expression of *COL2A1, COL10A1, BCL2, MMP13* were too low to detect.

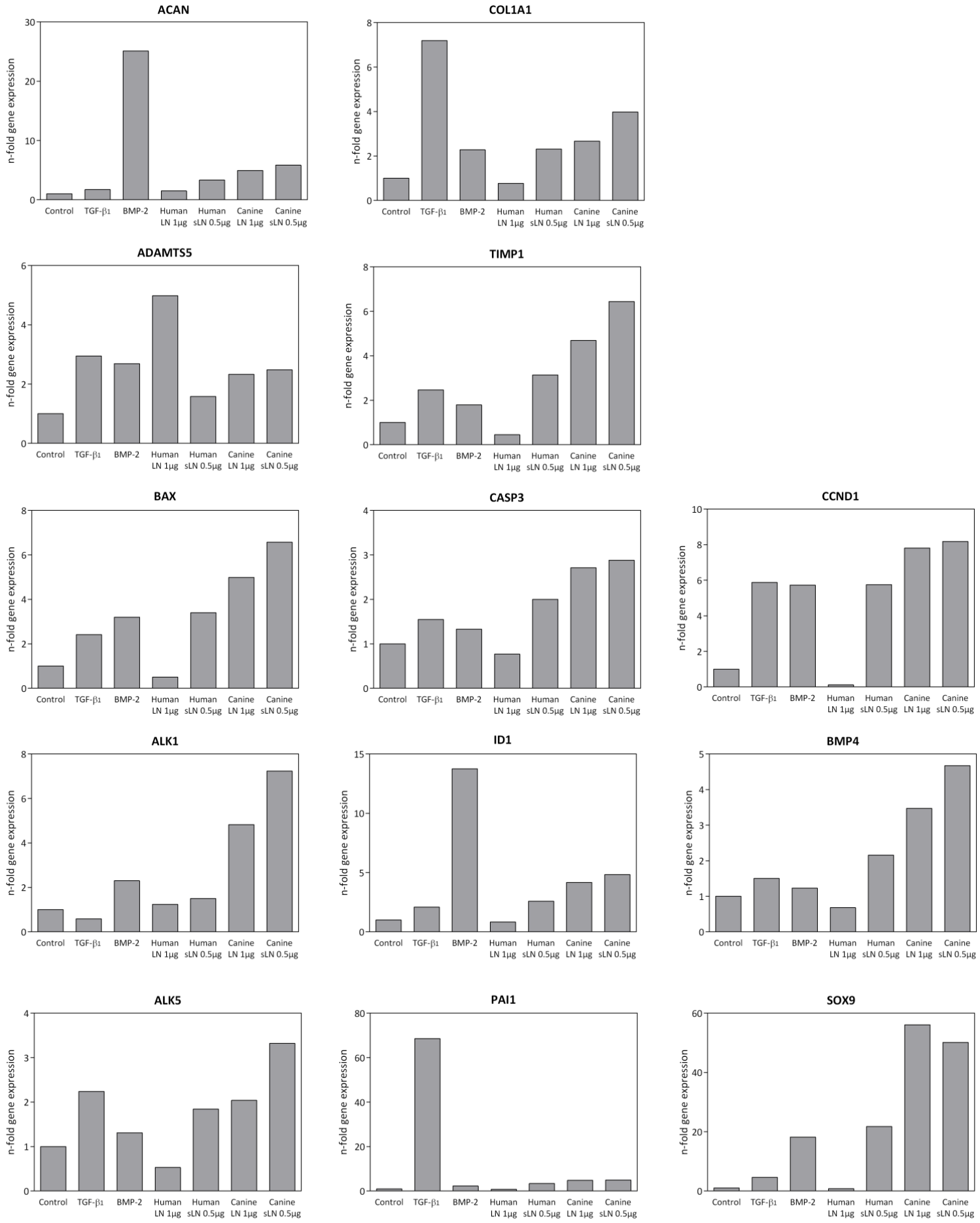

Fig. 35: Relative *ACAN, COL1A1, ADAMTS5, TIMP1, BAX, CASP3, CCND1, ALK1, ID1, BMP4, ALK5, PAI1* and *SOX9* gene expression of human nucleus pulposus cells, derived from degenerated intervertebral discs, cultured in monolayers and stimulated for 24 hours under hypoxic conditions (5%, O_2). TGF-β_1 and BMP-2 treatment were taken along as positive control for the activation of the signaling pathways. The untreated control micro-aggregates were set at 1. *n* = 1 (pool of 6 donors).

BOVINE NPCS

Human and canine (s)Link-N slightly increased pSMAD1 and pSMAD2 of the bovine NPCs after 24 hours compared with the untreated controls. This minimal increase was, however, too small to be significantly different (Fig. 36a, b). As positive control for the BMP signaling pathway, BMP-2 treatment induced the highest pSMAD1 levels ($p < 0.05$; Fig. 36a). Though not significant, an increase in pSMAD2 compared with the untreated controls was also detected after BMP-2 stimulation (Fig. 36b). Most pSMAD1 was detected after TGF-β_1 treatment, however this increase was not significant due to large donor variations and small sample size (Fig. 36b). The ratio between pSMAD1/2 was the highest after BMP-2 treatment, whereas TGF-β_1 treatment resulted in a significantly lower ratio compared with the untreated controls ($p < 0.05$; Fig. 36c).

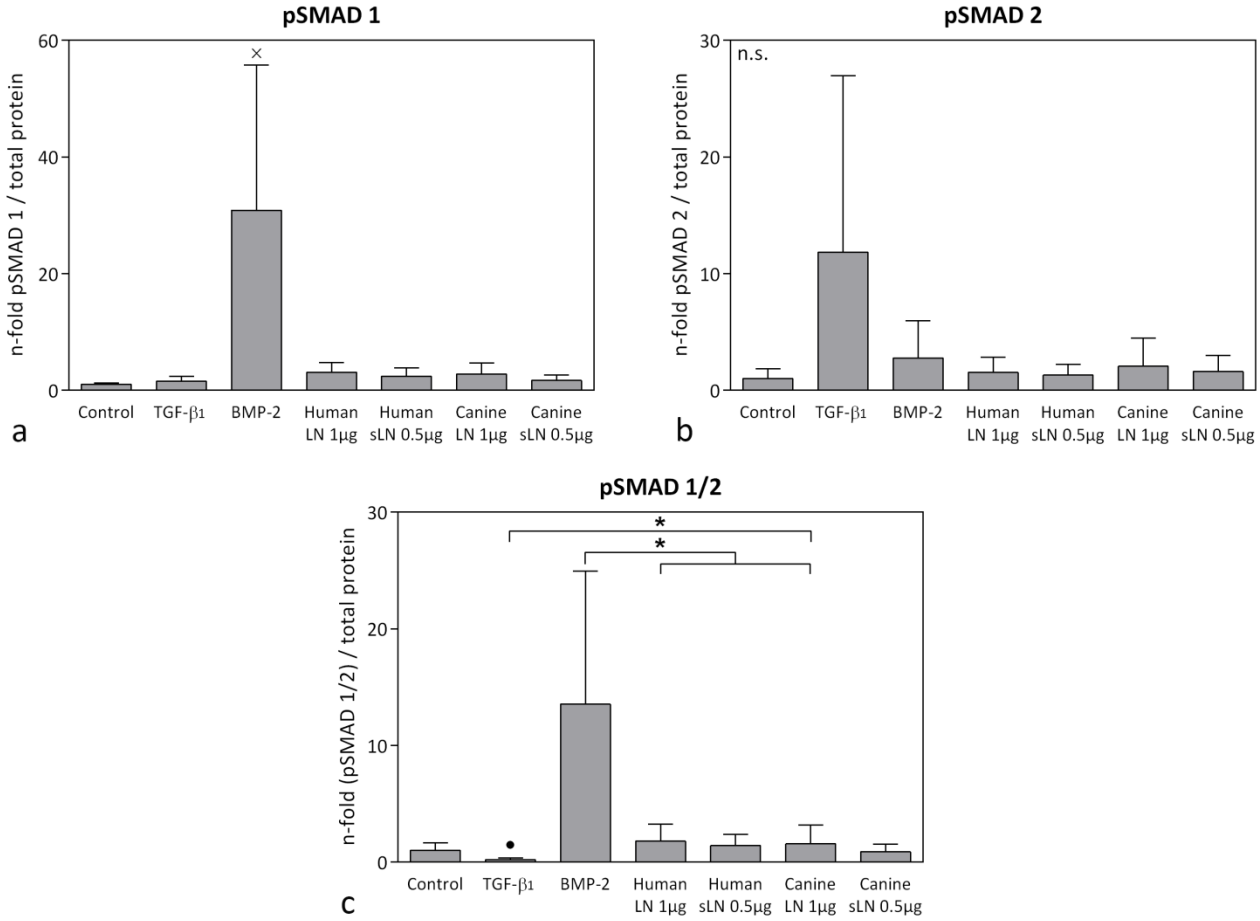

Fig. 36: A slight, not significant, increase in pSMAD1 and 2 was obtained by treatment with human and canine (s)Link-N Relative pSMAD levels (mean + SD) of bovine NPC monolayers stimulated for 24 hours in hypoxia (5% O_2) with different treatments. (**a**) Relative pSMAD1 levels. (**b**) Relative pSMAD2 levels. (**c**) Relative pSMAD1/2 levels.. TGF-β_1 and BMP-2 treatment were taken along as positive control for SMAD activation. The untreated control micro-aggregates were set at 1. $n = 5$ *per* condition. ∗: $p < 0.05$. ×: significantly different ($p < 0.05$) from all other conditions. ● : significantly different ($p < 0.05$) from the untreated controls.

CANINE NPCS

Treatment of the canine NPCs with both the human and canine variant of (s)Link-N resulted in a slight, but significant, increase in SMAD1 phosphorylation compared with the untreated controls ($p < 0.05$; Fig. 37a). BMP-2 treatment resulted in most SMAD1 activation, but due to small sample size and large donor variations this increase was only borderline significant ($p = 0.06$; Fig. 37a). Treatment with TGF-β_1 also induced significantly more SMAD1 activation compared with the untreated controls ($p < 0.05$; Fig. 37a). In general, the SMAD1 pathway was more potently activated in canine NPCs compared with human and bovine NPCs (Fig. 34a, Fig. 36a, Fig. 37a). Treatment with canine Link-N induced increased pSMAD2 levels compared with the untreated controls ($p < 0.05$; Fig. 37b) and TGF-β_1 treatment induced significantly more SMAD2 signaling compared with all (s)Link-N treatments ($p < 0.05$; Fig. 37b). The ratio between SMAD1/2 activation was, as expected, the highest in the NPCs treated with BMP-2 ($p < 0.05$; Fig. 37c). TGF-β_1 treatment resulted in a significantly higher ratio compared with the untreated controls ($p < 0.05$; Fig. 37c).

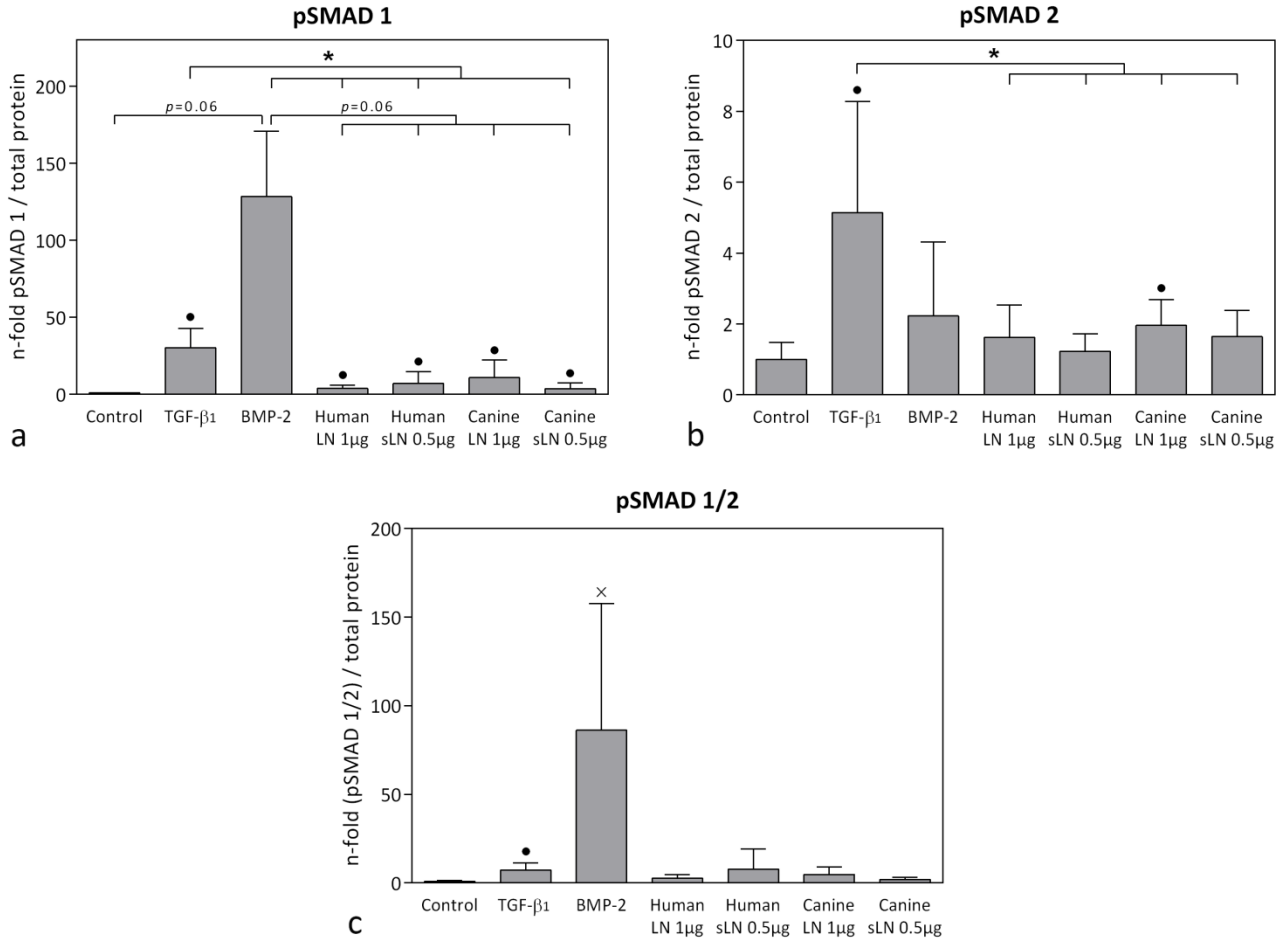

Fig. 37: No significant increase in pSMAD1, 2 or 1/2 is obtained by treatment with human and canine (s)Link-N. Relative pSMAD levels (mean + SD) of human NPC monolayers stimulated for 24 hours in hypoxia (5% O_2) with different treatments. (**a**) Relative pSMAD1 levels. (**b**) Relative pSMAD2 levels. (**c**) Relative pSMAD1/2 levels. TGF-β_1 and BMP-2 treatment were taken along as positive control for SMAD activation. The untreated control micro-aggregates were set at 1. $n = 5$ *per* condition. ∗: $p < 0.05$. × : significantly different ($p < 0.05$) from all other conditions. ● : significantly different ($p < 0.05$) from the untreated control.

DISCUSSION

The working mechanism by which Link-N exerts its regenerative effects has only been investigated on rabbit IVD cells. Since our previous studies showed differences in the effect of canine and human (s)Link-N on NPCs of different species, the present study focused on analyzing the activated SMAD signaling pathways by the human and canine variant of (s)Link-N in human, bovine and canine NPCs as a first step towards delineating the working mechanism of Link-N.

HUMAN AND CANINE LINK-N SLIGHTLY INCREASE BOTH SMAD1 AND 2 SIGNALING IN HUMAN, BOVINE NPCS

Although not significant, the results of the present study revealed that the human and canine variant of (s)Link-N slightly induced both SMAD1 and 2 signaling of human and bovine NPCs after 24 hours of stimulation. The study by Wang *et al.* (2013) showed that human Link-N increased phosphorylation of SMAD1/5 within 2 hours, peaking by 24 hours, and remaining at peak steady-state levels for at least 48 hours. No significant effect on SMAD2/3 levels was observed in their study at any time point.[40] In contrast with our study, the Wang *et al.* study used Western Blot to determine pSMAD1/5 and pSMAD2/3 levels, and maybe this technique was not sensitive enough to detect the (mild) increase in pSMAD2/3 levels. Furthermore, the Wang *et al.* study treated rabbit IVD cells in alginate beads with 200 ng/mL human Link-N. Rabbit NPs still contain NCs, whereas NPs from human and canine patients with back pain due to IVD degeneration contain about 100% NPCs.[7] Therefore, the present study used human, bovine, and canine NPCs.[6] The Wang *et al.* study also revealed that Link-N targets the rabbit IVD cells through a direct peptide-protein association between Link-N and BMP receptor type II.[40] The increased pSMAD2 detected in the present study, however, indicates increased activation of the TGF-β_1 pathway by (s)Link-N as well. The exact molecular mechanism by which (s)Link-N promotes SMAD2 activation remains to be elucidated. Previous work indicated that BMPs (a) directly stimulated the SMAD2/3 pathway via ALK3 in gonadotrope-like cells[95], (b) differentially activate SMAD2/3 in immortalized human granulosa cells[96] and (c) signal through the canonical TGF-responsive SMAD2 and 3, by stimulating complex formation between the BMP-binding TGF-superfamily receptors ALK3/6, and the SMAD2/3 phosphorylating receptors ALK5/7.[97] Further research using biotinylated canine and human (s)Link-N on human, bovine, and canine NPCs should elucidate whether Link-N also directly interacts with the TGF receptor or induces SMAD2 signaling only via interaction with the BMP receptor.

In the studies described in chapter 1 and 3, in which the regenerative effect of human and canine (s)Link-N on human, bovine and canine NPCs was investigated, gene expression profiling of BMP-2 (*ALK1, ID1*) and TGF-β_1 (*ALK5, PAI1*) pathway markers at day 7 was performed. In that study, no increased gene expression of the aforementioned pathway markers was encountered by human or canine Link-N treatment. In contrast, in human and bovine NPCs, we did detect potent regenerative effects of human (s)Link-N treatment on protein level after 28 days of treatment. This indicated that we missed upregulation of the pathway genes at day 7 or that there were other pathways involved that we did not take into account. Therefore, in this study, we investigated the gene expression of the human NPCs after 24 hours of stimulation. These results indicate that beside the markers of the BMP-2 pathway (*ALK1, ID1, BMP4*) also markers of the TGF-β_1 pathway (*ALK5, PAI1*) were activated by treatment with mainly canine (s)Link-N. However, since the samples used for gene expression

profiling consisted of only 1 pool of human donors (*n* = 1) per condition, it was not possible to perform statistic on the data. Therefore, all these upregulations are not proven to be significantly different. So further research using a bigger sample size, should confirm or reject these results.

Beside the human variant, also the canine variant of (s)Link-N induced SMAD1 and 2 phosphorylation in human and bovine NPCs. This was in contrast with results of chapter 3, in which no/very mild regenerative effects of canine (s)Link-N were encountered on human and bovine NPCs cultured in micro-aggregates for 28 days. Thus, in human and bovine NPCs, the human variant of (s)Link-N exerted a more potent regenerative effect on protein level (it induced GAG deposition; chapter 1), but did not induce more SMAD signaling than canine (s)Link-N (which exerted no/very mild regenerative effects; chapter 3). This indicates that there are other pathways involved in the working mechanism of human (s)Link-N. However, it must be taken into account that SMAD signaling in the current study was tested on NPC monolayers stimulated for 24 hours, whereas our previous studies used a 3D micro-aggregates culture system for 28 days. Additionally, only 5 donors per species were tested, so future research with more donors should confirm/reject the results obtained in this study.

Beside SMAD signaling, the MAPK pathway is also reported to be associated with the expression of *SOX9*.[88, 94] In the study of Wang *et al.* (2013), treatment of the rabbit IVD cells with human Link-N failed to significantly promote activation of ERK1/2 and AKT signal transduction pathways, but a modest activation of p38 was observed.[95] Results of the study of Petit *et al*. (2011) also suggest that JNK, ERK, and AKT protein kinase signaling pathways are not required to initiate Link-N signaling to regulate the expression of collagen type II in NPCs.[46] But in their study, decreased instead of increased p38 was detected in human NPCs treated with 100 ng/mL human Link-N for 10 and 30 minutes.[46] So, in the future, beside the activation of SMAD signaling, also MAPK signaling should be taken into account when investigating the working mechanism of Link-N.

The different response of the bovine NPCs to the human variant compared with the canine variant of (s)Link-N implied that they induced different pathways. Human Link-N mainly increased GAG production (chapter 1), whereas canine Link-N increased collagen type II production (chapter 3). However, this difference could also be dependent on the O_2-saturation since the collagen type II deposition in the first study (chapter 1) was determined in normoxic conditions (21% O_2), while the third study (chapter 3) was performed under hypoxic conditions (5% O_2). Although the study of Mwale *et al.* (2011) reported that the O_2 levels (ranging from 1% to 21%) had little effect on the collagen type I (*COL1A2*) and II (*COL2A1*) mRNA levels of bovine NPCs cultured in alginate[98], further studies should investigate the activated pathways by both human and canine (s)Link-N treatment under different O_2 levels.

HUMAN AND CANINE LINK-N SLIGHTLY INCREASE BOTH SMAD1 AND 2 SIGNALING IN CANINE NPCS

The results of our previous studies indicated that both human and canine (s)Link-N induced only mild/no regenerative effects on canine NPCs (chapter 1 and 3). However, the present study reveals that human and canine (s)Link-N induced even more SMAD1 signaling in canine NPCs than in human and bovine NPCs (in which human Link-N was far more potent). So the question remains why these elevated pSMAD1 levels in canine NPCs did not result in as much matrix production as detected in

the human and bovine NPCs. A recent study of Luo *et al.* (2014) assumed that SMAD2/3 signaling has a down-regulatory effect on BMP/BMP receptor type II signaling via the miR-17-92 cluster.[99] Since canine Link-N simultaneously increased both pSMAD2 and pSMAD1 levels in canine NPCs, it could be that activated SMAD2/3 signaling inhibited SMAD1/5/8 signaling and subsequent ECM production. A second explanation could be that the moderately elevated pSMAD1 levels induced by (s)Link-N treatment are still too low to induce matrix production, and that in the human and bovine NPCs other matrix inducing pathways are activated as well. As described above, an experiment with biotinylated Link-N could elucidate whether (s)Link-N interacts with the BMP type II and/or potential other receptors. A third possibility could be that human and/or canine (s)Link-N are more rapidly degraded by canine NPCs than by human and bovine NPCs. The study of Nicolas *et al.* (2004) suggests that SMADs shuttle between the cell's cytoplasm and nucleus during TGF-β signaling and that this acts as a mechanism whereby SMADs continuously monitor receptor activity.[100] So it is possible that, when Link-N is rapidly broken down, the receptor activity ceases and pSMADs are subsequently dephosphorylated and shuttled out of the nucleus. Given that the present study only determined SMAD activation after 24 hours of stimulation, it could be that at later time point not enough Link-N is present anymore to sustain SMAD signaling and therefore no effect on matrix production after 28 days is detected. Future studies looking into the degradation of human and canine (s)Link-N by human, bovine and canine NPCs in culture (*e.g.* by targeted mass spectrometry[45]) could confirm/reject this hypothesis.

In our previous studies (chapter 1-3) a more potent response of the canine NPCs to TGF-β_1 treatment was detected compared with the human and bovine NPCs. Interestingly, in this study we found that TGF-β_1 treatment induced less SMAD2 signaling in canine NPCs but prominently more SMAD1 signaling compared to human and bovine NPCs. So this could play a role in the more potent effect of TGF-β_1 treatment on canine NPCs. Although it is generally accepted that TGF-β induces SMAD2/3 phosphorylation, several studies have shown that TGF-β can also induce SMAD1/5 phosphorylation in several cell types.[101, 102] Additionally, by using chemical inhibitors specific for the TGF-β and BMP type I receptors, their results showed that in some cell types, TGF-β induced SMAD1 phosphorylation independently of BMP type I receptors.[103] Thus, TGF-β-mediated SMAD1 phosphorylation appears to occur via different receptor complexes in a cell type specific manner.

LIMITATIONS

In the current study, only SMAD1 and 2 signaling were taken into account at one time point (24 hours). Since there are more subtypes (*e.g*. SMAD3, 4, 5, 8) involved in the different pathways, future studies should also include those subtypes and more different time points. Beside the SMAD mediated pathways other pathways, *e.g.* the MAPK pathway, could be involved in the working mechanism of (s)Link-N which were not taken into account in the present study. Furthermore, the cells were cultured in 2D monolayers because 3D micro-aggregates do not contain enough protein for performing an ELISA. Since almost all cells in the *in vivo* environment are surrounded by other cells and ECM in a 3D fashion, 2D cell culture does not adequately take into account the natural 3D environment of cells. As a result, experiments using 2D cell culture sometimes provide misleading and non-predictive data for *in vivo* responses.[67] The small sample size used in this study hampered profound analyses of the obtained data, so future studies should include samples of more donors.

CONCLUSIONS

The human and canine variant of (s)Link-N induced a slight increase in both SMAD1 and SMAD2 signaling in human, bovine, and canine NPCs, indicating activation of both the TGF-β_1 and BMP-2 pathway. This does not explain the interspecies differences in the potency/direction of the regenerative effects that human and canine (s)Link-N exert on the NPCs. Further research investigating the receptor interactions and degradation of human and canine (s)Link-N by the NPCs of different species will hopefully provide more decisive answers.

GENERAL DISCUSSION

The experiments described in this paper aimed at determining the efficacy of Link-N as treatment for intervertebral disc (IVD) degeneration in canine patients. Herewith we aimed to contribute to the search for a new regenerative treatment for IVD degeneration in canine patients, but also for a suitable large animal model for *in vivo* testing of Link-N. In the first study, the used culture system was validated (using human and bovine nucleus pulposus cells (NPCs)) and the regenerative effects of human (s)Link-N treatment on canine NPCs were delineated. In the second study, the additional regenerative effects of canine mesenchymal stromal cells (MSCs) in combination with (s)Link-N treatment on canine NPCs were investigated. The third study aimed at delineating the effect of species differences in the amino-acid sequence of (s)Link-N on its efficacy in human, bovine, and especially canine NPCs. The last study was performed to elucidate the working mechanism of human and canine (s)Link-N on human, bovine, and canine NPCs.

INTERSPECIES-DIFFERENCES IN NPC RESPONSE TO HUMAN AND CANINE (S)LINK-N TREATMENT

In agreement with previous studies[38, 41, 43], the human variant of (s)Link-N induced regenerative effects on human and bovine NPCs. These results validated the Link-N batch and 3D micro-aggregate culture system which was used in the current studies. The canine variant of (s)Link-N exerted no regenerative effects on the human NPCs at all, which was most likely due to the species difference in amino acid sequence and subsequent improper docking on the receptor(s).

Interestingly, we detected a more potent effect of the human variant compared with the canine variant of (s)Link-N on GAG deposition of bovine NPCs, while the amino acid sequence of bovine Link-N is more comparable to that of canine Link-N and the amino acid sequence of bovine sLink-N is the same as that of canine Link-N. Moreover, canine (s)Link-N induced abundant collagen type II deposition in bovine NPCs, in contrast to the human variant of (s)Link-N. So the different amino acid(s) in the canine (s)Link-N sequence versus the human (s)Link-N sequence likely resulted in the activation of other pathways in the bovine NPCs. However, the effect of human (s)Link-N was tested in normoxia (chapter 1) while the study investigating the effect of canine (s)Link-N was performed under hypoxic conditions (chapter 3). Thus, although the study of Mwale *et al.* (2011) reported that the O_2 levels (ranging from 1% to 21%) had little effect on the collagen type I (*COL1A2*) and II (*COL2A1*) mRNA levels of bovine NPCs cultured in alginate[98], the influence of the O_2-staturation on collagen type II protein deposition should be determined first.

Unfortunately, both canine and human (s)Link-N alone or combined with canine MSCs exerted no potent regenerative effect on canine NPCs. Under normoxic culture conditions a slight, non-biologically relevant, increase in GAG and DNA content by human (s)Link-N was detected, whereas in hypoxia no regenerative effects of Link-N treatment were detected at all. The canine variant of (s)Link-N did induce a small, significant increase in GAG and DNA content compared with the untreated controls when cultured under hypoxic conditions. This increase, however, was not biologically relevant and mainly due to less performing control micro-aggregates in this study rather than a more potent effect of the canine variant of (s)Link-N. Since NPCs derived of four similar but two other canine donors were used for determining the effect of human compared with canine

(s)Link, this discrepancy in performance of the control condition could be due to donor differences. However, the response to TGF-β_1 treatment was comparable between the two studies. Altogether, it could be stated that there are interspecies differences in the NPC response to human and canine (s)Link-N treatment (Table 14). Our last study (chapter 4), investigating the working mechanism of the two (s)Link-N variants on the NPCs of differences species, aimed at elucidating the reasons for these differences in NPC response. Unfortunately, no distinct differences in activation of SMAD1 and SMAD2 signaling were detected between the NPCs of the different species. To further investigate the working mechanism, ongoing studies focus on elucidating the receptor interactions of human and canine sLink-N with NPCs of the different species. Therefore, immunofluorescence and confocal microscopy will be performed on human, bovine, and canine NPCs stimulated with biotinylated human and canine sLink-N, as described in the paper of Wang *et al*. (2013).[40]

Another reason for the mild response of the canine NPCs to Link-N treatment could be the hampered regenerative capacity of the NPCs. Although the obtained effects of Link-N on human and bovine NPCs validated the efficacy of our Link-N batch and used micro-aggregate culture system, it could still be that canine NPCs reside better in a hydrogel culture and require such an environment to be able to react to (s)Link-N treatment more potently. Therefore, future studies should determine the regenerative effects of (s)Link-N on canine NPCs when cultured in hydrogels which are preferably already used in clinical practice for intradiscal cell transplantation. In the study of Benz *et al*. (2012) they injected *in vitro* expanded disc cells which were supported by a polyethylene glycol-crosslinked serum albumin/hyaluronan hydrogel in ovine discs which resulted in IVD repair.[73]

As it has been suggested that notochordal cells (NCs) play an important role in maintaining the healthy NP by synthesizing new ECM[104] and by regulating ECM production of other cells[105], further studies testing the regenerative effects of human and canine (s)Link-N on canine NCs should determine whether these cells are able to respond more potently to Link-N treatment. However, then it still needs to be determined why canine NPCs are less responsive to (s)Link-N treatment than human and bovine NPCs. A possibility could be that canine NPCs degrade (s)Link-N rapidly so that it cannot exert its effect optimally. Therefore, follow up studies will focus on studying the differences in degradation rate of human and canine (s)Link-N by human, bovine, and canine NPCs. Medium samples, collected at different time points from micro-aggregate cultures, will be analyzed by targeted mass spectrometry, as performed in the paper of Gawri *et al.* (2014).[45]

Table 14: Overview of the effects of human and canine (s)Link-N treatment on NPC of different species

	Human NPCs		**Bovine NPCs**		**Canine NPCs**		
	Human (s)Link-N	Canine (s)Link-N	Human (s)Link-N	Canine (s)Link-N	Human (s)Link-N		Canine (s)Link-N
DNA	-	-	++	-	Nx: +	Hx: -	+
GAG	++	-	++	+	Nx: +	Hx: -	+
Col1	++	-	++	++	-		-
Col2	-	-	-	++	-		-
pSMAD1	+	+	+	+	+		+
pSMAD2	+	+	+	+	+		+

The symbols indicate no (-), mild (+) , or prominent (++) effects

OVINE INSTEAD OF CANINE MODEL FOR *IN VIVO* TESTING OF (S)LINK-N

As described above, we encountered that (s)Link-N treatment did not induce potent regenerative effects on canine NPCs and the human NPCs responded differently to (s)Link-N treatment than the canine NPCs. These *in vitro* results imply that the dog cannot serve as a suitable large animal model for translation of Link-N treatment into human patients. As human (s)Link-N is still considered as promising new regenerative treatment for back pain due to IVD degeneration in human patients, the search for a suitable large animal model should be continued. Human (s)Link-N exerted comparable effects on bovine NPCs as on human NPCs. Therefore bovine could be considered as a suitable large animal option, however considerations of size, housing, and reproduction limit their use as animal model.[75] Interestingly, an ovine model for IVD degeneration is already widely used as *in vivo* model for studying new treatments for human IVD degeneration.[106-108] Additionally, similar to cows and chondrodystrophic dog breeds, sheep resemble humans in that their NPs may contain some NCs at birth, but the numbers decrease rapidly.[7, 79]

To determine the potential of an ovine model for studying the regenerative effects of the human and canine variant of (s)Link-N, a pilot study with NPCs of one ovine donor was performed. NPCs were harvested and, after expansion, cultured for 28 days in micro-aggregates under hypoxic conditions as described in chapter 1. Either human or canine Link-N (1 µg/mL or 10 ng/mL) or sLink-N (0.5 µg/mL or 5 ng/mL) were added to the culture medium twice a week.

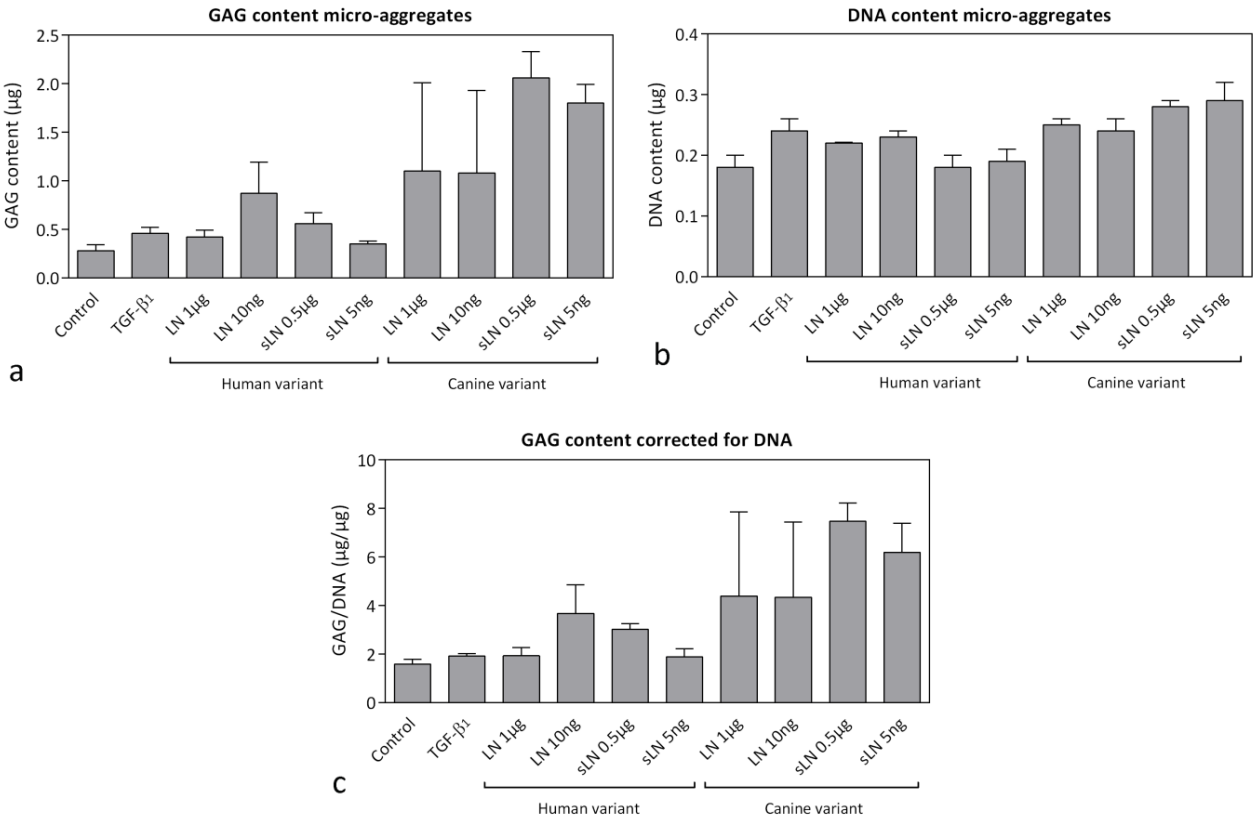

Fig. 38: Human but prominently canine (s)Link-N shows to have a regenerative effect on the ovine nucleus pulposus cells (NPCs). (**a**) GAG content (mean + SD) of the micro-aggregates. GAG and DNA content (mean + SD) of ovine NPC micro-aggregates cultured for 28 days in hypoxia (Hx, 5%O_2) in different conditions (**b**) DNA content (mean ± SD) of the micro-aggregates. (**c**) GAG content corrected for the DNA content.

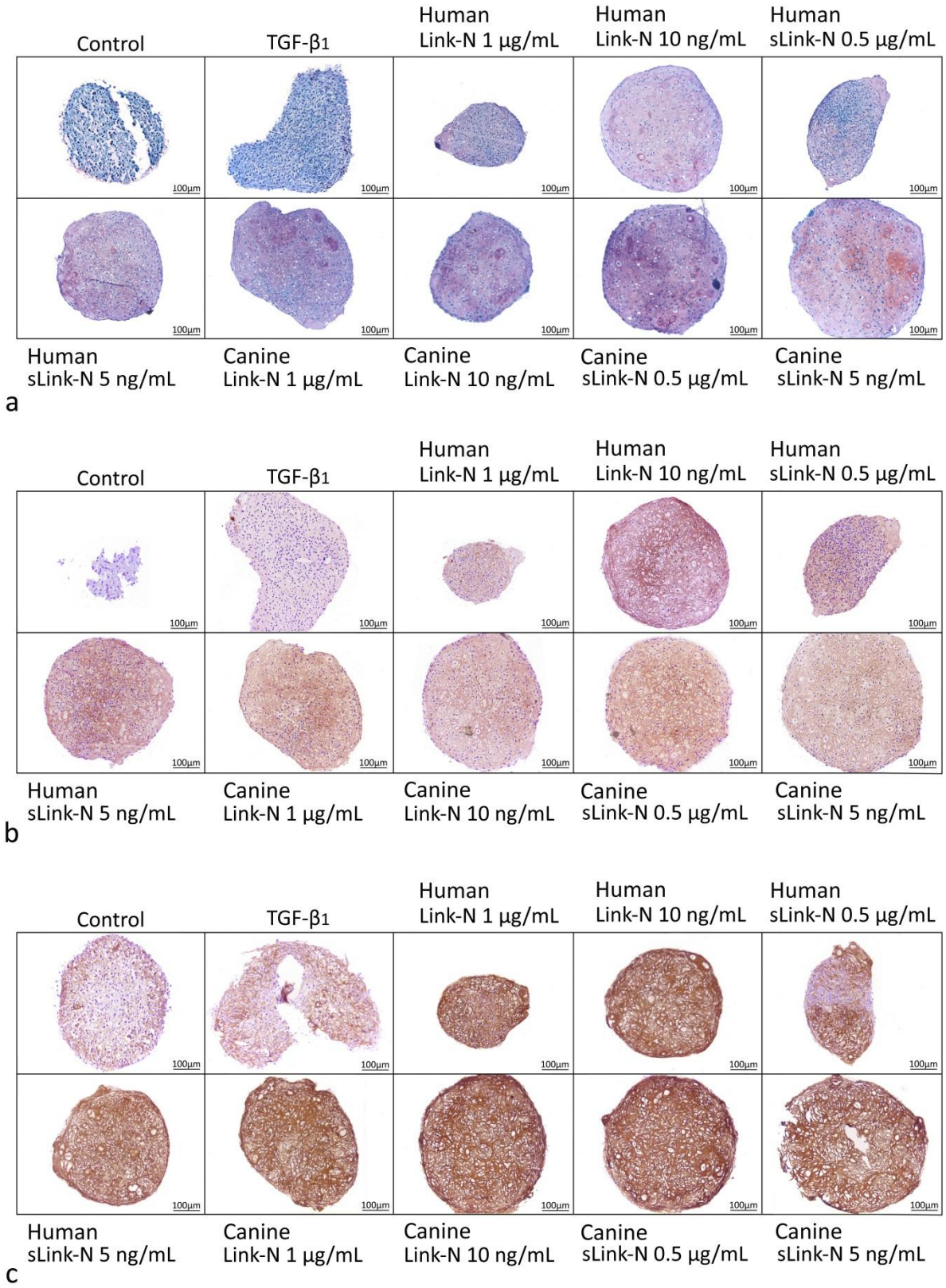

Fig. 39: Histological images of ovine nucleus pulposus cells (NPCs) cultured in micro-aggregates in hypoxia (5% O_2) for 28 days stained with Safranin O/Fast Green (**a**) and immunohistochemical staining for collagen type I (**b**) and II (**c**). GAG, collagen type I and II deposition were increased by all canine and human (s)Link-N treatments compared with the untreated control and TGF-β_1 condition.

The results indicated that human and prominently canine (s)Link-N increased GAG production, corrected and not corrected for the DNA content, of the ovine NPCs compared with the untreated controls (Fig. 38a,c). These results were confirmed by the Safranin O/Fast Green staining (Fig. 39a), which also showed most GAGs present within the micro-aggregates treated with canine and human (s)Link-N. The increase in DNA content by all treatments compared with the untreated controls was negligible (Fig. 38b).

The sheep donor used for this pilot study was a cross breed between a Swifter and a Texel species. The Texel species is known for its remarkable muscle development and leanness.[109] A mutation in the myostatin or growth and differentiation factor (GDF) 8 gene is likely to be the genetic cause of the muscular phenotype of this breed of sheep.[109] Clop *et al.* (2006) identified a single G to A transition in the 3' untranslated region of myostatin (g+6723G-A) as the probable causal polymorphism. This transition creates a target site for microRNA-1 and microRNA-206, which leads to translational inhibition of myostatin.[110] Since myostatin is a negative regulator of muscle growth, impaired function results in a doubling of skeletal muscle mass.[111] Studies of both humans and laboratory animals have reported a strong, positive correlation between muscle mass and bone mass.[112, 113] Additionally, results from the study of Hamrick *et al*. (2003) suggested that increased muscle mass in mice lacking myostatin is associated with increased bone mass as well as degenerative changes in the IVD.[114] Furthermore, myostatin is a member of the transforming growth factor-β superfamily and the signaling pathways it transduces can be divided into SMAD-mediated and non-SMAD pathways.[115] The SMAD-mediated pathway starts with the binding of the mature C-terminal dimer of myostatin to one of the two activin receptors type II.[114] The receptor recruits, phosphorylates, and thereby activates the activin receptors type I (ALK4 and ALK5) which in turn leads to the phosphorylation and activation of SMAD2/3.[115, 116] Aside from the SMAD-mediated signal pathway, also the MAPK signaling pathways have been shown to be involved in myostatin signal transduction.[115] Thus, since the myostatin mutation may influence the state of the ovine IVD and signaling pathways that are also involved in the working mechanism of Link-N, it is important to check whether the sheep donors used for determining the effect of Link-N are carriers of this mutation.

The sheep donor used in this pilot study appeared to be heterozygous for the myostatin mutation. McPherron *et al.* (2002) found that the effect of myostatin is dose-dependent, as mice heterozygous for the disrupted myostatin sequence have muscle weights that are intermediate between those of normal mice and mice homozygous for the myostatin mutation.[117] The study of Johnson *et al.* (2005) stated that it remains equivocal for some traits whether the effect of the A allele is additive or non-additive in sheep.[118] However, it is definite that the maximal benefit in muscling is seen in animals carrying 2 copies of the A allele.[119] So further studies testing the effect of human and canine (s)Link-N on homozygote positive and negative sheep donors for the myostatin mutation, should determine the influence of this mutation on the response to Link-N treatment. Furthermore, since the ovine model should be as similar to the human situation as possible, myostatin mutation negative sheep should be used for testing Link-N treatment.

Altogether, the preliminary results of testing Link-N treatment on ovine NPCs are promising but further research using NPCs of more ovine donors (that do not possess the myostatin mutation) should determine whether the sheep can indeed function as suitable large animal model as bridge between the *in vivo* studies in rabbits and clinical trials in human patients.

GENERAL CONCLUSIONS

The studies we have performed imply that human (s)Link-N induced an anabolic response in human and bovine nucleus pulposus cells (NPCs), which validated the used batch of Link-N and 3D culture system. In addition, the results indicate that human and canine Link-N alone or combined with canine mesenchymal stromal cells lack the potency to be used as new regenerative therapy for intervertebral disc (IVD) degeneration in dogs. Experiments investigating the species differences in signaling pathways activated by human and canine (s)Link-N did not result in a decisive explanation for the mild response of the canine NPCs to Link-N treatment, so this remains to be further elucidated. Furthermore, our studies indicate that the dog cannot serve as a suitable large animal model for translation of the treatment with (s)Link-N into human patients. However, since Link-N is still a promising peptide to be used as treatment for IVD regeneration in human patients, the search for a suitable large animal model should continue, *e.g.* by looking into the ovine species.

FUTURE PERSPECTIVES

Although the obtained effects of Link-N on human and bovine nucleus pulposus cells (NPCs) validated the efficacy of our Link-N batch and used micro-aggregate culture system, it could still be that canine NPCs reside better in a hydrogel culture and require such an environment to be able to react to (s)Link-N treatment more potently. Therefore, future studies should determine the regenerative effects of (s)Link-N on canine NPCs when cultured in hydrogels.

As it has been suggested that notochordal cells (NCs) play an important role in maintaining the healthy nucleus pulposus by synthesizing new matrix[104] and by regulating matrix production of other cells[105], follow up studies testing the regenerative effects of human and canine (s)Link-N on canine NCs should determine whether these cells are able to respond more potently to Link-N treatment.

To further investigate the working mechanism of Link-N, ongoing studies focus on elucidating the receptor interactions of human and canine sLink-N with NPCs of the different species. Therefore, immunofluorescence and confocal microscopy will be performed on human, bovine, and canine NPCs stimulated with biotinylated human and canine sLink-N, as described in the paper of Wang *et al*. (2013).[40]

Since it is possible that canine NPCs degrade (s)Link-N rapidly so that it cannot exert its effect optimally, follow up studies will focus on studying the inter species differences in degradation rate of human and canine (s)Link-N. Medium samples, collected at different time points from human, bovine and canine NPCs micro-aggregate cultures, will be analyzed by targeted mass spectrometry, as performed in the paper of Gawri *et al.* (2014).[45]

The preliminary results of testing Link-N treatment on ovine NPCs are promising but further research using NPCs of more ovine donors (that do not possess the myostatin mutation) should determine whether the sheep can indeed function as suitable large animal model as bridge between the *in vivo* studies in rabbits and clinical trials in human patients.

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APPENDIX

ATTENDED COURSES AND CONGRESSES

Followed Courses:

- 'Introductory Course Statistics with R/Rstudio' lectured by Hans Vernooij and Jan van den broek
- 'Scientific Writing' lectured by Mrs. Linda McPhee
- 'Giving Effective Oral Presentations' lectured by Mrs. Margo de Wolf
- Monthly manuscript meeting during which a recently published article was discussed with the orthopedics research team

Attended Congresses:

- 'Veterinary Science Day' 2015
- 'European Veterinary Conference Voorjaarsdagen' 2016
    - Gave an oral presentation titled: 'The regenerative effect of Link-N on degenerated canine intervertebral disc cells'
- 'Annual meeting Nederlandse Vereniging voor Matrix Biologie' 2016
    - Gave an oral presentation titled: 'The regenerative effect of Link-N on degenerated canine intervertebral disc cells'
- Lecture titled: 'Paradoxical roles of prolyl hydroxylases in HIF-1 regulation in nucleus pulposus' given by Makarand v. Risbud at the 'RM additional siminar' 2016
- 'Symposium Musculoskeletal Regeneration Research Network: The Joint as an Organ' 2016