The Effect of Link-N, sLink-N and Mesenchymal Stem Cells on Canine Intervertebral Disc Degeneration

A study on the effectiveness of Link-N vs sLink-N and additional effect of BMSC's by means of qPCR

Oskam, J. (Janneke) 2/2/2016 Supervisors: Mw. Dr. M. A. Tryfonidou (Marianna) and Drs. F.C. Bach (Frances)

Key words: Canine intervertebral disc degeneration, nucleus pulposus cells, Link-N, short Link-N, mesenchymal stem cells

Contents

	Page
Abstract	2
Introduction	3
Intervertebral disc disease	3
Treatment and regenerative medicine	3
Link-N	5
Aim of the study	6
Expected results	6
Materials and methods	7
Study design	7
Materials	7
Methods	9
 Crushing of the micro-aggregates 	9
 RNA extraction 	9
 cDNA synthesis 	10
RT-qPCR	10
Statistics	10
Results	11
Discussion	<u> 19</u>
Conclusion	20
References	21

Abstract

BACKGROUND Intervertebral disc disease (IVDD) is a progressive degenerative disease in humans and dogs. Current treatments only relieve the pain on the short term. Several regenerative treatment strategies employ growth factors and mesenchymal stem cells (MSC's). A newly developed synthetic peptide – Link-N – has been reported to be promising for intervertebral disc (IVD) regeneration. It has a positive effect on the production of collagen type I, II and X and proteoglycan by nucleus pulposus (NP) cells and stimulates cell proliferation. This effect may also be obtained by the biologically active part of Link-N (1-16), short Link-N (1-8).

AIM OF THE STUDY Define the effect of Link-N, sLink-N and Link-N and sLink-N in combination with MSC's on canine IVDD.

HYPOTHESIS sLink-N will be as effective as Link-N on NP cells. MSC's will have an additive effect on the (s)Link-N effect.

STUDY DESIGN Real time quantitative PCR was performed on micro-aggregates of nucleus pulposus (NP) cells and micro-aggregates of NP cells and MSC's of Beagle donors without treatment or treated with TGF-β1, Link-N or sLink-N. The gene expression of *collagen type 1, II and X, aggrecan, SOX 9, BMPR 2, MMP-9, MMP-13, TIMP-1, ADAMTS 5, ID 1, Pai 1, ALK 1, ALK 5, Cyclin-D1, caspase 3, BCL 2* and *BAX* were evaluated to examine the effect of (s)Link-N on degenerated NP cells.

RESULTS Treatment with sLink-N showed no statistically significant changes in gene expression compared to untreated NP cells. BMSC's showed statistically significant upregulation of *collagen I* and *MMP-9* compared to cultures without BMSC's. No statistically significant changes in gene expression were seen in other culture conditions.

CONCLUSION Human Link-N and sLink-N have no effect on degenerated canine NP cells. Combining human Link-N or sLink-N with canine BMSC's does not exert an additive trophic effect on canine NP cells.

Key words: Canine intervertebral disc degeneration, nucleus pulposus cells, Link-N, short Link-N, mesenchymal stem cells

Introduction

Intervertebral disc disease

The intervertebral disc (IVD) forms a joint between two vertebrae. It allows confined movement of the spine. The elasticity of the disc makes it possible to absorb shocks. The healthy IVD consists of the nucleus pulposus (NP) surrounded by the annulus fibrosus (AF) with a transitional zone (TZ) in between. The NP mainly contains notochordal cells (NC's) and chondrocytes which produce an extracellular matrix (ECM) rich in proteoglycans, mainly aggrecan, and collagen type I and II. The collagen forms a fibrous network important for the integrity of the disc (Mwale et al., 2003). The TZ consists of chondrocyte like cells (CLC's) and the AF contains CLC's and fibrocytes (Hansen, 1952). In the healthy IVD small nutrients like oxygen and glucose reach the cells of the non-vascularized inner AF and NP from the outer vascularized parts of the AF through diffusion and osmosis. Bigger molecules are transported by bulk fluid flow created by the negative charge of proteoglycans in the ECM (Holm et al., 1981).

Intervertebral disc disease (IVDD) is a progressive degenerative disease in humans and dogs. In dogs suffering from IVDD there is a degeneration of (parts of) the IVD. IVDD is characterized by cellular changes in the NP and AF and changes of their associated ECM. In essence NC's from the NP are replaced by CLC's from the TZ (Hansen, 1952). In the ECM glycosaminoglycan (GAG, a side chain of proteoglycans) decreases and collagen type I increases. As a result the ECM becomes less hydrated. This compromises the nutrients supply through diffusion and bulk fluid flow causing further cell degeneration and ECM changes (Bergknut et al., 2013). The degeneration process causes structural changes of the IVD. The disc loses its elasticity. By normal physical pressure the disc bulges outwards and the NP can extrude through ruptures in the AF (Hansen, 1952) (fig. 1). As a consequence the dislocated disc can lead to compression of the spinal cord and thus cause pain and neurological problems (Bergknut et al., 2013).

Treatment and regenerative medicine

Current treatments for IVDD are pain management and/or anti-inflammatory therapy or surgical removal of the degenerated tissue, replacement with prosthesis and vertebral bone fusion. Surgical treatments can relief the pain on the short term, however they also alter the spine biomechanics resulting in adjacent problems on the long term (Hoogendoorn et al., 2008; Mwale et al., 2014). Therefor medical treatment of IVDD is desired. There have been studies on the regeneration of the IVD. For cell regeneration several methods can be used such as cell based therapy, recombinant gene therapy or therapy with growth factors, proteins and/or cytokines (Hoogendoorn et al., 2008).

For cell based therapy several strategies have been studied. CLC's can be used to retard IVD degeneration. However when added to a severe degenerated disc, the CLC's may lose specific characteristics due to degeneration making them less effective (Watanabe et al., 2010). A second type of cells are NC's. They have a regenerative potential and a great restorative capacity for other cells like CLC's and MSC's. They can stimulate CLC's or can differentiate in CLC's, hereby influencing the NP cell population to maintain a healthy NP. It is also possible that NC's produce growth factors that stimulate the synthesis of ECM in the NP (Korecki et al., 2010). IVDD is associated with the loss of NC's (Smolders et al., 2013). Also multipotent NP progenitor cells have been reported (Bach et al., 2014; Erwin et al., 2013). However more research is necessary to know if they are suitable for treatment.

A third method is based on mesenchymal stem cells (MSC's). These cells can differentiate into NP cells through direct or indirect communication with NP cells in a degenerated disc through paracrine

mediators. Also collagen type II in the ECM of the NP can induce chondrogenic differentiation (Hoogendoorn et al., 2008). These new cells can produce healthy ECM and help repair the degenerated disc (Masuda et al., 2004; Hiyama et al., 2008). In addition MSC's can act immuno-suppressive by secreting certain factors (Meirelles et al., 2009). This is very promising for IVD regeneration. Disadvantages are however that the cells must be harvested and proliferated causing discomfort for the patient and take time (Hoogendoorn et al., 2008).

Recombinant gene therapy is also mentioned in studies. In theory genetically modified cells which are able to produce a desired product is a good idea. However due to several disadvantages it has not been used in a clinical setting yet (Bach et al., 2014). The sudden onset of cell proliferation can lead to formation of tumors (Watanabe et al., 2010). Also incorrect administration of the products have been associated with several negative effects like paralysis and death (Vadalà et al., 2007). So this type of therapy is not ready for clinical use.



Figure 1. The degenerative events of the process of IVDD in a schematic overview. On the top several factors are shown which can increase the risk of developing IVDD. These factors cause a viscous cycle in which ECM degeneration and cellular changes occur, resulting in different lesions shown on the bottom (Bergknut et al., 2013).

Cytokines can help regenerate the IVD by stimulating growth factors that inhibit catabolic and/or stimulate anabolic processes in the IVD. Growth factors like transforming growth factor β (TGF- β), bone morphogenetic proteins (BMP's) and growth and differentiation factors (GDF's) can increase production of SOX 9 (a protein which is active during chondrogenic differentiation), collagen type II and aggrecan. However BMP's and GDF's can cause inflammatory reactions and collapse of the IVD (Walsh et al., 2004). TGF- β can also stimulate cell proliferation (Masuda et al., 2004). Other growth factors like insulin-like growth factor 1 (IGF-1) and epidermal growth factor (EGF) enhance cell proliferation (Walsh et al., 2004). Anti-inflammatory factors like tissue inhibitor of metalloproteinase 1 (TIMP-1) are involved in matrix remodeling by production of catabolic matrix metalloproteinases (MMP's) which break down ECM (Hoogendoorn et al., 2008). Before applying as a treatment more research is necessary to determine the efficacy, duration of action, safety and adverse effects in vivo of these factors (Bach et al., 2014). Although promising as a regenerative medicine for IVDD more research is necessary and also the costs of this kind of therapy is quite high (Mwale et al., 2003).

Link-N

Synthetic peptides could be used as an alternative treatment which is cheaper and easier to apply. Currently a new synthetic peptide called Link-N (amino acid sequence: DHLSDNYT LDHDRAIH) is subject of many studies. It's an N-terminal peptide of a link glycoprotein in human cartilage. This link protein occurs naturally in IVD's where it stabilizes aggrecan in the ECM by creating large proteoglycan aggregates. Link-N, the product of proteolysis of this link protein, is thought to be an indicator to IVD cells that ECM degeneration is taking place. This way it stimulates the cells to increase ECM synthesis to compensate for the degeneration. In adults lesser amounts of this link protein are found and more Link-N fragments are found in cartilage. This suggests it plays a role in IVDD which occurs more with increasing age (McKenna et al., 1998; Mwale et al., 2003).

This peptide can also be harvested in vitro from human cartilage proteoglycan aggregates. It is produced by proteolysis by proteases (stromeolysins, gelatinases and collagenases). It has already been shown that synthetic Link-N can act as a growth factor and stimulate proteoglycan synthesis in human articular cartilage (McKenna, et al., 1998; Liu et al., 2000). A study on bovine tails showed that Link-N stimulates the synthesis of proteoglycans and type II and X collagen (Mwale et al., 2003). Other studies have shown that Link-N has a positive effect on the regeneration of IVD cells in humans and rabbits by stimulating the production of collagen type I, II and X and proteoglycans by NP cells and promoting cell proliferation (Mwale et al., 2003, 2011; Petit et al., 2011). Link-N promotes production of ECM by binding directly to bone morphogenetic protein receptor type 2 (BMPR 2) and activating the bone morphogenetic protein (BMP) signaling pathway or the SMAD 1/5/8 cascade (Wang et al., 2013).

A recent study on Link-N reported that the peptide is cleaved in the AF of the IVD. They show that a part of the native Link-N is biological active. This part consists of the first eight amino acids of the peptide of sixteen amino acids. This shorter version is called Link-N 1-8 (and the native version Link-N 1-16) or short Link-N, abbreviated sLink-N (and the native version Link-N) (Gawri et al., 2014). This active sLink-N and Link-N may both potentially have great regenerative effects in a degenerated IVD. Benefits of sLink-N, when it is proven to be as effective as Link-N, would be that it forms a smaller burden for the amino acid metabolism of the patient, it is easier to apply and cheaper to produce than Link-N due to the smaller size.

Aim of the study

The overall aim of this study was to define the effect of Link-N and sLink-N separately and in combination with bone marrow derived MSC's (BMSC's) on canine IVDD. These effects were studied with the aid of gene expression profiling in the presence of Link-N or sLink-N (and BMSC's) in canine NP cells. Following genes were included based on their role in selected cell functions which are affected by IVD degeneration (table 1).

Cell function	Protein gene expression		Protein role	
Matrix production	Collagen type I, II and X	Col I/II/X	Components of the ECM	
and chondrogenic differentiation	Aggrecan	ACAN	Component of the ECM which helps to withstand compression	
	SRY box 9	SOX 9	DNA transcription regulator, activates expression of <i>Col II</i> and <i>ACAN</i>	
	Bone morphogenetic protein receptor type 2	BMPR 2	Serine/threonine kinase, a receptor for BMP stimulating SOX 9 and collagen II and aggrecan synthesis. Link-N is known to interact with this receptor and activate the signaling pathway (Wang, et al., 2013).	
Matrix degradation	Matrix metallopeptidase 9	MMP-9	Degradation of collagen IV and V	
(remodeling)	Matrix metallopeptidase 13	MMP-13	Degradation of collagen II	
	Tissue inhibitor of	TIMP-1	Matrix metallopeptidase inhibitor	
	metallopeptidase 1			
	Disintegrin and metalloproteinase with thrombospondin motifs	ADAMTS 5	Aggrecanase; proteolysis of aggrecan	
Signaling pathways	DNA-binding protein inhibitor 1	ID 1	DNA transcription inhibitor, stimulates the BMP signaling pathway for activation of <i>SOX 9</i> and production of collagen II and aggrecan	
	Plasminogen activator inhibitor 1	Pai 1	Stimulates the TGF-β signaling pathway for activation of MMP's	
	Activin receptor-like kinase 1	ALK 1	Receptor for BMP signaling pathway (SMAD 1/5/8)	
	Activin receptor-like kinase 5	ALK 5	Receptor for TGF-β signaling pathway (SMAD 2/3)	
Cell proliferation	Cyclin-D1	Cyclin-D1	CDK4-6 kinase regulator	
Apoptosis	Caspase 3	Casp 3	Pro-apoptotic protein	
	B-cell CLL 2	BCL 2	Anti-apoptotic protein	
	BCL2-associated X protein	BAX	Pro-apoptotic protein	

Table 1. Included genes in this study sorted by function

Expected results

The hypothesis is that sLink-N is as effective as Link-N, because it contains the biologically active part of the peptide (Gawri et al., 2014). BMSC's are suspected to have an additional effect on the (s)Link-N effect, because it has been shown that single BMSC treatment has a positive effect on IVD degeneration (Masuda et al., 2004).

The BMP signaling pathway will be activated by (s)Link-N and the TGF- β signaling pathway will not be affected. Matrix production and chondrogenic differentiation will be stimulated by (s)Link-N. Matrix degradation will also be increased because of increased matrix remodeling. Cell proliferation is suspected to be increased and apoptosis to be decreased.

Materials and methods

Study design

Before the onset of this experiment the NP cells were cultured in a chondrogenic medium with and without BMSC's in hypoxic conditions. This was done in micro-aggregates of 35.000 NP cells per culture for the cultures without BMSC's and with 17.500 NP cells and 17.500 BMSC's per culture for the cultures with BMSC's. Each culture was treated with a) nothing (control), b) 10 μ g/mL TGF- β 1, c) 1 μ g/mL human Link-N, d) 0,5 μ g/mL human sLink-N or e) 5 ng/mL human sLink-N, resulting in five different treatment groups as shown in figure 2. Culturing was done in duplicate.

On day 7 of culture the micro-aggregates were extracted from the culture medium. Each duplicate micro-aggregate was put together as one sample, resulting in 60 samples processed for RNA analysis and subsequent cDNA synthesis. Thereafter, with the aid of RT-qPCR the gene expression of *collagen type I, II* and *X, aggrecan, SOX 9, BMPR 2, MMP-9, MMP-13, TIMP-1, ADAMTS 5, ID 1, Pai 1, ALK 1, ALK 5, Cyclin-D1, caspase 3, BCL 2* and *BAX* was evaluated.

PLATE 1 NPC					Donor nr
Control	TGF-β1	Link-N (1µg/mL)	sLink-N (0,5µg/mL)	sLink-N (5ng/mL)	617
Control	TGF-β1	Link-N (1µg/mL)	sLink-N (0,5µg/mL)	sLink-N (5ng/mL)	151
PLATE 2 NPC					
Control	TGF-β1	Link-N (1µg/mL)	sLink-N (0,5µg/mL)	sLink-N (5ng/mL)	679
Control	TGF-β1	Link-N (1µg/mL)	sLink-N (0,5µg/mL)	sLink-N (5ng/mL)	859
PLATE 3 NPC					
Control	TGF-β1	Link-N (1µg/mL)	sLink-N (0,5µg/mL)	sLink-N (5ng/mL)	013
Control	TGF-β1	Link-N (1µg/mL)	sLink-N (0,5µg/mL)	sLink-N (5ng/mL)	156
PLATE 4 NPC + MSC					
Control	TGF-β1	Link-N (1µg/mL)	sLink-N (0,5µg/mL)	sLink-N (5ng/mL)	617
Control	TGF-β1	Link-N (1µg/mL)	sLink-N (0,5µg/mL)	sLink-N (5ng/mL)	151
PLATE 5 NPC + MSC					
Control	TGF-β1	Link-N (1µg/mL)	sLink-N (0,5µg/mL)	sLink-N (5ng/mL)	679
Control	TGF-β1	Link-N (1µg/mL)	sLink-N (0,5µg/mL)	sLink-N (5ng/mL)	859
PLATE 6 NPC + MSC					
Control	TGF-β1	Link-N (1µg/mL)	sLink-N (0,5µg/mL)	sLink-N (5ng/mL)	013
Control	TGF-β1	Link-N (1µg/mL)	sLink-N (0,5µg/mL)	sLink-N (5ng/mL)	156

Figure 2. Study design of the culture conditions of the NP cells and BMSC's from which the micro-aggregates were collected.

Materials

NP cells from degenerated IVD's of six male Beagle donors were readily available from the Biobank TR group orthopaedics of the Faculty of Veterinary Medicine of Utrecht University (FVM- UU). They have been cultured and collected as micro-aggregates before onset of this experiment.BMSC's of one female Beagle donor were also readily available from the Biobank TR group orthopaedics (FVM- UU). They have been cultured with NP cells and collected as micro-aggregates before onset of this experiment.

Gene	Sequence (Forward/Reverse primer)	Exon	Product size	Annealing temp (°C)
Target gene	S			
Col I	F: GTGTGTACAGAACGGCCTCA	F: 2	109	61
	R: TCGCAAATCACGTCATCG	R: 2		
Col II	F: GCAGCAAGAGCAAGGAC	F: 52	150	60,5-65
	R: TTCTGAGAGCCCTCGGT	R: 53		
Col X	F: CCAACACCAAGACACAG	F: 1	80	61
	R: CAGGAATACCTTGCTCTC	R: 2		
ACAN	F: GGACACTCCTTGCAATTTGAG	F: 13/14	110	61-62
	R: GTCATTCCACTCTCCCTTCTC	R: 14		
SOX 9	F: CGCTCGCAGTACGACTACAC	F: 6	105	62-63
	R: GGGGTTCATGTAGGTGAAGG	R: 6		
BMPR 2	F: GTCTTCACAGTATGAACATGATGG	unknown	150	64-65
	R: AACACTTTCACAGCAACTGG			
MMP-9	F: GCATGACATCTTCCAGTACCA	unknown	74	63
	R: CCGAGAATTCACACGCCAGTA			
MMP-13	F: CTGAGGAAGACTTCCAGCTT	unknown	250	65
	R: TTGGACCACTTGAGAGTTCG			
TIMP-1	F: GCGTTATGAGATCAAGATGAC	F: 2	120	66
	R: ACCTGTGCAAGTATCCGC	R: 3		
ADAMTS 5	F: CTACTGCACAGGGAAGAG	F: 5	148	61
	R: GAACCCATTCCACAAATGTC	R: 6		
ID 1	F: CTCAACGGCGAGATCAG	F: 2	135	59,5
	R: GAGCACGGGTTCTTCTC	R: 3		
Pai 1	F: AAACCTGGCGGACTTCTC	F: 5	98	61,5
	R: ACTGTGCCACTCTCATTCAC	R: 6		
ALK 1	F: CCTTTGGTCTGGTGCTGTG	F: 9	107	61
	R: CGAAGCTGGGATCATTGGG	R: 10		
ALK 5	F: GAGGCAGAGATTTATCAGACC	F: 4	116	59,5
	R: ATGATAATCTGACACCAACCAG	R: 5		
Cyclin-D1	F: GCCTCGAAGATGAAGGAGAC	F: 2	117	60
	R: CAGTTTGTTCACCAGGAGCA	R: 3		
Casp 3	F: ATCACTGAAGATGGATGGGTTGGTT	F: 8	139	58
	R: TGAAAGGAGCATGTTCTGAAGTAGCACT	R: 8		
BCL 2	F: GGATGACTGAGTACCTGAACC	F: 2	80	61,5-63
	R: CGTACAGTTCCACAAAGGC	R: 3		
BAX	F: CCTTTTGCTTCAGGGTTTCA	F: 2/3	108	58-59
	R: CTCAGCTTCTTGGTGGATGC	R: 3		
Reference g	enes			
SDHA	F: GCCTTGGATCTCTTGATGGA	F: 6	92	61
	R: TTCTTGGCTCTTATGCGATG	R: 6		
GAPDH	F: TGTCCCCACCCCCAATGTATC	F: 2	100	58
	R: CTCCGATGCCTGCTTCACTACCTT	R: 2		
HPRT	F: AGCTTGCTGGTGAAAAGGAC	F: 5/6	104	56-58
	R: TTATGTCAAGGGCATATCC	R: 7		
RPS 19	F: CCTTCCTCAAAAAGTCTGGG	F: 2/3	95	61
	R: GTTCTCATCGTAGGGAGCAAG	R: 3		

Table 2. Primers used in this study for qPCR of <u>target genes</u> collagen type I, II and X (Col I/II/X), aggrecan (ACAN), SRY box 9 (SOX 9), bone morphogenetic protein type 2 receptor (BMPR 2), matrix metallopeptidase 9 and 13 (MMP-9 and -13), tissue inhibitor of metallopeptidase 1 (TIMP-1), disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS 5), DNA-binding protein inhibitor 1 (ID 1), plasminogen activator inhibitor (Pai 1), activin receptor-like kinase 1 and 5 (ALK 1 and 5), cyclin D1 (Cyclin-D1), caspase 3 (Casp 3), B-cell CLL 2 (BCL 2) and BCL2-associated X protein (BAX) and <u>reference genes</u> succinate dehydrogenase complex subunit A (SDHA), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), hypoxanthine-guanine phosphoribosyltransferase (HPRT) and ribosomal protein S19 (RPS19).

Human Link-N (1-16) and human sLink-N (1-8) had already been shipped from the McGill University in Montreal, Canada to the Faculty of Veterinary Medicine of Utrecht University (FVM-UU) by Fackson Mwhale and added to NP (and BMSC's) cultures before onset of this experiment.

Q-PCR primers for mentioned genes, *collagen type I, II* and *X, aggrecan, SOX 9, BMPR 2, MMP-9, MMP-13, TIMP-1, ADAMTS 5, ID 1, Pai 1, ALK 1, ALK 5, Cyclin-D1, caspase 3, BCL 2* and *BAX* and previously determined reference genes *SDHA, GAPDH, HPRT* and *RPS 19* for qPCR were obtained from the Primer bank (FVM-UU, JDV)(table 2).

Methods

Crushing of the micro-aggregates and RNA extraction

For crushing of the micro-aggregates and extraction of RNA from the samples the RNeasy[®] micro kit with RLT (QIAGEN©) was used. Prior to RNA isolation, micro-aggregates were crushed. This was done according to the protocol. The RLT buffer, a lysis buffer, was made by adding 10 μ L β -mercapto-ethanol to 1 mL RLT from the kit. The micro-aggregates were frozen in liquid nitrogen and then crushed by hand with a pellet pestle. Freezing and crushing was repeated four to six times until the micro-aggregates were fully crushed. 350 μ l RLT + β -mercapto-ethanol was added. The crushed micro-aggregates were transferred to micro tubes and vortexed. The micro tubes were dipped in liquid nitrogen and put on dry ice during the process. The tubes were stored in a freezer (-70 °C) to preserve overnight until the RNA could be extracted.

For the RNA extraction the crushed micro-aggregates from the freezer were vortexed until the ice was melted and then centrifuged shortly to make sure the crushed micro-aggregates were on the bottom of the tube. The RNA extraction was done with DNAse according to the protocol. RPE^+ buffer, a washing buffer, was made by adding 96% ethanol to RPE concentrate from the kit in ratio 1:4. Therefor 28 mL was added to 20 mL RPE⁺ concentrate. A second washing buffer, RW1 buffer, was ready for use from the kit. To make 70% ethanol 6,5 mL RNAse free water was added to 17,5 mL 96% ethanol. And to make 80% ethanol 5,6 mL RNAse free water was added to 28 mL 96% ethanol. DNAse was made by adding 315 μ l re-constituted DNAse (RDD) to 45 μ l DNAse.

350 μ l 70% ethanol was added to each crushed micro-aggregate with 350 μ l RLT (ratio 1:1). This solution was transferred to an RNeasy[®] MinElute spin column from the kit and then centrifuged for 30 seconds on the maximum speed (13200 rpm/16100 rcf) with a closed lid. Centrifuging was always done on the maximum speed and with a closed lid. Each time after centrifuging the flow through was thrown away. The RNA was washed with 350 μ l RW1 buffer and centrifuged. 80 μ l DNAse was pipetted on the membrane in the spin column. The whole was incubated for 15 minutes at room temperature. Then the RNA was washed with 350 μ l RW1 buffer and centrifuged and washed with 500 μ l RPE⁺ and centrifuged. 500 μ l 80% ethanol was added and the solution was centrifuged for two minutes. The used 2 mL tubes were replaced by new tubes and centrifuged for five minutes with the lid open to let the columns dry. The spin column was transferred to a 1,5 mL tube. 17 μ l RNase free water was added and the solution was pulled away and pipetted again on the membrane and centrifuged for one minute. The flow-through was pulled away and pipetted again on the membrane and centrifuged for one minute. The crushed samples were stored in the freezer (-70 °C) overnight.

cDNA synthesis

For cDNA synthesis the iScriptTM cDNA Synthesis Kit (Bio-Rad Laboratories[©]) was used. From each sample 15 μ l isolated RNA was collected in tube strips. If not enough RNA was available nuclease free water from the kit was supplemented to a maximum volume of 15 μ l. During the process the

samples and strips were stored on ice. Next a mix of iScriptTM reverse-transcription reaction mix with iScriptTM reverse transcriptase (ratio 4:1) was made by adding 260 μ l of the reaction mix to 65 μ l reverse transcriptase. 5 μ l of the mix was added to each tube. This way a reaction volume of 20 μ l was attaint for the qPCR. The solutions were incubated for five minutes at 25 °C, 30 minutes at 42 °C and 5 minutes at 85 °C. They were stored at 4 °C until the qPCR could be started.

RT-qPCR

The necessary primers were dissolved in Milli-Q[®], purified water (Merck Millipore Corporation, Darmstadt, Germany). The cDNA samples were diluted 10 times with Milli-Q[®]. From the 10 times diluted cDNA a part was diluted again 5 times (so in total 50 times) to use for the qPCR, the other part was stored in the freezer as a backup.

A standard line dilution of a pool of all the samples was made to determine the reaction efficacy of the qPCR. The standard dilution was made of the 10 times diluted cDNA. No template controls (NTC) were performed to test the used mix solution and Milli-Q[®] for DNA contamination and to assess for primer dimers.

On a 384 wells plate a maximum of four genes could be imbedded. For the plate set up the sample maximization method was used to be able to compare the expression level of a particular gene between different samples without technical run-to-run variation between samples. Because not all genes could fit on one plate multiple runs were executed. Therefor the genes were sorted by matching annealing temperature for the plate set up (table 2).

For each gene 6,0 μ l of a mix of 83,9 μ l Milli-Q[®], 456,0 μ l iTaq[™] universal SYBR[®] Green Super Mix (Bio-Rad Laboratories[©]) and 3,6 μ l forward and reverse primers was pipetted in the wells of a 384 wells plate and 4,0 μ l of the 50 times diluted cDNA was added to each well. iTaq[™] universal SYBR[®] Green Super Mix was used as a buffer solution, DNA polymerase and dye. The plate was covered with a seal and centrifuged for 30 seconds on 5.000 rpm. Then the plate was placed in a Bio-Rad I-cycler (Bio-Rad Laboratories[©]).

Analysis of qPCR was done as follows. Primer-dimers and other products which melted at different temperatures were excluded from the analysis. The samples which peaked at another temperature than the melt peak of a gene in a melt curve were removed by hand. Relative quantification was performed by employing the Livak ($\Delta\Delta$ Ct) method in which the expression ratio was calculated. Therefore the amplification efficacy (E) of the target and reference genes was determined. Then the threshold cycles (Ct values) of each target gene were normalized by the mean Ct value (Δ Ct) of four reference genes, GAPDH, RPS19, SDHA and HPRT. The Ct value is the cycle at which a product accumulates to an extent it has a detectable fluorescent signal. The Δ Ct of all conditions for each target gene was used as a calibrator ($\Delta\Delta$ Ct). For the samples in which no signal was detected a Ct value of 40 was manually entered, as forty cycles were executed in one qPCR run. This way the n-fold change was calculated. Additional the standard deviation (st. dev.) for each n-fold change was calculated. For combining the results of NPC's cultures with the results of NPC's and BMSC's cultures the standard deviation was corrected for changes in the n-fold change relative to the non-combined deviation.

Statistics

The $\Delta\Delta$ CT for individual target genes was used as a parameter for the statistical analysis. For the statistical analysis SPSS software (SPSS, Inc., Chicago, IL, USA) was employed. The distribution was determined per target gene using the Shapiro-Wilk normality test. Based on the distribution,

parameters were analyzed according to the one way analysis of variance (ANOVA) (normal distribution; p > 0,05) or Mann Whitney U test (skewed data; p < 0,05). Post hoc correction for multiple comparisons was done according to the Benjamin-Hochberg correction. p < 0,05 was considered statistically significant. Data with 0,1 > p > 0,05 were interpreted as a tendency.

Results

Effect of (s)Link-N on the expression of genes promoting matrix production. Link-N and sLink-N did not have statistically significant effects on genes involving matrix production compared to the control group cultured in plain chondrogenic medium. No statistically significant changes in expression of *collagen I* and *II* and *Sox 9* were found in any of the treatment groups in comparison to the untreated control group (fig. 3A, B and D). Expression of *aggrecan* tended to increase in the groups treated with Link-N and sLink-N compared to the micro-aggregates treated with 10 µg/mL TGF- β 1 (p = 0,065), but not compared to the untreated control group (fig. 3C). Micro-aggregates treated with 5 ng/mL sLink-N showed higher expression of *BMPR 2* compared to the group treated with 10 µg/mL TGF- β 1 (p < 0,05) (fig. 3E). *Collagen X* expression was undetectable.

Effect of (s)Link-N on expression of genes promoting matrix degradation. Link-N and sLink-N did not have statistically significant effects on genes involving matrix degradation compared to the control group cultured in plain chondrogenic medium. Treatment with Link-N and sLink-N did however significantly increase expression of *MMP-9* and *MMP-13* (p < 0,05) as well as expression of *ADAMTS 5* (p < 0,01) compared to treatment with TGF- β 1 (fig. 4A-C). No statistically significant changes were found in expression of *TIMP-1* (fig. 4D).

(s)Link-N did not have effect on cell proliferation. There was no statistically significant effect on expression of cyclin D1 in the cultures treated with Link-N, sLink-N nor TGF-β1 compared to the control group cultured in plain chondrogenic medium.

Effect of (s)Link-N on expression of anti- and pro-apoptotic genes. Link-N and sLink-N did not have statistically significant effects on apoptosis compared to the control group cultured in plain chondrogenic medium. Expression of the anti-apoptotic gene *BCL 2* did however decrease significantly when NP cells were treated with sLink-N in comparison to NP cells treated with 10 μ g/mL TGF- β 1 (p < 0,05). Expression of the pro-apoptotic gene *BAX* tended to increase in presence of Link-N and sLink-N (p = 0,084). The *BCL 2 : BAX* ratio decreased when NP cells were treated with sLink-N compared to treatment with TGF- β 1 (fig. 5A and B). Expression of the pro-apoptotic gene *caspase 3* decreased significantly when NP cells were treated with Link-N and sLink-N compared to NP cells treated to NP cells treated to NP cells treated to NP cells treated with 10 μ g/mL TGF- β 1 (p < 0,05) (fig. 5C).

(s)Link-N acts through BMP signaling pathway. Expression of *ID* 1 was significantly upregulated in NP cells treated with Link-N and sLink-N compared to the NPC cultures treated with 10 µg/mL TGF- β 1 (p < 0,05) (fig. 6A). Whereas expression of *Pai* 1 was significantly decreased in NP cells treated with 1 µg/mL Link-N (p < 0,01) and 5 ng/mL sLink-N (p < 0,001) compared to NP cells treated with 10 µg/mL TGF- β 1 (fig. 6B). *ALK* 1 expression was undetectable and changes in expression of *ALK* 5 were not statistically significant.





Figure 4. Gene expression of *matrix metallopeptidase 9* and 13 (*MMP-9* and *MMP-13*), *ADAMTS 5* and *TIMP-1* in NP cells to examine the effect of (s)Link-N on matrix degradation on NP cells isolated from degenerated canine intervertebral discs. It was shown that Link-N and sLink-N did not had effect on matrix degradation in degenerated canine NP cells. * indicates p < 0,05 and ** indicates p < 0,01.





pathway (s)Link-N acts. Link-N and sLink-N both activated the BMP signaling pathway and did not affect the TGF signaling pathway. * indicates p < 0,05, ** indicates p < 0,01 and *** indicates p < 0,001.

Additional effects of BMSC's on expression of genes promoting matrix production. Significantly higher expression of collagen I was seen in all cultures with BMSC's compared to the NPC cultures (p < 0,01). In the cultures treated with 10 µg/mL TGF- β 1 collagen I expression was significantly lower than in the other cultures with and without BMSC's (p < 0,05) (fig. 7A). Significantly lower expression of collagen II, aggrecan and SOX 9 was seen in the cultures with BMSC's treated with 5 ng/mL sLink-N compared to this treatment group without BMSC's (p < 0,05). Between the treatment groups no significant differences were found (fig. 7B-D).

Additional effects of BMSC's on expression of genes promoting matrix degradation. Expression of *MMP-9* was significantly higher in all cultures with BMSC's compared to the NPC cultures (p < 0,05), except in the culture treated with 10 µg/mL TGF- β 1 (fig. 8A). *MMP-13* (p < 0,05) and *TIMP-1* (p < 0,01) expression was lower in the culture with BMSC's treated with 5 ng/mL sLink-N compared to this treatment group without BMSC's (fig. 8B and D). In the TGF- β 1 treatment group expression of *MMP-13* was significantly lower compared to the other treatment groups (p < 0,05) (fig. 8B). Differences in *ADAMTS 5* expression between the groups with and without BMSC's were not statistically significant. However the group treated with 5 ng/mL sLink-N had significantly higher *ADAMTS 5* expression compared to the control group cultured in plain chondrogenic medium (p < 0,05) (fig. 8C).

BMSC's have no additional effect on cell proliferation. There were no statistically significant changes in expression of *cyclin D1* between NPC and NPC:MSC cultures regardless of the treatment. Likewise between the different treatment groups no statistically significant differences were found.

Additional effects of BMSC's on apoptosis. Differences in expression of the anti-apoptotic gene *BCL 2* between the cultures with and without BMSC's were not statistically significant. However in the cultures treated with 10 µg/mL TGF- β 1 expression of *BCL 2* was significantly higher than in the control group cultured in plain chondrogenic medium (p < 0,05) and the cultures treated with sLink-N (p < 0,01) (fig. 9A). Expression of the pro-apoptotic gene *BAX* tended to be higher in the cultures with BMSC's compared to those without BMSC's (p = 0,084) (fig. 9B). Expression of pro-apoptotic gene *caspase 3* tended to be higher in the culture with BMSC's treated with 5 ng/mL sLink-N compared to this treatment group without BMSC's (p = 0,093). The cultures treated with 10 µg/mL TGF- β 1 showed significantly higher expression of *caspase 3* compared to the untreated control groups and the cultures treated with sLink-N (p < 0,05) (fig. 9C).

Additional effects of BMSC's on activation of signaling pathways. Expression of *ID* 1 was lower in the NPC:BMSC culture than in the NPC culture when treated with 0,5 µg/mL sLink-N (p < 0,05). The expression of *ID* 1 was significantly lower in the cultures treated with 10 µg/mL TGF- β 1 (p < 0,01), 1 µg/mL Link-N (p < 0,05) and 0,5 µg/mL sLink-N (p < 0,01) compared to the control groups cultured in plain chondrogenic medium (fig. 10A). Expression of *Pai* 1 in the culture treated with 1 µg/mL Link-N was significantly higher in the culture with BMSC's than in the culture without BMSC's (p < 0,01) (fig. 10B). The cultures treated with sLink-N did not show statistical significant differences in expression of *Pai* 1.



and in presence (NPC:MSC) of BMSC's to examine additional effects of BMSC's on the effect of (s)Link-N on matrix production on NP cells isolated from degenerated canine intervertebral discs. BMSC's upregulated *collagen I* expression, but did not had positive synergistic effects with Link-N nor sLink-N on matrix production in degenerated canine NP cells. * indicates p < 0,05, ** indicates p < 0,001 between the NPC culture and NPC:MSC culture within a treatment group. o indicates p < 0,05 between treatment groups.



0,05 and ** indicates p < 0,01 between the NPC culture and NPC:MSC culture within a treatment group. o indicates p < 0,05 between treatment groups.





Figure 9. Gene expression of *BCL 2, BAX* and *caspase 3* in NP cells in absence (NPC) and in presence (NPC:MSC) of BMSC's to examine additional effects of BMSC's on the (anti-) apoptotic effects of (s)Link-N on NP cells isolated from degenerated canine intervertebral discs. Link-N nor sLink-N had an (anti-)apoptotic effect on degenerated canine NP cells in combination with BMSC's. o indicates p < 0.05 and oo indicates p < 0.01 between treatment groups.



Figure 10. Gene expression of *ID* 1 and *Pai* 1 in NP cells in absence (NPC) and in presence (NPC:MSC) of BMSC's to examine additional influences of BMSC's on the activation of pathways by (s)Link-N in NP cells isolated from degenerated canine intervertebral discs. *ID*1 expression was downregulated in the NPC:MSC culture when treated with sLink-N. *Pai* 1 expression was upregulated in the NPC:MSC culture when treated with sLink-N. *Pai* 1 expression was upregulated in the NPC:MSC culture when treated with Link-N. * indicates p < 0,05 and ** indicates p < 0,01 between the NPC culture and NPC:MSC culture within a treatment group. o indicates p < 0,05 and oo indicates p < 0,01 between treatment groups.

Discussion

In this study the effect of Link-N and sLink-N was evaluated as a first step towards translating a treatment strategy based on human (s)link-N for veterinary patients suffering from IVD disease. We demonstrated that (s)Link-N increased *ID* 1 expression and decreased *Pai* 1 expression significantly compared to TGF- β 1, confirming (s)Link-N acts through BMP signaling pathway as was found in a study on Link-N in rabbit cells (Wang et al., 2013).

Nevertheless, treatment with human Link-N nor sLink-N showed statistically significant changes in expression of *collagen I* and *II* in canine degenerated NP cells compared to untreated control NP cells. This is in contrast with other studies with human, bovine and rabbit IVD cells which report that Link-N has an effect on gene expression involving matrix production (Mwale et al., 2003, 2011; Petit et al., 2011). Also treatment with human Link-N nor sLink-N showed statistically significant changes in gene expression of *MMP-9, MMP-13* and *ADAMTS 5*. Contrary to the present study, other studies report changes in gene expression involving matrix degradation (Mwale et al., 2011). Although treatment with TGF-β1 did not cause significant upregulation of *collagen II, SOX9* and *aggrecan* as expected (Thompson et al., 1991), TGF-β1 did significantly inhibit gene expression involving matrix degradation. This indicates that the NP cells were responsive to catabolic stimuli. To the contrary, (s)Link-N upregulated of *MMP-9* and *-13* and *ADAMTS 5* compared to TGF-β1, indicating increased matrix degradation, which might be due to active matrix remodeling (unpublished data, M. Tryfonidou, Faculty of Veterinary Medicine of Utrecht University).

Furthermore, the effect of Link-N and sLink-N in combination with BMSC's was examined in this study. Treatment with human (s)Link-N and canine BMSC's did not show an additive trophic effect on degenerated canine NP cells. In contrast a study on rabbit NP cells cultured with BMSC's of the same rabbits report that BMSC's cultured with NP cells do have trophic effects on ECM synthesis and cell proliferation (Yamamoto et al., 2004). However in the co-cultures of the present study the same gene expression involving matrix production was found as in the NPC cultures alone, while these co-cultures contain only half of the NP cells. So it cannot be excluded that BMSC's do have a trophic effect. Also RT-qPCR was done at day 7 of culture, based on previous studies. However we cannot exclude that at this time point the changes in gene expression were the most outspoken.

Others have reported that Link-N can enhance chondrogenic differentiation of BMSC's in combination with a chondrogenic medium (Antoniou et al., 2012). However Link-N on itself in a serum free medium did not stimulate chondrogenic differentiation of BMSC's in the same manner as TGF- β did. Antoniou et al. also reports that differentiated BMSC's in presence of Link-N express higher levels of *aggrecan, SOX 9* and *collagen II*. The different results found in this study may be because human Link-N was used in combination with canine NPC's and BMSC's. Human and canine Link-N and sLink-N have a different sequence (table 3), resulting into a docking mismatch on the BMP2 receptor (unpublished data, Frances Bach, Faculty of Veterinary Medicine of Utrecht University).

It was expected that *collagen I* expression would be down regulated in the cultures with BMSC's treated with (s)Link-N or TGF-β1, because TGF-β1 decreases *collagen I* expression and increases *collagen II* expression (probably because BMSC's differentiate) (Chen, Hu, & Lü, 2000). For the same reason expression of *collagen II, SOX* 9 and *aggrecan* was expected to be up regulated.

	Link-N (amino acids 1-16)	sLink-N (amino acids 1-8)
Human	DHLSDNYT LDHDRAIH	DHLSDNYT
Canine	DHHSDNYT LNYDRVIH	DHHSDNYT

Table 3. Amino acid sequences of human and canine Link-N and sLink-N. Distinct amino acids are marked red.

Undifferentiated BMSC's produce a high collagen I content, while chondrogenically differentiated BMSC's produce collagen II when treated with TGF- β 1 or (s)Link-N (Antoniou et al., 2012). This study showed a higher *collagen I* expression in the cultures with BMSC's. However contrary to this concept, the present study showed no significant upregulation of *collagen II* expression in the TGF- β 1 treatment group, nor in the groups treated with (s)Link-N. This could be because BMSC's do not differentiate as well in a medium without growth factors as employed in this study.

In this study co-culturing with BMSC's also showed no statistically significant upregulation in expression of *SOX 9* and *aggrecan* compared to the NP cell cultures. However in the cultures treated with sLink-N *SOX 9, collagen II* and *aggrecan* expression was lower in the cultures with BMSC's. This in contrast with a study on Link-N and human BMSC's (Antoniou et al., 2012). Furthermore, low collagen I production could indicate few numbers of BMSC's in a culture due to a low survival rate. This is not likely as the *collagen I* expression in the other treatment groups is high, indicating the BMSC's can survive. Also there are studies that report MSC's can survive in canine IVD's (Hiyama et al., 2008). And a study on rabbit cells reported enhanced survival of NP cells in degenerated discs when treated with BMSC's and TGF- β 1 (Yang et al., 2010). Supportive of this theory expression of pro-apoptotic genes did not increase in cultures with BMSC's.

In the current study results concerning matrix degradation were variable. *MMP-13* expression was lower in the culture with BMSC's treated with 5 ng/mL sLink-N. This is in accordance with a study on the effect of MSC's in a rat model which reported that *MMP-13* expression is inhibited by BMSC's (Tang et al., 2015). However *MMP-9* expression was increased and *TIMP-1* expression was decreased in most cultures with BMSC's. Based on these results we can not exclude that BMSC's do have a trophic effect on degenerated NP cells in combination with Link-N.

One of the goals of this study was to determine whether sLink-N is as effective in repair of degenerated NP cells as Link-N. Unfortunately effects of low (5 ng/mL) or high (5µg/mL) doses sLink-N nor Link-N did differ significantly from each other or from the untreated control group in contrast to other studies. A study on human AF cells showed Link-N was cleaved by the cells and able to significantly upregulate proteoglycan synthesis in AF and NP cells indicating that sLink-N contains the active part of Link-N (Gawri et al., 2014). In contrast a study on human IVD cells reported that if Link-N is truncated with three, four or six amino acids it loses its effectiveness, even though one of the three truncated versions they tested contained the amino acid sequence of sLink-N (Wang et al., 2012).

Conclusion

Human Link-N and sLink-N have no effect on canine NP cells, likewise sLink-N does not appear to be more effective than Link-N. Additional human Link-N or sLink-N in combination with canine BMSC's appear to have no trophic effects on canine NP cells. So a combination of (s)Link-N and BMSC's does not amplify their regenerative potentials on degenerated NP cells. A subsequent research is started on the effect of canine Link-N on degenerated canine NP cells.

References

- Antoniou, J., Wang, H. T., Alaseem, A. M., Haglund, L., Roughley, P. J., & Mwale, F. (2012). The effect of Link N on differentiation of human bone marrow-derived mesenchymal stem cells. *Arthritis Research & Therapy*, *14*(6), R267. http://doi.org/10.1186/ar4113
- Bach, F. C., Willems, N., Penning, L. C., Ito, K., Meij, B. P., & Tryfonidou, M. A. (2014). Potential regenerative treatment strategies for intervertebral disc degeneration in dogs. *BMC Veterinary Research*, 10(1), 3. http://doi.org/10.1186/1746-6148-10-3
- Bergknut, N., Smolders, L. A., Grinwis, G. C. M., Hagman, R., Lagerstedt, A.-S., Hazewinkel, H. A. W., ... Meij, B. P. (2013). Intervertebral disc degeneration in the dog. Part 1: Anatomy and physiology of the intervertebral disc and characteristics of intervertebral disc degeneration. *Veterinary Journal (London, England : 1997), 195*(3), 282–91. http://doi.org/10.1016/j.tvjl.2012.10.024
- Chen, Y., Hu, Y., & Lü, Z. (2000). [Regulating effects of transforming growth factor-beta (TGF-beta) on gene expression of collagen type II in human intervertebral discs]. *Zhonghua Wai Ke Za Zhi* [*Chinese Journal of Surgery*], *38*(9), 703–6. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11832145
- Gawri, R., Ouellet, J., Nnerfjord, P., Alkhatib, B., Steffen, T., Heinegård, D., ... Haglund, L. (2014). Link N is Cleaved by Human Annulus Fibrosus Cells Generating a Fragment With Retained Biological Activity. *Journal of Orthopaedic Research*, *32*, 1189–1197. http://doi.org/10.1002/jor.22653
- Hansen, H. J. (1952). A pathologic-anatomical study on disc degeneration in dog, with special reference to the so-called enchondrosis intervertebralis. *Acta Orthopaedica Scandinavica.* Supplementum, 11, 1–117. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/14923291
- Hiyama, A., Mochida, J., Iwashina, T., Omi, H., Watanabe, T., Serigano, K., ... Sakai, D. (2008).
 Transplantation of mesenchymal stem cells in a canine disc degeneration model. *Journal of Orthopaedic Research : Official Publication of the Orthopaedic Research Society*, 26(5), 589– 600. http://doi.org/10.1002/jor.20584
- Holm, S., Maroudas, A., Urban, J. P., Selstam, G., & Nachemson, A. (1981). Nutrition of the intervertebral disc: solute transport and metabolism. *Connective Tissue Research*, 8(2), 101–19. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/6453689
- Hoogendoorn, R. J. W., Lu, Z. F., Kroeze, R. J., Bank, R. a, Wuisman, P. I., & Helder, M. N. (2008). Adipose stem cells for intervertebral disc regeneration: current status and concepts for the future. *Journal of Cellular and Molecular Medicine*, *12*(6A), 2205–16. http://doi.org/10.1111/j.1582-4934.2008.00291.x
- Liu, H., McKenna, L. A., & Dean, M. F. (2000). An N-terminal peptide from link protein can stimulate biosynthesis of collagen by human articular cartilage. *Archives of Biochemistry and Biophysics*, 378(1), 116–22. http://doi.org/10.1006/abbi.2000.1758
- Masuda, K., Oegema, T. R., & An, H. S. (2004). Growth factors and treatment of intervertebral disc degeneration. *Spine*, *29*(23), 2757–69. http://doi.org/10.1097/01.brs.0000146048.14946.af
- McKenna, L. A., Liu, H., Sansom, P. A., & Dean, M. F. (1998). An N-terminal peptide from link protein stimulates proteoglycan biosynthesis in human articular cartilage in vitro. *Arthritis and Rheumatism*, 41(1), 157–62. http://doi.org/10.1002/1529-0131(199801)41:1<157::AID-ART19>3.0.CO;2-J
- Meirelles, L. da S., Fontes, A. M., Covas, D. T., & Caplan, A. I. (2009). Mechanisms involved in the therapeutic properties of mesenchymal stem cells. *Cytokine & Growth Factor Reviews*, *20*(5-6), 419–27. http://doi.org/10.1016/j.cytogfr.2009.10.002

- Mwale, F., Demers, C. N., Petit, A., Roughley, P., Poole, a R., Steffen, T., ... Antoniou, J. (2003). A synthetic peptide of link protein stimulates the biosynthesis of collagens II, IX and proteoglycan by cells of the intervertebral disc. *Journal of Cellular Biochemistry*, *88*(6), 1202–13. http://doi.org/10.1002/jcb.10479
- Mwale, F., Masuda, K., Pichika, R., Epure, L. M., Yoshikawa, T., Hemmad, A., ... Antoniou, J. (2011). The efficacy of Link N as a mediator of repair in a rabbit model of intervertebral disc degeneration. *Arthritis Research & Therapy*, *13*(4), R120. http://doi.org/10.1186/ar3423
- Mwale, F., Wang, H. T., Roughley, P., Antoniou, J., & Haglund, L. (2014). Link N and mesenchymal stem cells can induce regeneration of the early degenerate intervertebral disc. *Tissue Engineering. Part A*, *20*(21-22), 2942–9. http://doi.org/10.1089/ten.TEA.2013.0749
- Petit, A., Yao, G., Rowas, S. Al, Gawri, R., Epure, L., Antoniou, J., & Mwale, F. (2011). Effect of synthetic link N peptide on the expression of type I and type II collagens in human intervertebral disc cells. *Tissue Engineering. Part A*, 17(7-8), 899–904. http://doi.org/10.1089/ten.TEA.2010.0494
- Tang, J., Cui, W., Song, F., & Zhai, C. (2015). Effects of mesenchymal stem cells on interleukin-1 β treated chondrocytes and cartilage in a rat osteoarthritic model. *Molecular Medicine Reports*, *12*, 1753–1760. http://doi.org/10.3892/mmr.2015.3645
- Vadalà, G., Sowa, G. A., & Kang, J. D. (2007). Gene therapy for disc degeneration. *Expert Opinion on Biological Therapy*, 7(2), 185–96. http://doi.org/10.1517/14712598.7.2.185
- Walsh, A. J. L., Bradford, D. S., & Lotz, J. C. (2004). In vivo growth factor treatment of degenerated intervertebral discs. *Spine*, *29*(2), 156–63. http://doi.org/10.1097/01.BRS.0000107231.67854.9F
- Wang, Z., Hutton, W. C., & Yoon, S. T. (2013). Effect of link protein peptide on human intervertebral disc cells. *Spine*, *38*(17), 1501–7. http://doi.org/10.1097/BRS.0b013e31828976c1
- Wang, Z., Weitzmann, M. N., Sangadala, S., Hutton, W. C., & Yoon, S. T. (2013). Link protein Nterminal peptide binds to bone morphogenetic protein (BMP) type II receptor and drives matrix protein expression in rabbit intervertebral disc cells. *The Journal of Biological Chemistry*, 288(39), 28243–53. http://doi.org/10.1074/jbc.M113.451948
- Watanabe, T., Sakai, D., Yamamoto, Y., Iwashina, T., Serigano, K., Tamura, F., & Mochida, J. (2010).
 Human nucleus pulposus cells significantly enhanced biological properties in a coculture system with direct cell-to-cell contact with autologous mesenchymal stem cells. *Journal of Orthopaedic Research : Official Publication of the Orthopaedic Research Society*, 28(5), 623–30.
 http://doi.org/10.1002/jor.21036
- Yang, H., Wu, J., Liu, J., Ebraheim, M., Castillo, S., Liu, X., ... Ebraheim, N. A. (2010). Transplanted mesenchymal stem cells with pure fibrinous gelatin-transforming growth factor-??1 decrease rabbit intervertebral disc degeneration. *Spine Journal*, *10*(9), 802–810. http://doi.org/10.1016/j.spinee.2010.06.019