The Effect of Caveolin-1 on Transforming Growth Factor β1 Treatment of Degenerated Canine Nucleus Pulposus Cells

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Abstract

Introduction. Intervertebral disc (IVD) degeneration is a common disorder that affects a significant proportion of the human population. Spontaneously occurring IVD degeneration is also a common problem in dogs, and therefore, the dog is considered an unique animal model for IVD degeneration research. The current purpose of a treatment is to alleviate pain. Because the current treatments do not solve the problem, there is increasing interest in regenerative therapies, like treatment with TGF-β. In this study, we have investigated whether caveolin-1, acknowledged for its physiological importance in tissue repair and fibrosis, has an additive effect on TGF-β treatment of degenerated canine nucleus pulposus cells (chondrocyte-like nucleus pulposus cells, CLCs).

Materials and methods. Degenerated nucleus pulposus cells (CLCs) were used from twelve canine donors; the donors were all chondrodystrophic dogs (Beagles), aged 19 – 32 months, eight females and four males. The CLC pellets were cultured for 28 days in standard chondrogenic medium without (negative control) or with 2 or 10 ng/mL TGF-β1 (positive controls), with and without caveolin-1 supplementation. At day 0, 1, 7 and 28, cell proliferation was determined by measuring the pellets' DNA content. Furthermore, the glycosaminoglycan (GAG) content was determined using a DMMB assay. At day 7 and day 28, gene expression was determined using RT-qPCR. Histology was performed at day 28.

Results. TGF-β has a positive, dose-dependent effect on cell proliferation and matrix production of the CLC pellets. In general, no additive effect of caveolin-1 is observed on TGF-β treatment of the degenerated CLC pellets. But in one donor, a statistical significant additive effect of caveolin-1 on the CLC pellets GAG content is observed on day 28.

Discussion. Although no additive effect of caveolin-1 is observed in the mean values of the pellets, it is interesting that if the donors are observed independently, donor A seems to have an additive effect of caveolin-1 on TGF-β 10 ng/mL treatment on day 28 of cell culture suggesting that the effect of caveolin-1 is donor-dependent. When the different donors are compared to each other, no differences in age or breed are present, so it is not certain why this effect only in donor A is seen. Although there is an effect of caveolin-1 on *Axin2* expression observed, the exact role of caveolin-1 in the Wnt/β-catenin signaling is still not certain. There are a few hypotheses, but further research is necessary to find out the exact role of caveolin-1 in this pathway.

Conclusion. No additive effect of caveolin-1 is found on TGF-β treatment of degenerated CLC pellets, except for donor A on GAG content. Further investigations are necessary to understand the different pathways caveolin-1 regulates an which role caveolin-1 plays in intervertebral disc degeneration.

Contents

Introduction

Low back pain in humans and dogs

Low back pain is a common disorder that affects a significant proportion of the human population. One of the most important causes for low back pain is intervertebral disc degeneration (IVD). Spontaneously occurring IVD degeneration and disease are major health concerns in humans, but also in dogs. The symptoms of dogs with IVD disease can be lameness or paresis, toe dragging, low tail or neck carriage, difficulties with rising, sitting or lying down, reluctance to jump or climb, urinary or faecal incontinence, and hyperesthesia or self-mutilation. However, IVD degeneration can also occur without any symptoms (6). Similar pathological IVD changes are seen in humans and dogs. The most important differences between humans and dogs are the absence of growth plates in growing human vertebrae and the thicker cartilaginous endplates in humans. The thicker cartilaginous endplates are probably due to the fact that in humans, the vertebral growth mainly takes place in the interface between the cartilaginous endplates and the subchondral bone, whereas in dogs, the vertebral growth is primarily regulated through separate epiphyseal growth plates located within the vertebrae at both the cranial and caudal ends (9,10).

Animal models used for IVD degeneration research

In IVD degeneration research, different animal models are used, mostly with manually or chemically induced IVD degeneration (9). Spontaneously occurring IVD degeneration is a common problem in dogs, and the clinical presentation, macroscopic and microscopic appearance, diagnostics, and treatment are similar in both humans and dogs (1,9). Therefore, the dog is considered an unique animal model for IVD degeneration research.

Dog breeds can be classified into two groups, the chondrodystrophic (CD) and non-chondrodystrophic (NCD) breeds (see fig. 1 and 2). CD and NCD dogs are dissimilar with regard to the age of onset, frequency, and spinal location of IVD degeneration and IVD degenerative diseases. IVD degeneration is more common in CD dogs, because they have a genetic predisposition towards chondrodystrophy. CD dogs show a disturbed endochondral ossification, primarily of the long bones, resulting in disproportionally short limbs. IVD degenerative disease in CD dogs typically develops around 3–7 years of age, while in NCD dogs it develops around 6–8 years of age (2).

The healthy IVD

The biomechanical function of the IVD is to transmit compressive forces between vertebral bodies and to provide mobility as well as stability to the spinal segment (1). The IVD consists of a central nucleus pulposus (NP), an outer annulus fibrosus (AF), the transition zone (TZ) and the vertebral endplates (EPs) (see fig. 3) (6). The NP is a mucoid, translucent, bean-shaped structure, mainly composed of water, located slightly eccentrically in the IVD (1,8). Cations are attracted to the negatively charged proteoglycans and create a strong osmotic gradient. Therefore, water molecules draw into the NP (6). The main cell type in a healthy canine NP is the notochordal cell (NC), which is considered to be a potential NP progenitor cell (1,8). It is a large cell that is characterized by cytoplasmic vesicles. NCs are found in clusters and produce an amorphous basophilic matrix rich in proteoglycans and collagen type II (1).

Fig 1: Beagle, a chondrodystrophic dog breed (11)

Fig 2: German Shepherd, a non-chondrodystrophic dog breed (12)

The EPs play an essential role in NP nutrient delivery by enabling diffusion and permeability. This is necessary, because the NP has no direct blood supply. The NP contains a relatively large amount of cells and a small amount of extracellular matrix (ECM) during early development. In the mature NP, the ECM/cell ratio is high (6).

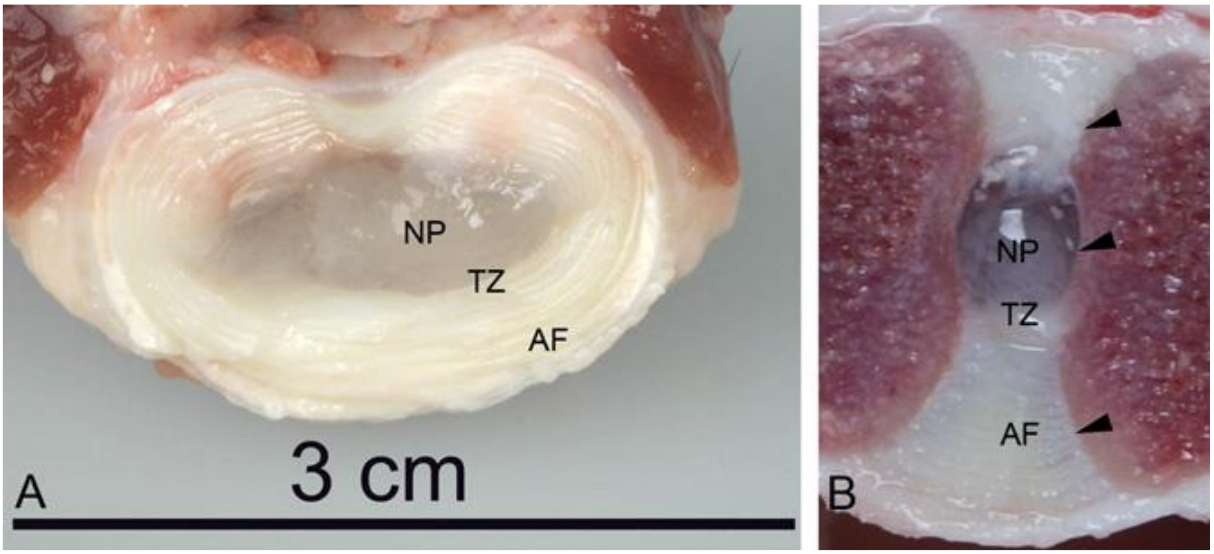

Fig. 3: (A) Transverse section through a intervertebral disc of a mature non-chondrodystrophic dog. The nucleus pulposus (NP), transition zone (TZ), annulus fibrosus (AF) are shown. (B) Sagittal section through an intervertebral disc of the same dog. Here the endplates (arrowheads) are also shown (1).

IVD degeneration

In early IVD degeneration, the changes closely resemble those of physiological ageing of the disc (1). The first alterations that can be noticed in IVD degeneration are the cellular changes within the NP. The large NCs are replaced by chondrocyte-like nucleus pulposus cells (CLCs) (see fig. 4). The replacement of NCs by CLCs and their ECM is called chondrification (1). In adult humans and CD dogs, the NCs are not found in the healthy IVD; they are only present in adult NCD dog NPs, in children NPs (<10 years of age) and CD dog NPs younger than 1 year of age (10). In NCD dogs, the NCs remains the predominant cell type of the NP during the majority of life (4). In CD dogs, the NCs differentiate into or are replaced by CLCs before 1 year of age (8).

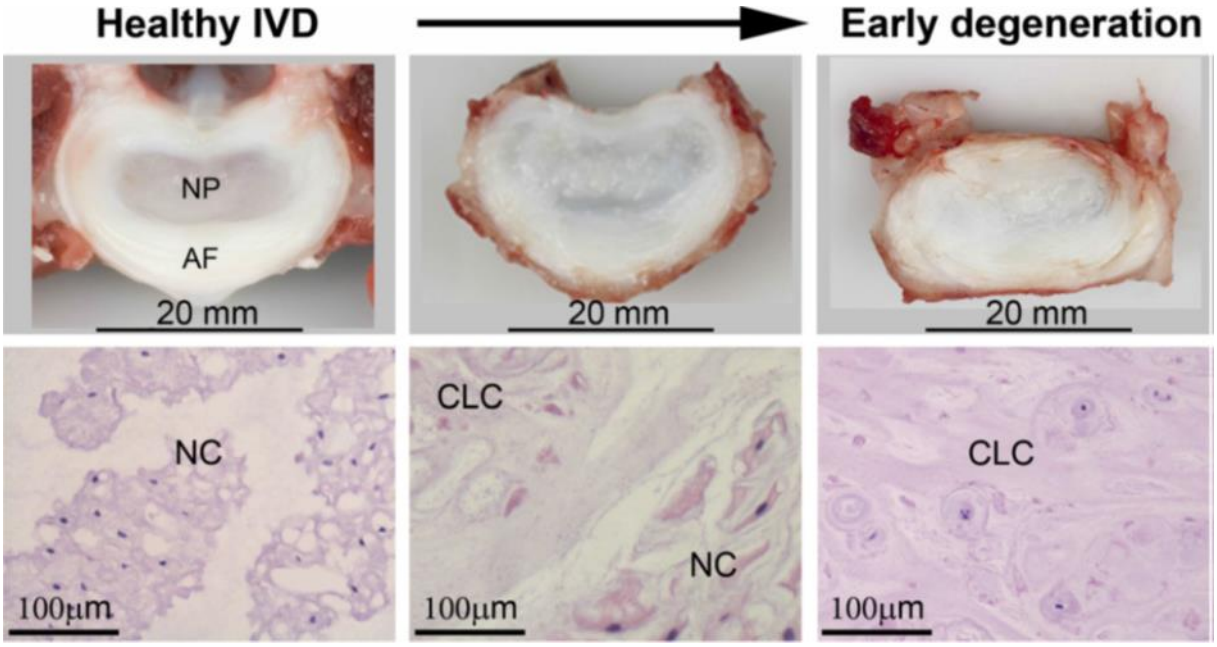

Fig. 4: The change from a healthy intervertebral disc (IVD) to early IVD degeneration. Upper left: Macroscopic image of a healthy IVD with the annulus fibrosus (AF) and the bean-shaped nucleus pulposus (NP). Upper middle and right: Macroscopic image during IVD degeneration, where the difference between AF and NP becomes less obvious. Lower left: Microscopic image of a healthy NP, where the notochordal cells (NCs) are the main cell type. Lower middle: Microscopic image of a changing NP, where there is a mix of cartilage-like cells (CLCs) and NCs. Lower right: Microscopic image of early IVD degeneration, where the NP consists mainly of CLCs (6).

The physiological function of the IVD is largely dependent on the quality of its ECM and therefore on the ability of its constituent cells to synthesize, remodel, and maintain a biochemically healthy matrix (1). In IVD degeneration, the synthesis of collagen type II declines, whereas the production of the less compliant collagen type I increases (26). Also the catabolic matrix proteinase (MMP) activity increases and glycosaminoglycan (GAG) content decreases. This results in weakening and increased vulnerability to damage, because matrix repair is impaired in the avascular IVD. There is a vicious circle of continuous damage and inadequate repair. When IVD degeneration has started, the disease is progressive (6).

Regenerative treatments for intervertebral disc disease

The current purpose of a treatment for IVD degeneration in dogs is to alleviate pain. It includes physiotherapy, anti-inflammatory/analgesic medication and surgery. Procedures for surgery include removal of the NP (nucleotomy) through fenestration of the AF alone or combined with partial removal of the vertebral roof (laminectomy) or vertebral body (ventral decompression). The aim of surgery is to relieve the compression of neural structures. However, the therapies mentioned above do not lead to repair of the IVD and may have considerable side-effects (6).

Because the current treatments do not solve the problem of the IVD, there is increasing interest in regenerative therapies (6). Their aim is to repair the degenerated disc matrix and in this way to restore the biomechanical function of the IVD by using adult stem or progenitor cells, growth factors, and/or gene therapy (6,8).

Regenerative medicines aim can be to stimulate the synthesis of NP matrix. However, stimulation of the remaining cells may be insufficient, because of the relatively few cells in the degenerated IVD and the impaired cell viability. To overcome this problem, cell-based therapies can be used. The most cell types used are CLCs, mesenchymal stromal (stem) cells (MSCs), and NCs. Exogenous growth factors can stimulate cell proliferation and/or matrix synthesis. This can alter IVD homeostasis by inhibiting catabolic or by stimulating anabolic processes. Most growth factors that have been tested *in vitro* have been shown to successfully decrease cell apoptosis and to increase chondrogenic matrix production, and to direct MSC differentiation towards a NP-like phenotype. It has also been proven that growth factors are effective *in vivo* in animal models with experimentally induced IVD degeneration. However, there have been no studies of growth factors tested in animal models with spontaneously occurring IVD degeneration (6).

Knowledge of the pathways involved in the pathogenesis of IVD degeneration is necessary to develop regenerative treatments for IVD diseases in dogs and humans (10). Furthermore, it is essential to identify IVD degeneration in an early stage, to prevent the occurrence of IVD disease. This can be accomplished by magnetic resonance imaging (MRI) studies, but in veterinary practice this diagnostic tool is only used when there are already clinical signs. The therapeutic effect of injected cell and growth factors may be influenced by the severity of IVD degeneration. It is also not known whether the cell-based IVD treatment strategies are able to regenerate NP tissue, since the current studies only showed a halting of degeneration. Furthermore, all regenerative strategies based on injectable treatments may cause considerable side effects (6).

The role of caveolin-1 in IVD degeneration and regeneration

The involvement of both environmental and genetic factors have been proposed as important causes for IVD degeneration. There is some evidence that many features of IVD degeneration originate at a cellular level. Also, there is a prolonged alteration in cellular activity observed during degeneration and it is possible that cellular senescence plays a major role in the pathogenesis. Senescence not only occurs with aging, but can also occur prematurely in response to stress. It is suggested that caveolin-1 and stress-induced premature senescence (SIPS) may play a prominent role in the pathogenesis of IVD degeneration (3). Senescent cells are unable to proliferate, but do remain viable and metabolically active for a long period of time. They do not longer respond to

external stimuli, such as growth factors. This may lead to reduced tissue function in ageing organs (16).

Caveolae are plasma membrane compartments. They are found abundantly in terminally differentiated cells such as fibroblasts. The mammalian caveolin gene family consists of three proteins, which are integral membrane proteins (caveolin-1, -2, and -3) essential for the structural integrity and function of caveolae (3). Caveolin-1 has been acknowledged for its physiological importance in tissue repair and fibrosis (5). It is known that caveolin plays a significant role in various cellular processes, including stem cell regulation and proliferation (4,7). SIPS can also be induced by an overexpression of caveolin-1 (3).

Variable effects of caveolin-1 on different cell types

The effects of caveolin-1 are variable and they seems to be dependent upon cell type and the stage of differentiation and/or degeneration. The inhibitory association of signaling molecules with caveolin-1 may be responsible for the inhibitory effects of caveolin-1 on cell proliferation. A decrease in caveolin-1 expression or low caveolin-1 activity may be required to activate cell proliferation and differentiation. In mouse embryonic stem cells caveolin-1 appeared to be required for cell renewal. Therefore, caveolin-1 may negatively regulate the proliferation of adult progenitor cells, but in embryonic stem cells it may be positively involved in proliferative signaling (7).

For tissue repair it may also be required that caveolin-1 expression is down-regulated. During tissue regeneration in skeletal tissue temporal changes in caveolin expression occurs. The down-regulation of caveolin-1 expression it a pre-requisite for cell proliferation, migration and differentiation to repair muscle wounds. However, it has been shown that caveolin-1 has a positive role in kidney regeneration and cutaneous wound healing. In cases where caveolin-1 is required for repair and regeneration, caveolin-1 probably inhibits the not necessary pathways. In this way, the beneficial pathways for the activation of repair are not inhibited by these other pathways. It thus seems to depend on the tissue type whether caveolin-1 expression has a positive or negative effect on tissue repair (see table 1) (7).

Celtype	Effect of caveolin-1
Mesenchymal stem cells	Cell proliferation ↓ Cell differentiation ↓
Embryonic stem cells	Cell renewal ↑ Cell proliferation ↑ Cell differentiation ↓
Brain	Neuronal differentiation ↓
Mammary gland	Prolactin signaling ↓
Skeletal muscle	Cell proliferation ↓ Cell migration ↓ Cell differentiation ↓
Heart	Maintenance of healthy tissue ↑ Formation of new blood vessels ↑ Cell proliferation ↑
Liver	Regeneration ↑

	Cell proliferation ↑
Kidney	Regeneration ↑
Skin	Wound healing ↑

Table 1: The effects of caveolin-1 in different types of cells (7)

Caveolin-1 in the IVD

Caveolin-1 gene expression has been shown to be higher in degenerated compared with healthy canine IVDs. Furthermore, caveolin-1 has been suggested to be an important player in NC physiology. Caveolin-1 KO mice showed relatively few healthy NC clusters in the NP. Most NP cells lacked the morphological characteristics of NCs and showed signs of apoptosis, and the NP contained abundant intercellular chondroid matrix. These results suggest that caveolin-1 is essential to maintain the NC/healthy NP, and that decreased caveolin-1 expression is an important factor in NC physiology and IVD degeneration (4).

The effect of caveolin-1 on signaling pathways

Growth factors stimulate cellular metabolism; they change the tissue homeostasis into an anabolic status (5,15). It is known that caveolin-1 regulates Wnt/β-catenin signaling, transforming growth factor-β (TGF-β) signaling and bone morphogenetic protein (BMP) signaling. All pathways can guide stem cell fate (7).

It has been shown that a down-regulation of caveolin-1 resulted into down-regulated canonical Wnt/β-catenin signaling. Caveolin-1 can stimulate Wnt signaling by activating integrin-linked kinases and through the accumulation of β-catenin to caveolae membranes, but it can also inhibit Wnt signaling by recruiting β-catenin to caveolae membranes (4). Canonical Wnt/β-catenin signaling is involved in tissue degeneration and regeneration and regulates notochord cell fate and stem cell renewal and apoptosis (4,8). It also plays a crucial role in maintaining the notochordal fate during embryogenesis (8). Decreased Wnt signaling may result in increased apoptosis and decreased self-renewal of NCs or NP stem cells, and ultimately in chondroid metaplasia of the NP (4). Canonical Wnt signaling is important for the promotion of the differentiation or maintenance of stem cells in a self-renewing state. It has been shown that during IVD degeneration canonical Wnt signaling increases, supporting the proliferation of cells and in this way trying to restore the IVD health (8).

TGF-β stimulates cell proliferation and differentiation in the IVD, and also stimulates ECM production. It has been shown that TGF-β has anabolic effects on proteoglycan synthesis in canine disc cells. However, it seemed that overexpression of TGF-β increased matrix metalloproteinase 13, which means that it had a deleterious effect on degenerated cartilage tissue (15). TGF-β binds to a serine-threonine kinase receptor complex. This complex consists of two distinct proteins; the type I and type II receptor. When TGF-β binds, it induces the type I and type II receptor to associate, which leads to phosphorylation of the type I receptor by the type II receptor. The type I receptor is now activated and signals to the SMAD family of intracellular mediators. However, SMAD-independent pathways of TGF-β do also exist (20).

There are two different TGF-β type I receptors; ALK5 and ALK1. Activation of ALK5 leads to activation of the SMAD2/3 pathway, which in turn leads to stimulation of synthesis of ECM components. ALK1 activation leads to stimulation of SMAD1/5/8, that leads to inhibition of SMAD2/3 and thus, the inhibition of new synthesis of ECM components. Besides, it also leads to the activation of MMP13 and thus, further IVD degeneration (13,15). Research has been shown that the ratio of ALK5/ALK1 depends on age; younger animals seems to have more ALK5 receptors. When animals grow older, the expression of ALK5 decreases significantly. Therefore a shift from ALK5 to ALK1 is observed and IVD degeneration continues in older animals (13).

There are observations that caveolin-1 regulates TGF-β1 signaling by directly interacting with the TGF-β receptors, so that binding of TGF-β1 to the ALK5 and ALK1 receptor is inhibited (5,14,20). Because there is a higher expression of ALK5 in IVD cells, the SMAD2 pathway is more sensitive and TGF-β1 seems to activate mainly SMAD2. In this way, ECM components can be synthesized and disc regeneration can occur (15). In a small pilot caveolin-1 culture experiment at FVM-UU, a positive effect of TGF-β treatment was indeed established on the DNA and GAG content of CLC pellets. Furthermore, caveolin-1 (1 μM) seemed to have an additive effect upon TGF-β treatment of these degenerated CLCs, concerning the DNA and GAG content of the CLC pellets. However, another experiment is needed to confirm these preliminary data (to obtain more statistical power).

Aim and hypothesis of the study

The overall purpose of this project is to find the best cell/growth factor-based regenerative strategy to restore the health and function of the degenerated IVD. The specific aim of this experiment is to determine the additive effect of caveolin-1 on TGF-β_1 treatment of degenerated canine CLCs. It is hypothesized that caveolin-1 has an additive effect on TGF-β_1 treatment of CLCs. Thus, a stronger positive effect on cell proliferation, DNA and GAG content is expected compared with TGF-β_1 treatment alone.

Materials and methods

Cell culture

Degenerated nucleus pulposus cells (CLCs) were used from twelve canine donors; the donors were all CD dogs (Beagles), aged 19 – 32 months, eight females and four males. The NP tissue was carefully isolated (aseptically) from the IVDs of the spines. The NP tissue was digested (using pronase and collagenase) to obtain single CLCs. The CLCs of the dogs were pooled (passage 2) to yield 5 different CLC pellet combinations (pooled donors A, B, C, D, and E). The CLC pellets contained 200.000 cells and were cultured for 28 days (5% CO_2, 20% O_2) in standard chondrogenic medium without (negative control) or with 2 or 10 ng/mL TGF-β_1 (positive controls), with and without caveolin-1 (0.01, 0.1 and 1 μM) supplementation. Nine different CLC pellet conditions were analysed during this experiment; (negative) control CLC pellets, TGF-β_1 (2 or 10 ng/mL) CLC pellets, TGF-β_1 (2 or 10 ng/mL) + caveolin-1 (0.01 μM) CLC pellets, TGF-β_1 (2 or 10 ng/mL) + caveolin-1 (0.1 μM) CLC pellets, and TGF-β_1 (2 or 10 ng/mL) + caveolin-1 (1 μM) CLC pellets.

DNA content

DNA content was determined at day 0, 1, 7, and 28 to determine cell proliferation. After rinsing with Hank's Balanced Salt Solution (HBBS), pellets were digested using 200 μL papain digestion solution (per mL of 2* papain buffer: 1,57 mg cysteine HCL (Sigma, C7880) + 250 μg papain (Sigma, P3125) and were incubated overnight at 60°C. The next day they were vortexed and incubated for an additional hour to ensure complete pellet digestion. DNA content was measured using the Qubit™ Assays according to the manufacturer's instructions. For the measurement, 195 μL of Working Solution was used and 5 μL of the sample was added.

Glycosaminoglycan content

To quantify glycosaminoglycans (GAGs) in the pellets and the medium, a Di-methyl methylene blue (DMMB) assay was used. The pellet GAG content was determined at day 0 (only control), 1, 7 and 28, and the medium GAG content was determined every week (week 1, 2, 3, and 4). Digested pellets (see above) and culture medium samples were 1:10 diluted in PBS-EDTA. 100 μL of the diluted samples was pipetted in the plate and 200 μL of DMMB staining solution (Sigma, 341088-1G) was added to each well. The extinction was measured as quickly as possible at 540 nm and 595 nm using a microplate reader. The total GAG in each sample was calculated by dividing the 540 nm measurement through the 595 nm measurement and correcting for the blank control samples.

Quantitative PCR (qPCR)

At day 7 and day 28, RNA was isolated from the pellets. The pellets were crushed using a pellet pestle (Archos technologies, 9551-901) to release the RNA. RNA isolation was performed with the RNeasy® microkit according to manufacturer's instructions. To ensure DNA removal, an additional DNA digestion step with DNase (RNAse-Free DNase Set, Qiagen, 79254) was included. Total RNA content was quantified with a Nanodrop1 ND-1000 spectrophotometer (Thermo

Scientific). For the generation of cDNA, the iScriptTM cDNA Synthesis Kit (170-8891, Bio-Rad, Veenendaal, The Netherlands) was used according to the manufacturer's instructions.

Primer sequences were already designed using PerlPrimer (http://perlprimer.sourceforge.net). All primers were purchased from Eurogentec (Maastricht, The Netherlands). An overview of the primer pairs used is given in table 2.

In order to normalize the relative expression of the target genes, a set of 4 reference genes was evaluated: *RPS19*, *SDHA*, *GAPDH* and *HPRT.* Undetermined values were given an arbitrary value of 40. The following genes were tested*: ACAN, VCAN, Col2a1, ADAMTS5, TIMP1, MMP-13, CyclinD1, Axin2, SOX9, Cav1, BMP-7, Pai1, Bcl2, Casp3, TGFβ1, ALK1, ALK5, ID1* and *BAX.*

RTqPCR was performed using the iQTM SYBR Green Supermix Kit (Bio-Rad, Veenendaal, The Netherlands) and the MyiQTM single color Real-Time PCR Detection System (Bio-Rad, Veenendaal, The Netherlands). Relative gene expression was calculated using the Pfaffl method (30).

Gene	Sequence	Tm annealing
GAPDH	5' TGTCCCCACCCCCAATGTATC 3'	58
	5' CTCCGATGCCTGCTTCACTACCTT 3'	
HPRT	5' AGCTTGCTGGTGAAAAGGAC 3'	56 + 58
	5' TTATAGTCAAGGGCATATCC 3'	
RPS19	5' CCTTCCTCAAAAAGTCTGGG 3'	61 + 63
	5' GTTCTCATCGTAGGGAGCAAG 3'	
SDHA	5' GCCTTGGATCTCTTGATGGA 3'	61
	5' TTCTTGGCTCTTATGCGATG 3'	
ACAN	5' GGACACTCCTTGCAATTTGAG 3'	61 - 62
	5' GTCATTCCACTCTCCCTTCTC 3'	
VCAN	5' TCTCACAAGCATCCTGTCTCAC 3'	65
	5' CCATCGGTCCAACGGAAGTC 3'	
Col2a1	5' GCAGCAAGAGCAAGGAC 3'	60.5 - 65
	5' TTCTGAGAGCCCTCGGT 3'	
ADAMTS5	5' CTACTGCACAGGGAAGAG 3'	61
	5' GAACCCATTCCACAAATGTC 3'	
TIMP1	5' GGCGTTATGAGATCAAGATGAC 3'	66
	5' ACCTGTGCAAGTATCCGC 3'	
MMP-13	5' CTGAGGAAGACTTCCAGCTT 3'	65
	5' TTGGACCACTTGAGAGTTCG 3'	
CyclinD1	5' GCCTCGAAGATGAAGGAGAC 3'	60
	5' CAGTTTGTTCACCAGGAGCA 3'	
Collagen I	5' GTGTGTACAGAACGGCCTCA 3'	61
	5' TCGCAAATCACGTCATCG 3'	
Axin2	5' GGACAAATGCGTGGATACCT 3'	60
	5' TGCTTGGAGACAATGCTGTT 3'	
SOX9	5' CGCTCGCAGTACGACTACAC 3'	62
	5' GGGGTTCATGTAGGTGAAGG 3'	
Cav1	5' CGCACACCAAGGAAATCG 3'	60
	5' AAATCAATCTTGACCACGTCG 3'	

BMP-7	5' CAACGCCATCTCTGTCCTCTACTTC 3'	59
	5' AGGCTCGGACGACCATGTTTCTGT 3'	
Pai1	5' AAACCTGGCGGACTTCTC 3'	61.5
	5' ACTGTGCCACTCTCATTCAC 3'	
Bcl2	5' GGATGACTGAGTACCTGAACC 3'	61.5 - 63
	5' CGTACAGTTCCACAAAGGC 3'	
Casp3	5' CGGACTTCTTGTATGCTTACTC 3'	61
	5' CACAAAGTGACTGGATGAACC 3'	
TGFβ1	5' AATGGCTGTCCTTTGATGTCAC 3'	64.5
	5' CTGGAACTGAACCCGTTAATGTC 3'	
ALK1	5' CCTTTGGTCTGGTGCTGTG 3'	61
	5' CGAAGCTGGGATCATTGGG 3'	
ALK5	5' GAGGCAGAGATTTATCAGACC 3'	59.5
	5' ATGATAATCTGACACCAACCAG 3'	
ID-1	5' CTCAACGGCGAGATCAG 3'	59.5
	5' GAGCACGGGTTCTTCTC 3'	
BAX	5' CCTTTTGCTTCAGGGTTTCA 3'	58 - 59
	5' CTCAGCTTCTTGGTGGATGC 3'	

Table 2: Genes with their associated sequences. All genes were tested with a two step at the Tm annealing temperature.

Histology

Histology was performed at day 28. 0.1% eosin in 4% neutral buffered formalin (Boom BV Memmel) was added to the pellets. After 24 hours, the pellets were embedded in 2.4% alginate and eventually into paraffin. After paraffin embedding, the pellets were sectioned into slices of 5 µm thickness using a microtome. Slides were stained using Safranin-O/Fast Green. After staining, pictures of all pellet slides were made using a Olympus/color CCD camera (Center for Cell Imaging, FVM-UU).

The slides of donor C, TGF-β 2 ng/ml + caveolin-1 0,01 µM and donor E, TGF-β 2 ng/mL and TGF-β 2 ng/mL + caveolin-1 0,1 µM were also stained using terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay kit (Millipore; ApopTag Plus Peroxidase In Situ Apoptosis Detection Kit S7101) according to manufacturer's instructions.

Statistical analyses

The corrected ΔCt method was used for determination of relative quantitative gene expression (17). ΔCt-values were determined for each time point by subtracting the mean reference gene Ct-value at the time point of interest from the target gene Ct-value at the same time point: $\Delta Ct = Ct_{target} - Ct_{mean\ ref}$.

Statistical analysis was performed using R Studio (version 0.96, http://www.rstudio.com) and R (version 2.15.2) (RCoreTeam,2012). A cox regression model was used to analyse RT-qPCR data. CT values corrected for the mean CT values of the reference genes were used as the dependent variable. Samples that remained below the RT-qPCR threshold were considered censored values. The cox regression model, defined by the explanatory variables "time", "culture condition" or/and

the interaction term "time:culture condition", was optimized for every gene. In each model, "donor" was taken as a fixed effect to take into account the correlation between measurements of the same donor.

Data of the DNA and DMMB measurements were checked for normality after which they were analysed using a linear mixed effect model. The fixed effects that were used were "time", "culture" and the interaction term "time:culture condition". To take into account the correlation between measurements of the same donor, the model included a random intercept.

Results

DNA content

The DNA content was measured using the the Qubit™ Assay at day 0, day 1, day 7 and day 28. In figure 5, the graph of the DNA content of the pellets of each condition is shown.

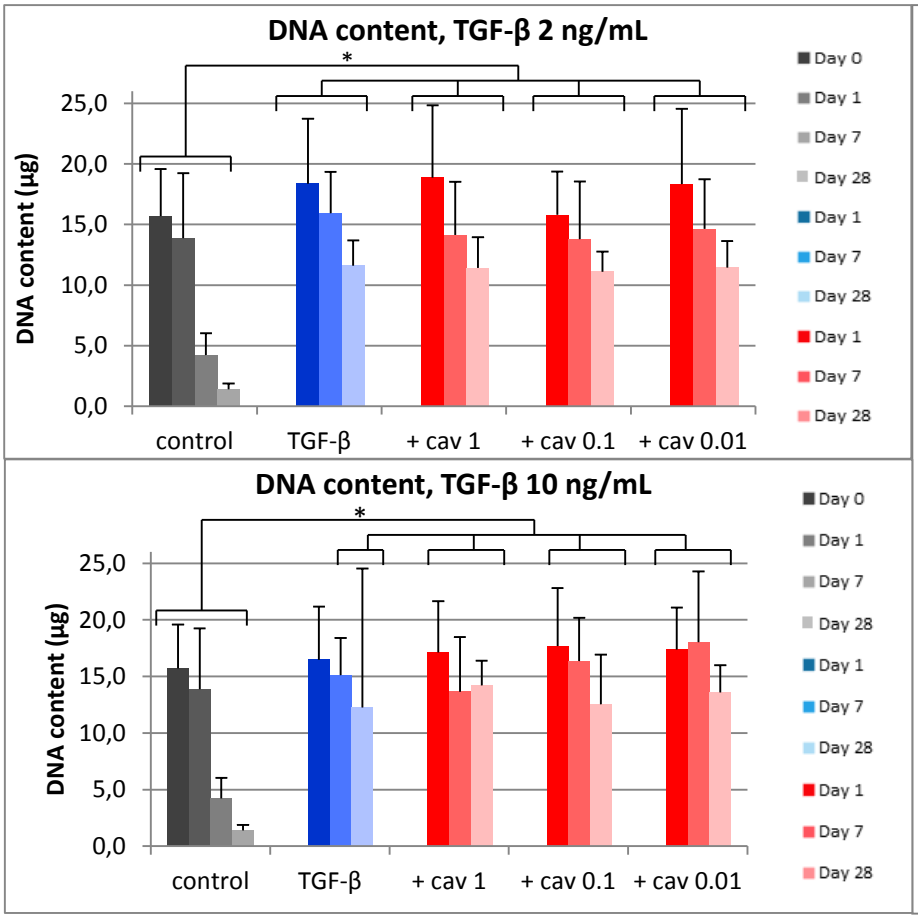

Fig. 5: Results of the DNA content of the different condition groups, with the two different dosages of TGF-β_1 (2 and 10 ng/mL). In all conditions, except for TGF-β 10 ng/mL at day 1, the DNA content is significantly higher when compared with the control group. No additive effect of caveolin-1 or dose-dependent effect of TGF-β is observed.

During the treatment of 28 days, the DNA content of the pellets of all treatment groups decreases over time. The DNA content of pellets treated with TGF-β_1 (with or without caveolin-1) is higher compared with the control group at every time point. Only a treatment with TGF-β 10 ng/mL alone on day 1 is borderline significantly different from the control (P value = 0.0638). There is no considerable difference between the pellets treated with TGF-β_1 2 ng/mL or 10 ng/mL concerning DNA content. Also, no additive effect of caveolin-1 is observed on TGF-β treatment of the degenerated canine CLC pellets.

Glycosaminoglycan content

Pellets

The glycosaminoglycan (GAG) content in the pellets was determined at day 0, day 1, day 7 and day 28. Figure 6 shows the results of the DMMB assay of all the conditions.

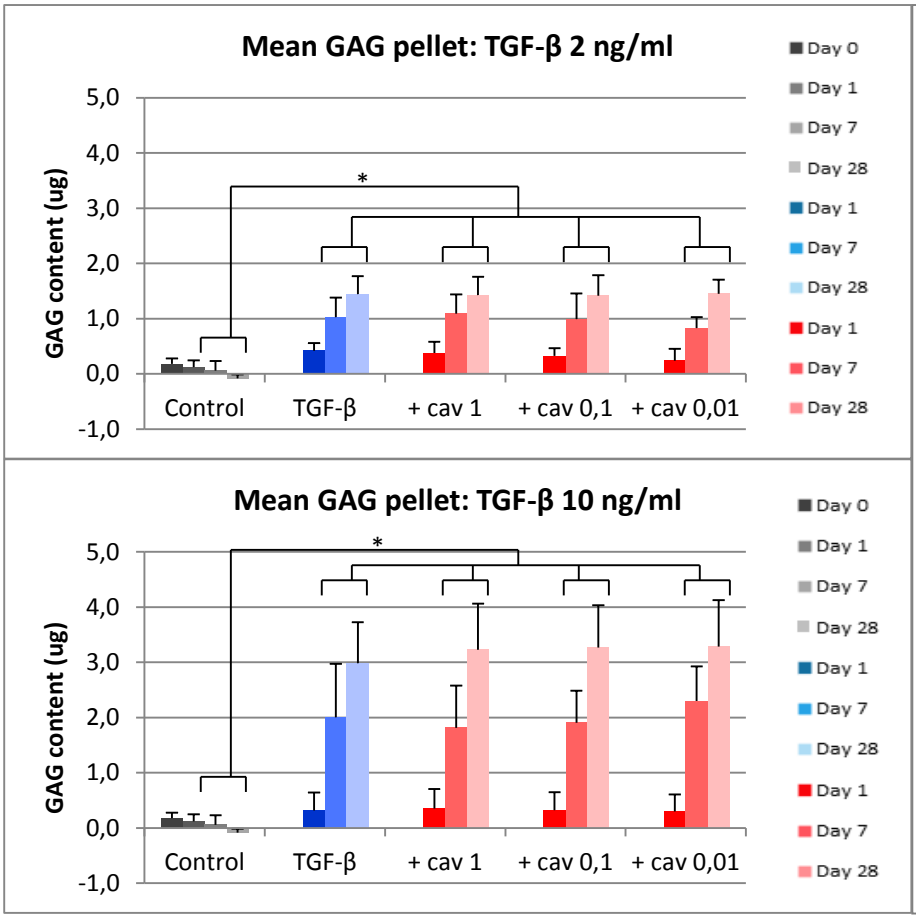

Fig. 6: Results of the GAG content on the pellets of all conditions. All the TGF-β-treated pellets have a higher GAG content than the control group ($P<0.05$). The GAG content of the pellets treated with TGF-β_1 10 ng/mL is significantly higher than the pellets treated with TGF-β1 2 ng/mL(with or without caveolin-1) at day 7 and day 28 ($P<0.05$). Again, no additive effect of caveolin-1 on TGF-β_1 treatment is observed.

The total amount of GAGs increases in time in the TGF-β-treated pellets, at day 28 the highest GAG content is observed. This is, however, not the case in the control pellets. The pellets receiving TGF-β_1 (with or without caveolin-1) have a higher GAG content compared with the control group at every time point, with statistical significance at day 7 and day 28 ($P<0.05$). The GAG content is higher in the 10 ng/mL TGF-β_1-treated pellets compared with the 2 ng/mL TGF-β_1-treated pellets (with or without caveolin-1) ($P<0.05$), suggesting a dose-dependent effect of TGF-β_1 on the pellets GAG content. This effect is also significant at day 7 and day 28 ($P <0.01$). No additive effect of caveolin-1 on TGF-β_1 treatment is observed in the mean pellet GAG values.

GAG pellet corrected for DNA

Not in all pellets, exactly the same amount of cells is present. Therefore, the total GAG content in the pellets is corrected for the total DNA that is present in the pellets. The results are shown in figure 7.

If the GAG content is corrected for DNA, the same pattern in time is observed as in the pellets GAG content; the total amount of GAGs increases in time. Just like the Mean GAG content per pellet, all the treatment groups have higher GAG content per DNA content than the control group at each time. However, at day 1 a treatment with TGF- β 10 ng/mL alone is not significant (borderline significant; P value = 0,0502). The GAG content per DNA seems to be higher in the TGF-β 10 ng/mL

pellets compared with the TGF-β 2 ng/mL pellets, but this is not statistically significant. Again, no additive effect of caveolin-1 on TGF-β treatment is observed.

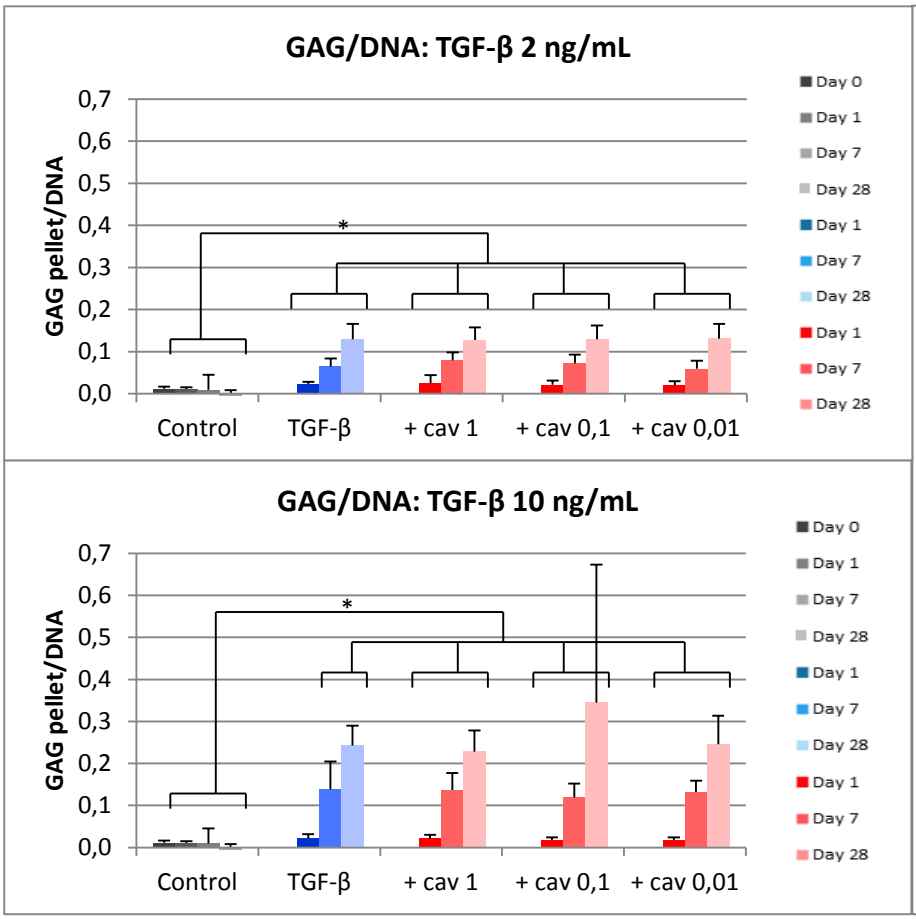

Fig. 7: Results of the pellets GAG content corrected for the pellets DNA content. The GAG content corrected for DNA of the pellets treated with TGF-β1 10 ng/mL is significantly higher than for the pellets treated with TGFβ 2 ng/mL (with or without caveolin-1), suggesting a dose-dependent effect of TGF-β1. Again, no additive effect of caveolin-1 on TGF-β treatment is observed.

Culture medium

Because GAGs are not only present in the pellets, but are also released in the medium, a DMMB assay of the medium of week 1 – 4 was performed. The results are shown in figure 8. In contrast to the GAG content of the pellets (whether or not corrected for DNA), no increase in GAG content in the medium is observed over time. Compared with the controls, the amount of GAGs is significantly higher in all TGF-β treatment groups in all 4 separate weeks as well as in total ($P<0,01$). TGF-β_1 10 ng/mL treatment increased the release of GAGs in the medium significantly more than TGF-β_1 2 ng/mL ($P<0,01$), so again a dose-dependent effect is observed here. Also, no additive effect of caveolin-1 on TGF-β treatment is observed.

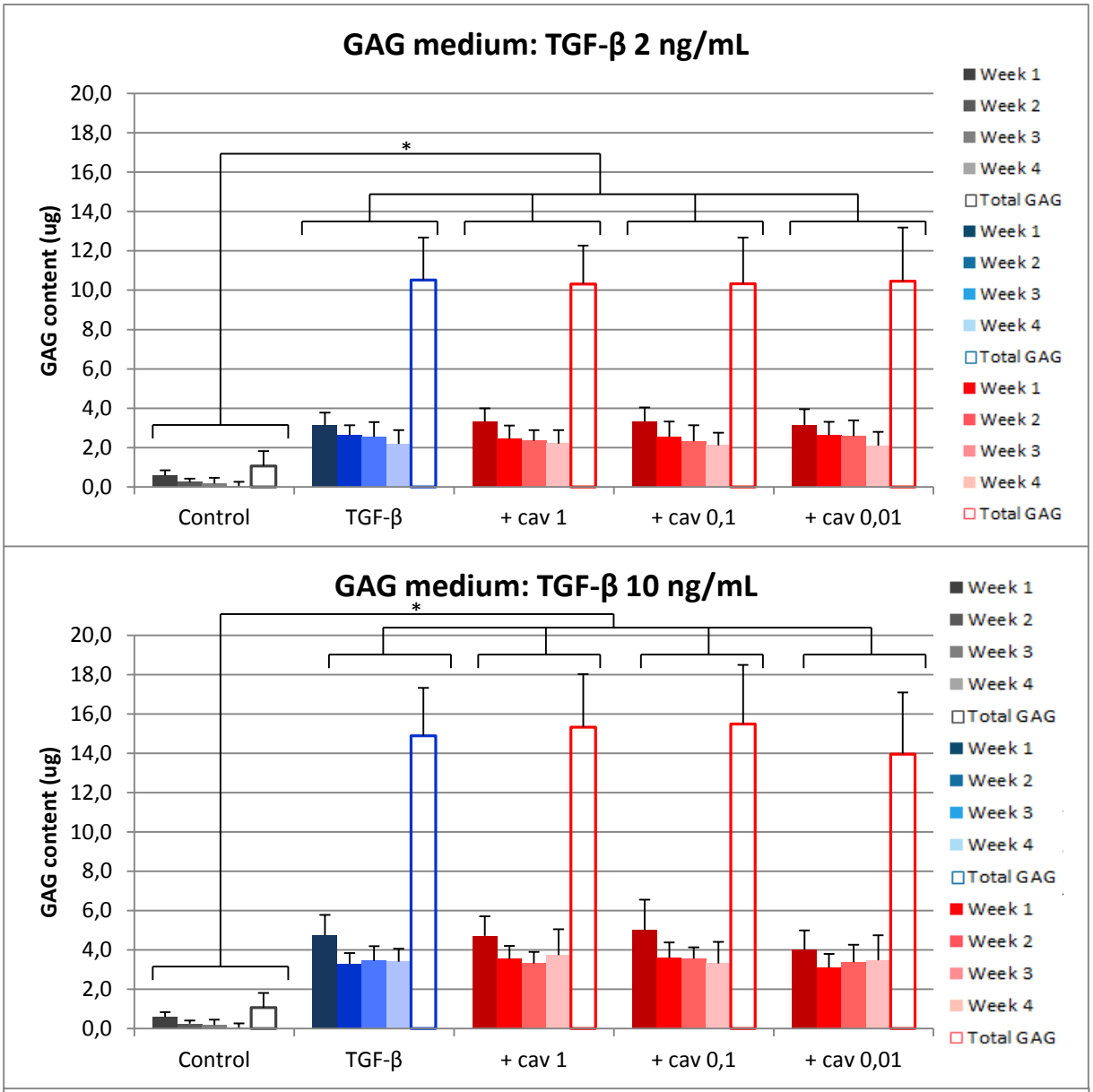

Fig 8: Results of the GAG content in the culture medium of the different treatment groups over week 1, 2, 3 and 4. The total of the 4 weeks together is shown as the Total GAG. No differences in the treatment groups are seen, except the dose-dependent effect of TGF-β. No effect of caveolin-1 is observed.

Quantitative PCR

The gene expression levels of different components of the pathways of IVD degeneration was determined using qPCR. The mean gene expression of aggrecan (*ACAN*), Collagen type 2 (*Col2a1*) and Collagen type 1 (*Col1*) of the five donors are shown in figure 9. Also the gene expression of versican (*VCAN*) was determined, but the results did not show any differences in treatment groups and is therefore not shown.

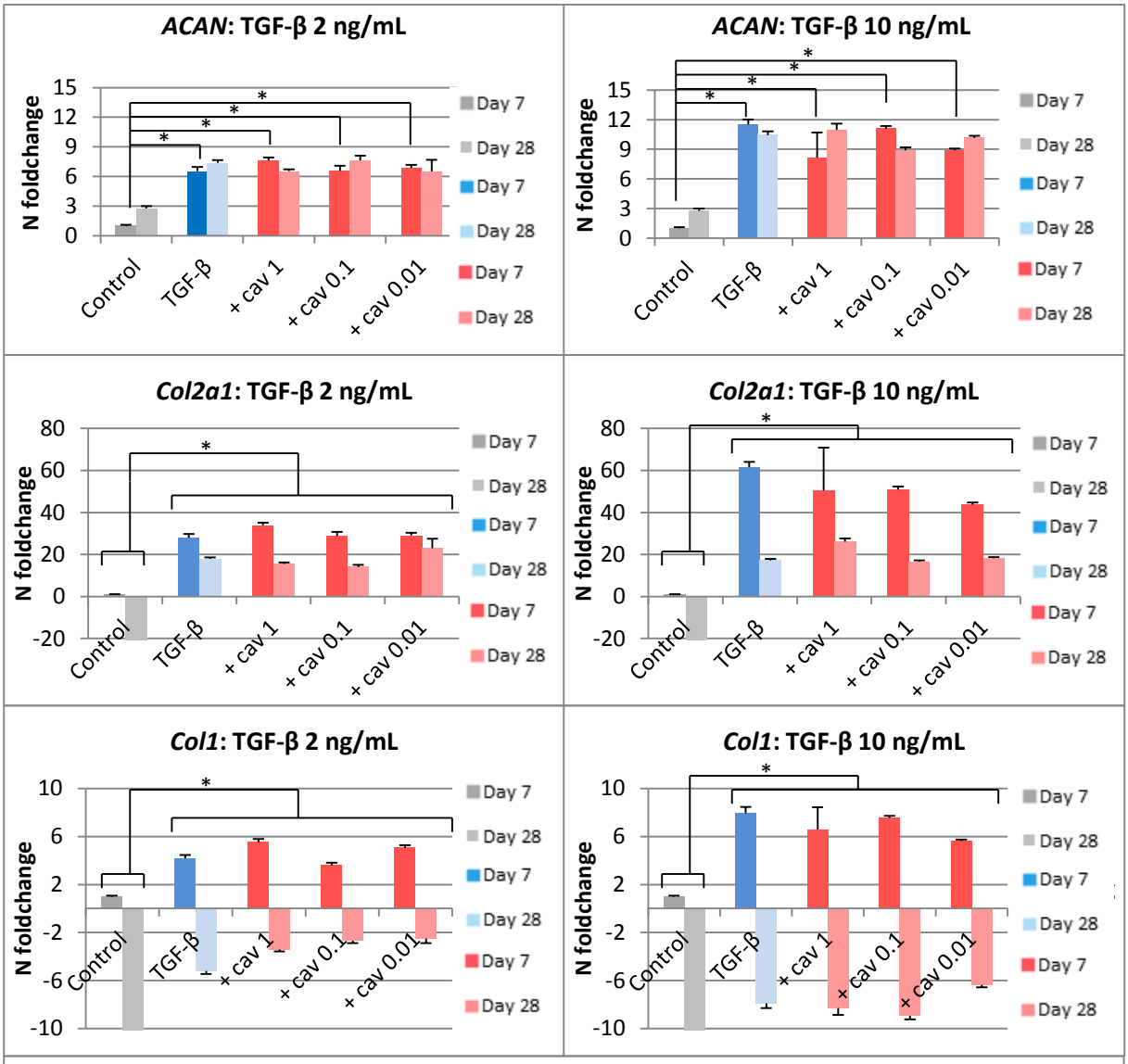

Fig. 9: qPCR results of the ECM genes *ACAN, Col2a1* and *Col1*. Aggrecan is upregulated, but only at day 7 all treatment groups differ significantly from the control. Day 7 differs significantly from day 28 (confidence interval; 3.184e+01 – 3.529e+03). For collagen type 2, it seems that at day 28 the upregulation is less than in day 7. All treatment groups differ significantly from the control at day 7 and day 28. Collagen type 1 is only upregulated at day 7 and downregulated at day 28. Again, all the treatment groups differ significantly from the control. For *ACAN* and *Col2a1*, a dose-dependent effect of TGF-β is significant (confidence interval; 1.791e-02 – 0.306012, 0.095941 – 0.66896, respectively). No additive effect of caveolin-1 can be noticed for all genes.

Aggrecan is upregulated in all TGF-β treatment groups compared with the control group, but even in the control group, gene expression of *ACAN* at day 28 is a little higher than at day 7. Only at day 7, all treatment groups differ significantly from the control. Also, day 7 differs significantly from day 28 (confidence interval; 3.184e+01 – 3.529e+03) in all groups. CLC pellets treated with TGF-β 10 ng/mL seems to give a higher upregulation than pellets treated with TGF-β 2 ng/mL at both days, with statistical significance at day 7 (confidence interval; 1.791e-02 – 0.306012) and day 28 (confidence interval 2.351e-02 – 0.409246).

Col2a1 is upregulated in the TGF-β treatment groups compared with the control group at day 7, but at day 28 the upregulation is lower than at day 7 (all groups), which suggests that after 7 days, the gene expression of collagen type 2 already decreases. However, the differences between the TGF-β treatment groups and the controls are significant at day 7 and day 28. Again a significant dose-dependent effect of TGF-β is observed (confidence interval; 0.095941 – 0.66896).

Collagen type 1 is upregulated at day 7, but strongly downregulated at day 28 compared with *Col1* gene expression of the control group at day 7. Gene expression in all treatment groups is again significantly different at both day compared to the control. There is no significantly dose-dependent effect on *Col1* gene expression. Caveolin-1 seems to have no additive effect at gene expression of all these genes.

To see if remodelling also occurs in the different treatment groups, the gene expression of *ADAMTS5, MMP13* and *TIMP1* was determined. These results are shown in figure 10. The results of the gene expression of the remodelling genes suggest that there is not much remodelling at day 7 and day 28. *ADAMTS5* gene expression is significantly different in all TGF-β treatment groups compared to the controls at both time points. At day 28, a treatment with TGF-β 10 ng/mL + caveolin-1 1 μM is significantly different from a treatment with TGF-β 10 ng/mL alone (confidence interval; 4.619e-02 – 8.833e-01). This suggests that at a dose of 1 μM, caveolin-1 maybe has an additive effect on gene expression of *ADAMTS5.* Also, a significant dose dependent effect of TGF-β is established at both days (confidence interval; 7.150e+02 – 7.960e+04 and 1.776 – 2.894e+01).

For the gene expression of *MMP13*, TGF-β 10 ng/mL and TGF-β 2 ng/mL + caveolin-1 1μM are significantly different from the controls at day 7 (confidence interval; 0.00529 – 0.1473 and 0.033282 – 0.6788). At day 28, all treatment groups differ significantly from the control. At day 7, all the conditions differ significantly from the treatment with TGF-β 10 ng/mL alone. This suggests that an effect of caveolin-1 and that again a dose-dependent effect of TGF-β is observed (confidence interval; 9.0838 – 264.759). On day 28, these effects are not significant anymore and only the control differs significantly from a treatment with TGF-β 10 ng/mL.

Gene expression of *TIMP1* was only significantly different in the control pellets compared with a treatment with TGF-β 10 ng/mL at both time points. Interestingly, gene expression of *TIMP1* differ significantly in pellets treated with TGF-β 10 ng/mL from all other groups. Again, this indicates that caveolin-1 does have an effect on TGF-β treatment of canine CLC pellets and a dose-dependent effect of TGF-β is observed for this gene.

CyclinD1 is a gene that encodes cell proliferation (18). Because all treatment groups showed an increase in *CyclinD1* gene expression compared with the control group, and no difference between the treatment groups was observed regarding *CyclinD1* gene expression, the results are not shown.

Axin2 gene expression is a read-out specific for canonical Wnt signaling and was therefore determined in this study (8). It is suggested that caveolin-1 can stimulate canonical Wnt signaling by activating intergrin-linked kinase (ILK), which inhibits glycogen synthase kinase 3-β (GSK3-β). GSK3-β phosphorylates β-catenin, which leads normally to the degradation of β-catenin (4). The gene expressions of *Axin2* and *ILK* are shown in figure 11.

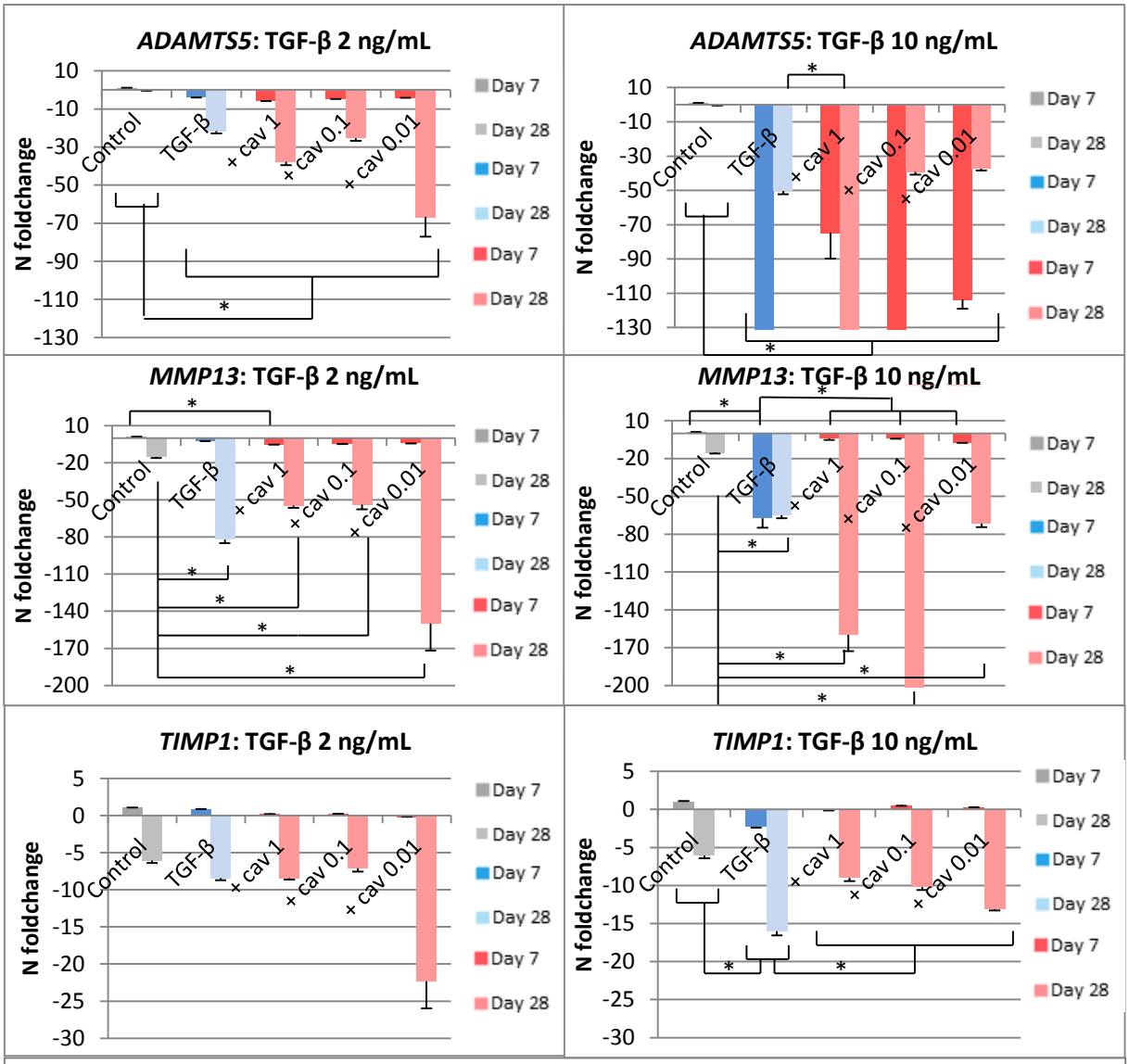

Fig. 10: qPCR results of *ADAMTS5, MMP13* and *TIMP1*. All the genes are downregulated in the treatment groups, but a stronger downregulation is seen at day 28. A dose-dependent effect of TGF-β is significant for all these genes. At both days, the gene expression of *TIMP1* is significantly higher when pelletes are treated with caveolin-1 in combination with TGF-β 10 ng/mL compared with a treatment with TGF-β 10 ng/mL alone. At day 28, *ADAMTS5* gene expression is significantly lower when TGF-β 10 ng/mL is combined with caveolin-1 1 µM in a confidence interval of 95%. For *MMP13* gene expression an effect of caveolin-1 is only observed on day 7; all treatment groups differ significant from a treatment with TGF-β 10 ng/mL alone.

Compared with the control pellets of day 7, *Axin2* is downregulated in all conditions. It seems that caveolin-1 has a dose-dependent effect on TGF-β treatment in both concentrations (2 and 10 ng/mL). In pellets treated with TGF-β 2 ng/mL, the effect of caveolin-1 seems to be additive on day 28; TGF-β downregulates *Axin2*, and with caveolin-1 the downregulation is even more. However, in pellets treated with TGF-β 10 ng/mL this effect seems to be contrary at both day 7

and 28. Here it seems that caveolin-1 inhibits TGF-β or stimulates *Axin2* expression. *ILK* does not show any effect of TGF-β or caveolin-1 on its gene expression.

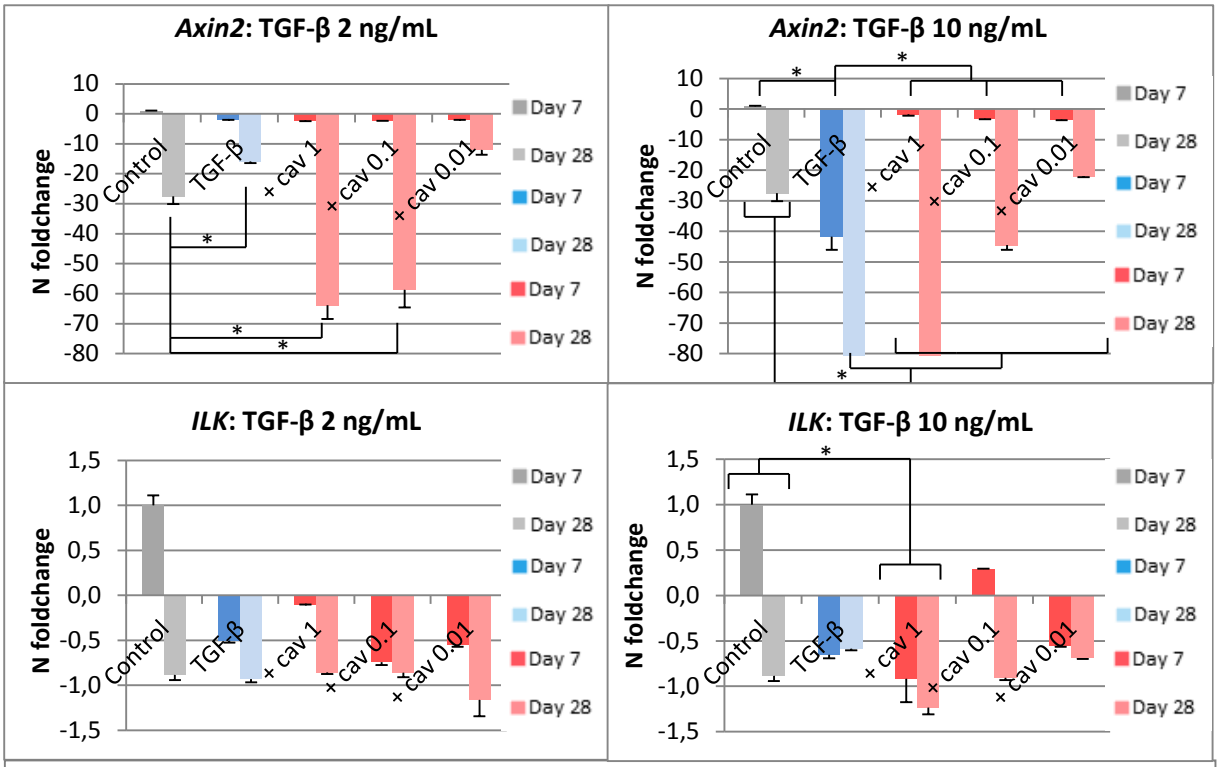

Fig. 11: Gene expressions of *Axin2* and *ILK. Axin2;* compared with the control of day 7, all conditions are downregulated. It seems that caveolin-1 has a dose-dependent effect on TGF-β treatment in both concentrations. At day 7, all conditions are significant different from a treatment with TGF-β 10 ng/mL, indicating that caveolin-1 inhibits TGF-β. However, in pellets treated with TGF-β 2 ng/mL seems the effect of caveolin-1 additive. No significance is found for this observation. *ILK;* there seems to be little downregulation compared with the control of day 7. Only a treatment with TGF-β 10 ng/mL + caveolin-1 1μM is significantly different from the control.

Only TGF-β 2 ng/mL + caveolin-1 0,01 μM is at day 28 not significantly different from the controls in *Axin2* gene expression. At day 7, *Axin2* gene expression of pellets treated with TGF-β 10 ng/mL + caveolin-1 1μM, TGF-β 2 ng/mL, TGF-β 2 ng/mL + caveolin-1 0,1 μM and TGF-β 2 ng/mL + caveolin-1 0,01 μM are not significantly different from the control. Interestingly, when all conditions are compared with TGF-β 10 ng/mL, it seems that all conditions differ significantly from this treatment at day 7. At day 28, only the control is different from TGF-β 10 ng/mL. This suggests that at day 7, the effect of caveolin-1 on TGF-β 10 ng/mL is significant, thus suggesting that caveolin-1 has an inhibiting effect on TGF-β 10 ng/mL treatment regarding *Axin2* gene expression. This effect disappears on day 28. Also, only at day 7 an significant dose-dependent effect of TGF-β treatment is observed (confidence interval; 13.1214 – 489.332). No dose-dependent effect of caveolin-1 is significant.

For *ILK* gene expression, only a treatment with TGF-β 10 ng/mL + caveolin-1 1μM is significantly different from the control (confidence interval; 0.1120 – 0.8153). This suggests that caveolin-1 in a dose of 1 μM maybe has an effect on integrated-linked kinase.

Sox9 encodes for chondrocyte differentiation, but was only a little upregulated in the TGF-β treated pellets on day 7 compared with the control (data not shown). Measurement at day 7 could even be too late for chondrocyte differentiation and, therefore, maybe it is necessary to determine this gene even more earlier (21).

To see if TGF-β (with or without caveolin-1) has an effect on both his own receptors, *ALK5* and *ALK1* gene expression were determined. Also the downstream pathways, respectively *Pai1* and *ID1*, were determined to see the effects of the treatments. Results are shown in figure 12. Although not much seems to change in the gene expression of *ALK5* compared to the control pellets at day 7, an upregulation of *Pai1* expression is observed in all conditions compared with controls. Statistics show that for *ALK5* gene expression, all conditions are not significantly different from the control group and for *Pai1* gene expression, all conditions are significantly different from the control group. In both genes there is no dose-dependent effect of TGF-β or an additive effect of caveolin-1 observed.

ID1 is downregulated in all conditions compared with the control, but *ALK1* gene expression only shows a downregulation on day 28; at day 7, there is even a little upregulation. Statistics show that for *ALK1* gene expression, all conditions are not significantly different from the control group and for *ID1* gene expression, all conditions are significantly different from the control group. Just like the gene expression of *Pai1*, no dose-dependent effect of TGF-β or an additive effect of caveolin-1 is observed.

In figure 13, the mean Ct ratio's of *ALK5/ALK1* and *Pai1/ID1* are shown. When Ct values are low, the gene expression is high, thus *ALK5* and *Pai1* gene expression are higher than *ALK1* and *ID1* gene expression respectively. Regarding the *ALK5/ALK1* ratio's, no difference between the TGF-β treatment groups and control group is observed. Also, no dose-dependent effect of TGF-β treatment or additive effect of caveolin-1 treatment is observed. However, on day 28 the ratio of *ALK5/ALK1* seems to be lower than on day 7; thus there is more *ALK5* or less *ALK1* gene expression on day 28. Also, on day 28 are the pellets treated with TGF-β 2 ng/mL + caveolin-1 0,1 μM significantly different from the control group, and from a treatment with TGF-β 2 ng/mL alone ($P=0.0296$ and 0.0142, respectively). This suggests that at day 28, a concentration of 0,1 μM caveolin-1 can have a positive effect on a treatment with a suboptimal dose of TGF-β (2 ng/mL). No additive effect of caveolin-1 is observed in a treatment with TGF-β 10 ng/mL, and also no differences in concentration of TGF-β treatment is observed.

In the *Pai1/ID1* ratio's, all TGF-β treatment groups are significantly different from the control group, suggesting that these groups, *Pai1* has an higher gene expression than *ID1.* Interestingly, when TGF-β 10 ng/mL + caveolin-1 0,01 μM is compared with TGF-β 10 ng/mL alone, a significant higher *Pai1* expression is observed ($P<0.05$). This indicates that caveolin-1 in a concentration of 0,01 μM can have a positive effect on TGF-β 10 ng/mL treatment, while at a suboptimal dose (2 ng/mL) this effect is not present.

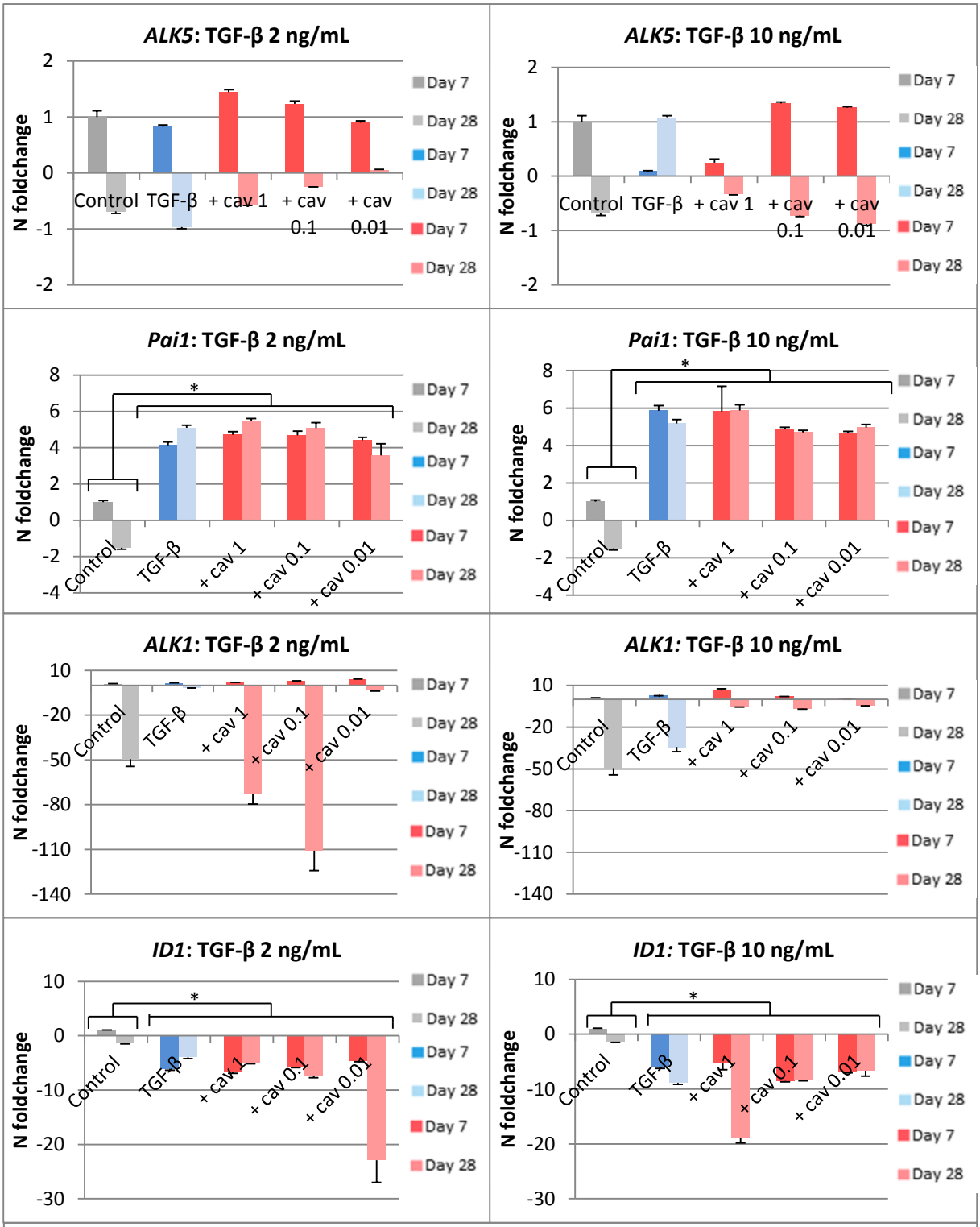

Fig. 12: Gene expressions of *ALK5, Pai1, ALK1* and *ID1*. Although not much seems to change in the gene expression of *ALK5*, an upregulation of *Pai1* is observed in all conditions compared with the control of this gene. This upregulation is statistically significant on day 28. *ID1* is downregulated in all conditions compared with the control on both days. This downregulation is significant for all conditions compared with the control. However, *ALK1* gene expression only shows a downregulation on day 28; at day 7, there is even a little upregulation.

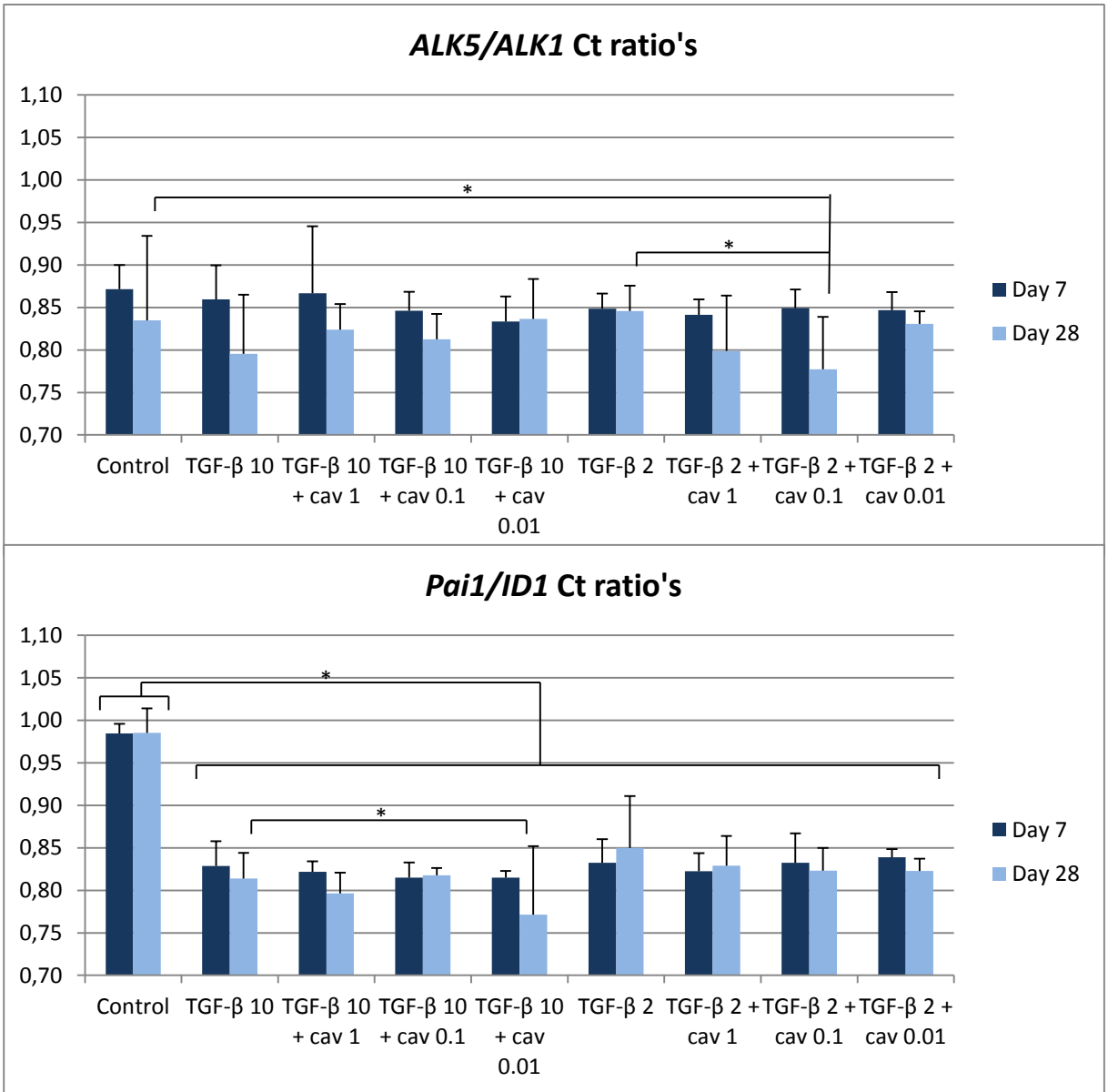

Fig. 13: CT ratio's of *ALK5/ALK1* and *Pai1/ID1.* Because CT values are used, the gene expression of *ALK5* and *Pai1* are higher than the gene expression of *ALK1* and *ID1. ALK5/ALK1*; there is no difference in ratio's between the treatment groups and the control observed. However, on day 28, a treatment with TGF-β 2 ng/mL + caveolin-1 0,1 μM seems to be significantly different from the control pellets and the pellets treated with TGF-β 2 ng/mL alone. This could indicate an additive effect of caveolin-1 in this concentration on a suboptimal dose of TGF-β. No difference in concentration of TGF-β treatment is observed.*Pai1/ID1*; compared with the control, all treated pellets have significantly more *Pai1* than *ID1* gene expression. No difference in concentration of TGF-β treatment is observed. However, when TGF-β 10 ng/mL + caveolin-1 0,01 μM is compared with TGF-β 10 ng/mL treatment alone on day 28, a significant difference is observed; this could also indicate for a additive effect of caveolin-1 in this low concentration on TGF-β treatment of degenerated CLCs.

To see if apoptosis occurs, *BAX* and *Bcl2* gene expression were determined. *BAX* encodes for apoptosis, while *Bcl2* encodes for anti-apoptosis. Results of the gene expression are shown in figure 14. *Bcl2* is upregulated in all treatment groups compared with the control of day 7. The

upregulation seems to be higher on day 28 than on day 7. At day 28, all conditions are significantly different from the control, while at day 7, only TGF-β 10 ng/mL + caveolin-1 0,1 µM differs significantly from the control. When all conditions are compared with TGF-β 10 ng/mL, it seems that at day 28 a dose-dependent effect of TGF-β is significant (confidence interval; 4.925e-02 – 7.901e-01).

BAX is downregulated in all TGF-β treatment groups compared with the control of day 7. No difference in time is observed in this gene expression. Statistics show that all conditions are significantly different from the control group at both days. No dose-dependent effect of TGF-β is observed in the *BAX* gene expression and in both genes, no additive effect of caveolin-1 is observed or significantly demonstrated.

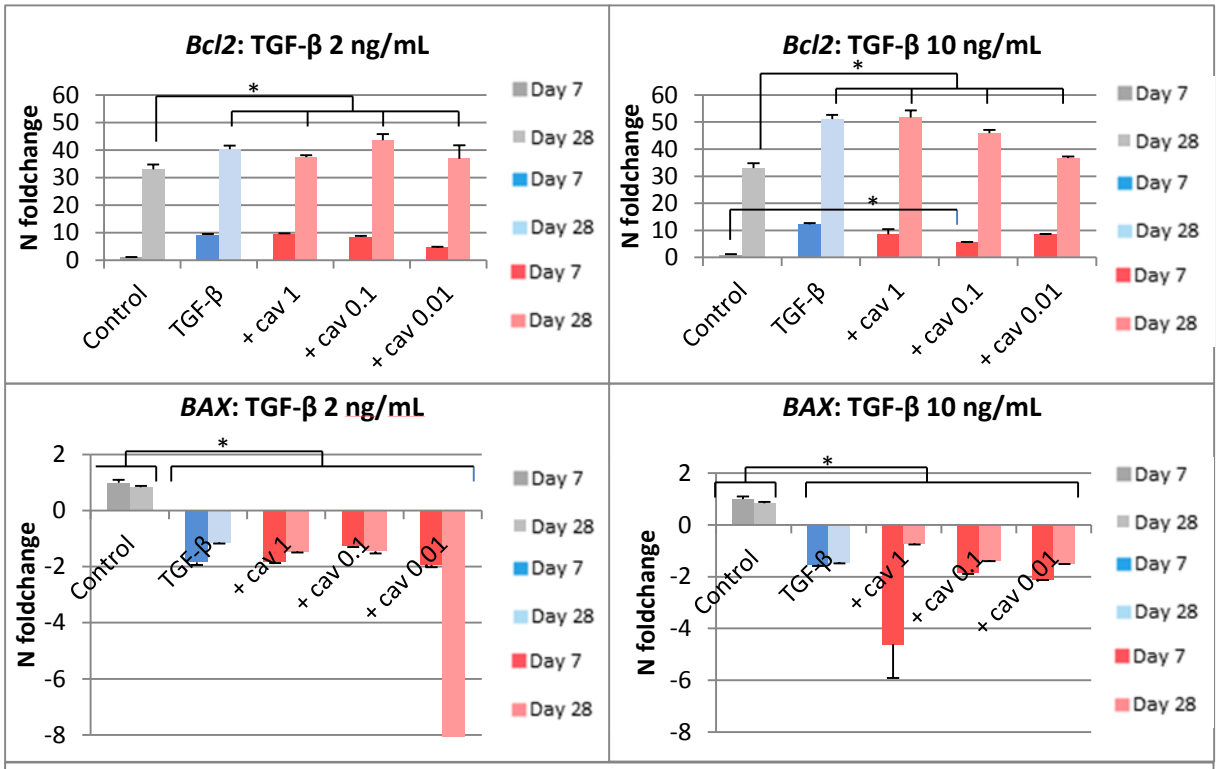

Fig. 14: Results of the gene expression of *Bcl2* and *BAX*. *Bcl2* is upregulated in all treatment groups at both days compared with the control of day 7. The upregulation is higher at day 28 compared with day 7. *BAX* is downregulated in all treatment groups on both days compared with the control of both day 7 and day 28. In both genes, no additive effect of caveolin-1 is observed.

To see whether apoptosis or anti-apoptosis is the main pathway which occurs in the cells, the Ct ratio's of *BAX/Bcl2* were calculated. In figure 15, the results are shown. Again, when Ct values are low, the gene expression of the gene is high. The ratio's of *BAX/Bcl2* show a significantly higher ratio at day 28 compared with day 7 ($P<0.01$). This means that at day 7, BAX gene expression is higher or Bcl2 gene expression is lower. At day 7, TGF-β 10 ng/mL + caveolin-1 1 µM is the only condition which is significantly higher than the control ($P<0.01$), while at day 28, TGF-β 2 ng/mL + caveolin-1 0,01 µM only is significantly higher than the control ($P<0.01$). When TGF-β 2 ng/mL + caveolin-1 0,01 µM on day 28 is compared with TGF-β 2 ng/mL alone, also a significant

difference is found (P<0.05), indicating that caveolin-1 0,01 µM can have an effect on TGF-β treatment, inhibiting apoptosis (*BAX*) and promoting anti-apoptosis (*Bcl2*).

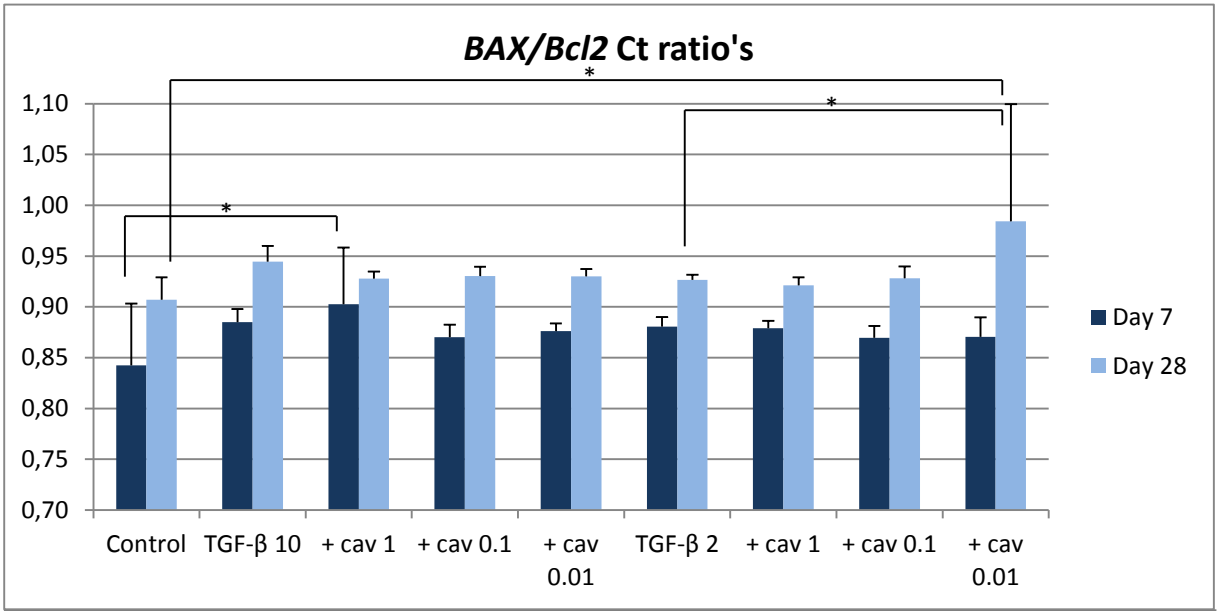

Fig. 15: *BAX/Bcl2* Ct ratio's. At day 7, TGF-β 10 ng/mL + caveolin-1 1 µM is the only condition which is significantly different from the control (higher ratio, thus lesser apoptosis, P<0.01). At day 28, this is TGF-β 2 ng/mL + caveolin-1 0,01 µM (P<0.01). When this last condition is compared with TGF-β 2 ng/mL alone, also a significantly difference is observed (P<0.05), indicating an effect of caveolin-1 on TGF-β treatment. At day 7, ratio's are significantly lower than at day 28 (P<0.01), thus more Bcl2 is expressed at day 28.

Histology

Histology was performed at day 28. Slides were stained using Safranin-O/Fast Green and thereafter, pictures of all pellet slides were made using a Olympus/color CCD camera (Center for Cell Imaging, FVM-UU). In figure 16, the pictures of all conditions of one donor (donor C) are shown.

The controls of all donors stained green, just like the picture of the control pellets in figure 16. This indicates that no or just a little amount of GAG is formed in the control group. In contrast, all other conditions of the donors showed the red staining seen in figure 16, which means a lot of GAGs are formed in this pellets. No dose-dependent effect of TGF-β treatment or additive effect of caveolin-1 treatment is observed.

Also, two pellets of donor E (TGF-β 2 ng/mL and TGF-β 2 ng/mL + caveolin-1 0,1 µM) showed the same 'hole' in the middle like donor C, TGF-β 2 ng/ml + caveolin-1 0,01 µM. No cells and/or GAGs were present in the middle of these pellets. To see if these 'holes' are due to cell apoptosis or necrosis, a TUNEL staining was made (see fig. 17). The picture of the TUNEL staining of donor C, TGF-β 2 ng/ml + caveolin-1 0,01 µM shows a brown colour on the edge of the 'hole' in the pellet. The two conditions of donor E were also stained and a same result is observed in these pellets. This suggests that apoptosis occurs at day 28 in the middle of the pellet.

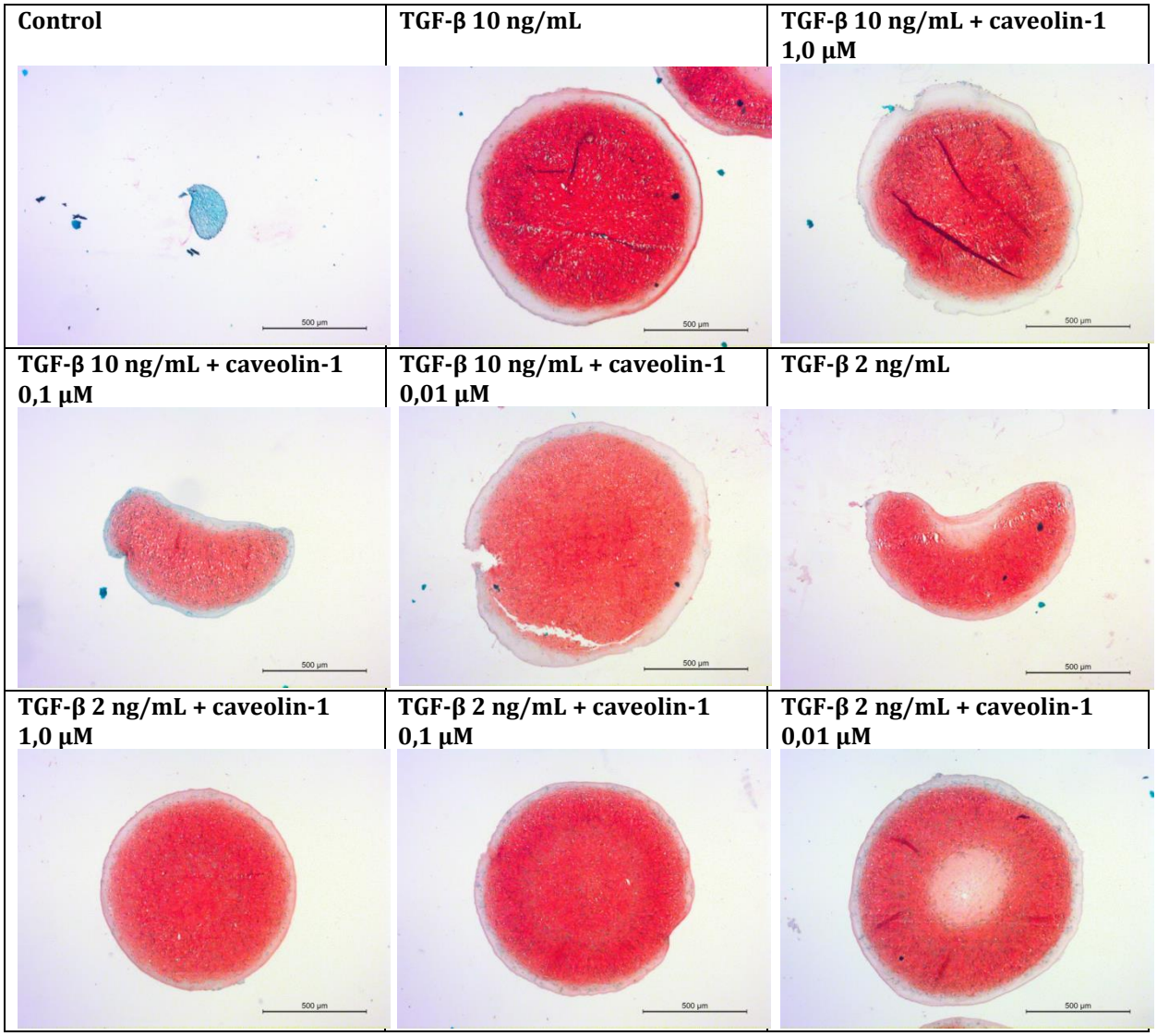

Fig. 16: Histology pictures of all conditions of donor C, 4x. The control stained only green; this means that no or only a little amount of GAGs are formed in the control group. All other conditions stained red, which indicates that a lot of GAGs are formed. No difference between TGF-β 10 ng/ml and TGF-β 2 ng/ml are observed, and also there is no effect of caveolin-1 visible in these stained pellets. The 'hole' in the middle of TGF-β 2 ng/ml + caveolin-1 0,01 µM was not expected; to see if this 'hole' is due to apoptosis or necrosis, a TUNEL staining was made.

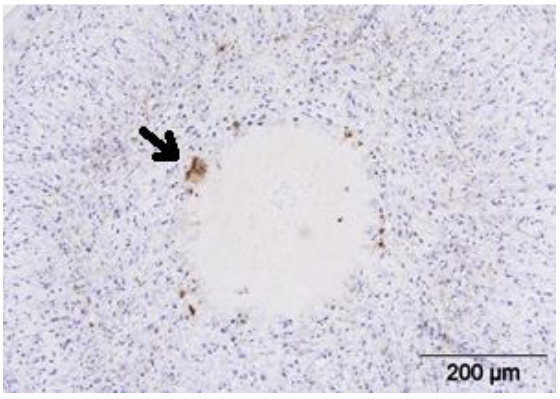

Fig. 17: TUNEL staining of donor C, TGF-β 2 ng/ml + caveolin-1 0,01 µM, 10x. On the edge of the 'hole' to the pellet (arrow), a brown colour is observed, which indicates for apoptosis.

Discussion

IVD degeneration is a major health concern in both humans and dogs. The aim of this study was to find the best cell/growth factor-based regenerative strategy to restore the health and function of the degenerated IVD. The specific aim of this experiment was to determine the additive effect of caveolin-1 on TGF-β1 treatment of degenerated canine CLCs. It is hypothesized that caveolin-1 has an additive effect on TGF-β1 treatment of CLCs in terms of cell proliferation and matrix production. Thus, a stronger positive effect on DNA and GAG content is expected compared with TGF-β1 treatment alone.

DNA content and ECM production

The pellets' DNA content decreases over time. In pellets treated with TGF-β (2 or 10 ng/mL), the DNA content stayed significantly higher at day 7 and day 28 compared with the control, possibly because growth factors stimulate cell proliferation, so more cells and thus more DNA is present in the pellets treated with TGF-β (6). Affirmatively, TGF-β is known to play an important role in chondrocyte proliferation (18). No dose-dependent effect of TGF-β treatment is observed for DNA content of the degenerated canine CLC pellets. This suggests that for DNA content, the a treatment with TGF-β 2 ng/mL is sufficient for canine CLC pellets. However, when the GAG contents are observed, the effect of TGF-β 10 ng/mL gives a significantly higher GAG content in the pellets and the pellets corrected for DNA than 2 ng/mL, suggesting that TGF-β 2 ng/mL is a suboptimal dose, and TGF-β 10 ng/mL is a better concentration to use as positive control in canine NPCs.

The GAG content of the control pellets decreases over time, while the GAG content of the TGF- β treated pellets (2 ng/mL or 10 ng/mL, with or without caveolin-1) increases. It is described that TGF-β is a multifunctional regulator of cellular proliferation, differentiation, and ECM production (15). As described in the introduction, TGF-β can signal through two different receptors: ALK5 and ALK1 (13,15,23). The two different pathways of these receptors are shown in figure 18.

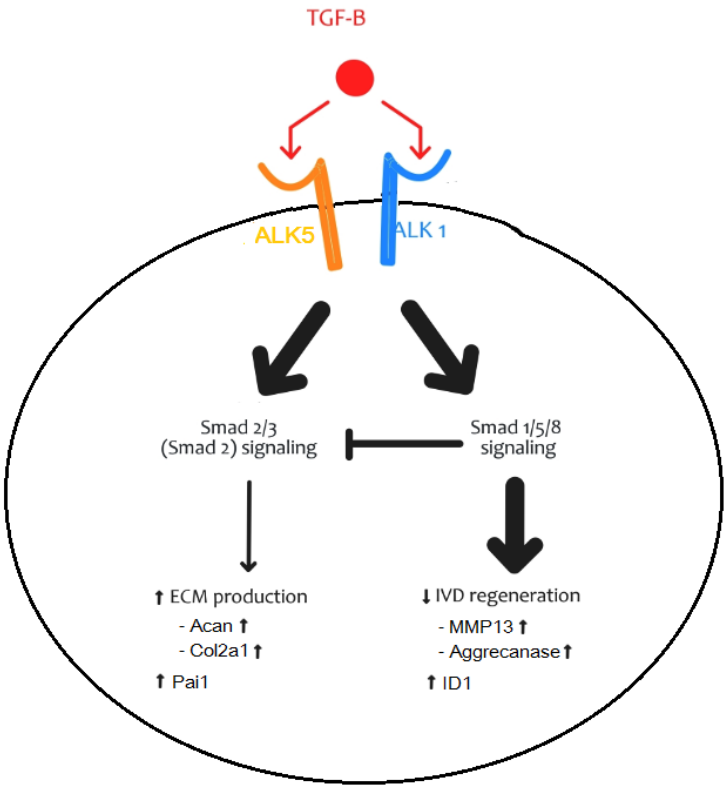

Fig. 18: Pathways of TGF-β signaling. Stimulation of the ALK5 receptors leads to Smad2/3 signaling. This results in ECM production and Pai1 signaling. Stimulation of ALK1 leads to Smad1/5/8 signaling, which inhibits Smad2/3 signaling and thus results in further IVD degeneration and stimulation of ID1.

The high GAG content in the pellets treated with TGF-β suggests that the Smad2/3 pathway is activated. Also, histology shows a lot of GAG was formed in the treated pellets. The gene expressions of *ACAN, Col2a1* and *Pai1* are all increased

compared with the control, and the ratio of *ALK5/ALK1* shows a higher gene expression of *ALK5* compared with *ALK1*. All these findings confirm the suggestion that Smad2/3 pathway is more activated than the Smad1/5/8 pathway. When the Smad1/5/8 pathway is activated, MMP13 is stimulated (13,23). The results of the *MMP13* gene expression show that there is a downregulation, which also confirm that the ALK1 pathway is less stimulated than the ALK5 pathway.

Additive effect of caveolin-1 on GAG content in Donor A

Although no additive effect of caveolin-1 is observed in the mean values of the pellets, it is interesting that if the donors are observed independently, there seems to be an additive effect of caveolin-1 on TGF-β 10 ng/mL treatment on day 28 of cell culture (P<0.01) in donor A. In figure 19, this effect in shown.

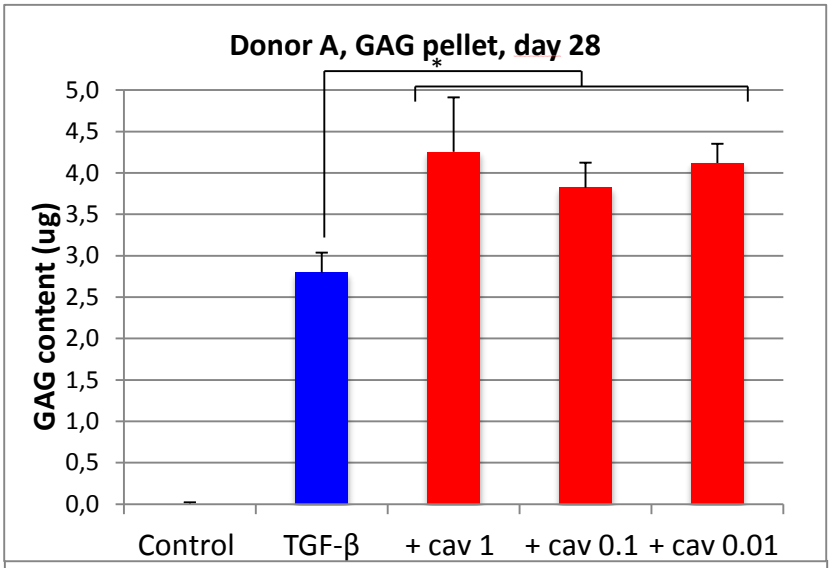

Fig. 19: Results of GAG content of the pellets of donor A, day 28. Caveolin-1 has an significant additive effect on TGF-β 10 ng/mL treatment (P<0,01).

The additive effect of caveolin-1 shown in the figure is significant (P<0.01). It is not known why only donor A shows an additive effect of caveolin-1 on TGF-β 10 ng/mL treatment. When the different donors are compared to each other, no differences in age or breed are present (see table 3). There are differences in gender, but only donor D and E contain male animals; this would suggest that donor B and C also should show the additive effect of caveolin-1, which is not the case.

	Donors	%	Breed	Average age (months)	Gender
A	509	75	Beagel	20	F
	151	25	Beagel		F
B	510	70	Beagel	19,3	F
	505	30	Beagel		F
C	499	70	Beagel	20	F
	505	30	Beagel		F
D	500	25	Beagel	22,5	F
	153	25	Beagel		M
	496	50	Beagel		F
E	679	20	Beagel	24,4	M
	150	55	Beagel		M
	155	10	Beagel		M
	498	15	Beagel		F

Table 3: Overview of all donors used. No differences in breed, age or gender are found for the explanation of the effect of caveolin-1 found in donor A.

To see if there is any difference in Smad signaling between the different donors, the ratio's of *ALK5/ALK1* and of *Pai1/ID1* are independently observed at day 28 (see figure 20). However, no significant difference in these ratio's, that could explain the additive reaction of donor A, is found. For the *ALK5/ALK1* ratio, it even seems that when caveolin-1 is added to a treatment with TGF-β, the ratio becomes higher. This suggests that in the presence of caveolin-1, more *ALK1* signaling is present compared with a treatment of TGF-β alone, which is contrary to the findings of the additive effect of caveolin-1 found in the GAG content (see figure 18). When the ratio of *Pai1/ID1* of donor A is compared with the ratio's of the other donors, no differences are found. Because there seems to be more *ALK1* in pellets treated with caveolin-1 on day 28, it was expected that the ratio of *Pai1/ID1* is also higher in these conditions, but this is not observed. Thus, it is still unclear why only Donor A shows a significant effect of caveolin-1 on GAG content.

Effect of caveolin-1 on *Axin2* gene expression

Dose-dependent effect?

In figure 11, the results of *Axin2* gene expression, a read out for Wnt signaling, is shown. It is known that TGF-β has an suppressive effect on the expression of *Axin2.* This is time-dependent, with increasing inhibition over time (19). The first thing noticed in our graphs, is the dose-dependent effect that caveolin-1 seems to have on the gene expression of *Axin2.* The higher the dose of caveolin-1, the higher the suppressive effect of TGF-β on *Axin2.* However, when statistics were performed, this effect was not significant. This can be due to the fact that statistics were performed on ΔCt values, and the graphs only show N-fold changes. However, at day 7, gene expression of *Axin2* was not as much downregulated when pellets were treated with TGF-β 10 ng/mL and caveolin-1 compared with TGF-β 10 ng/mL alone, and this difference is statistically

different. This indicates that caveolin-1 inhibits the inhibition of TGF-β on *Axin2* gene expression in NP cells.

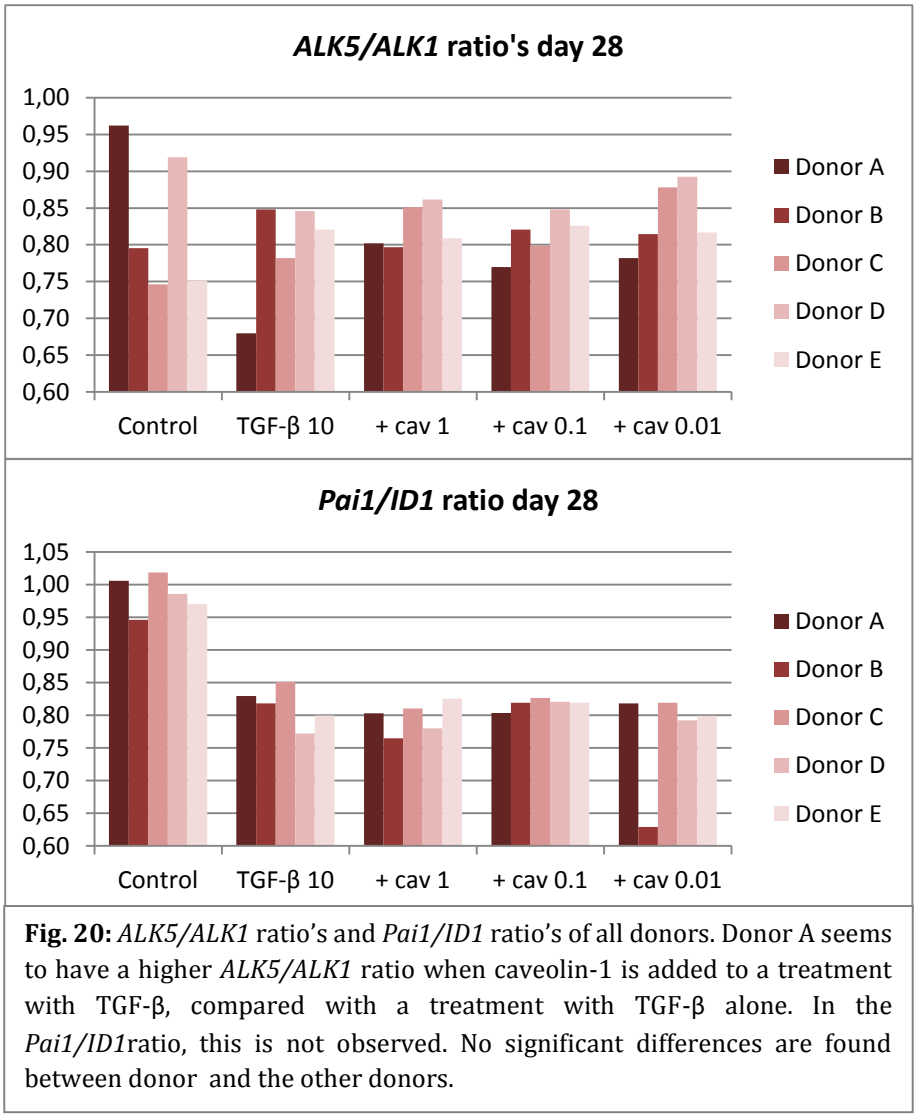

Fig. 20: *ALK5/ALK1* ratio's and *Pai1/ID1* ratio's of all donors. Donor A seems to have a higher *ALK5/ALK1* ratio when caveolin-1 is added to a treatment with TGF-β, compared with a treatment with TGF-β alone. In the *Pai1/ID1*ratio, this is not observed. No significant differences are found between donor and the other donors.

Effects of TGF-β and caveolin-1 on Wnt signaling

As already mentioned, TGF-β can bind to the ALK5 receptor, thereby stimulating the Smad2/3 pathway. In a study of Dao et al (2007) it is shown that Smad2/3 inhibits Wnt signaling (19). The TGF-β/Smad pathways and the Wnt/β-catenin pathways control both the proliferation and differentiation of chondrocytes (19).

β-catenin is a protein in the cytoplasm. Through interactions with cadherins, it plays a functional role in cell-cell adhesions (21). It is known that β-catenin can play an essential role in the control of cellular proliferation, differentiation, cell adhesion, apoptosis and tumorigenesis, by regulating cell-cell interactions and gene transcription (20). β-catenin is also a downstream intracellular molecule in the canonical Wnt pathway. Canonical Wnt signaling inhibits phosphorylation of β-catenin, in which way translocation to the nucleus can occur (21). Wnt/β-catenin signaling

regulates the expression of aggrecan and induces the expression of MMPs in NP cells. By inducing the expression of MMPs, it promotes cellular senescence and IVD degeneration (24).

When caveolin-1 is added to the pellets, a less inhibition of *Axin2* is observed in the pellets treated with TGF-β1. There are a few hypothesis about this phenomenon, all summarised in figure 21. The first hypothesis is the fact that the ALK5 receptor can be internalized by caveolin-1, thereby inhibiting the effect of Smad2/3 and so stimulating Wnt signaling (14).

Another possibility is the stimulating effect of caveolin-1 on integrin linked kinase (ILK). Overexpression of ILK results in the translocation of β-catenin to the nucleus (25). ILK is known to inhibit glycogen synthase kinase 3-β (GSK3β), an enzyme that phosphorylates β-catenin and in this way leads to the degradation of β-catenin. When degradation occurs, β-catenin cannot be translocated to the nucleus and no Wnt signaling will occur (4,25). However, in our results no significant stimulating effect of caveolin-1 on ILK is seen (see fig. 11). ILK is significantly downregulated with caveolin-1. So ILK is no explanation for the findings of less inhibition of Wnt signaling when caveolin-1 is added.

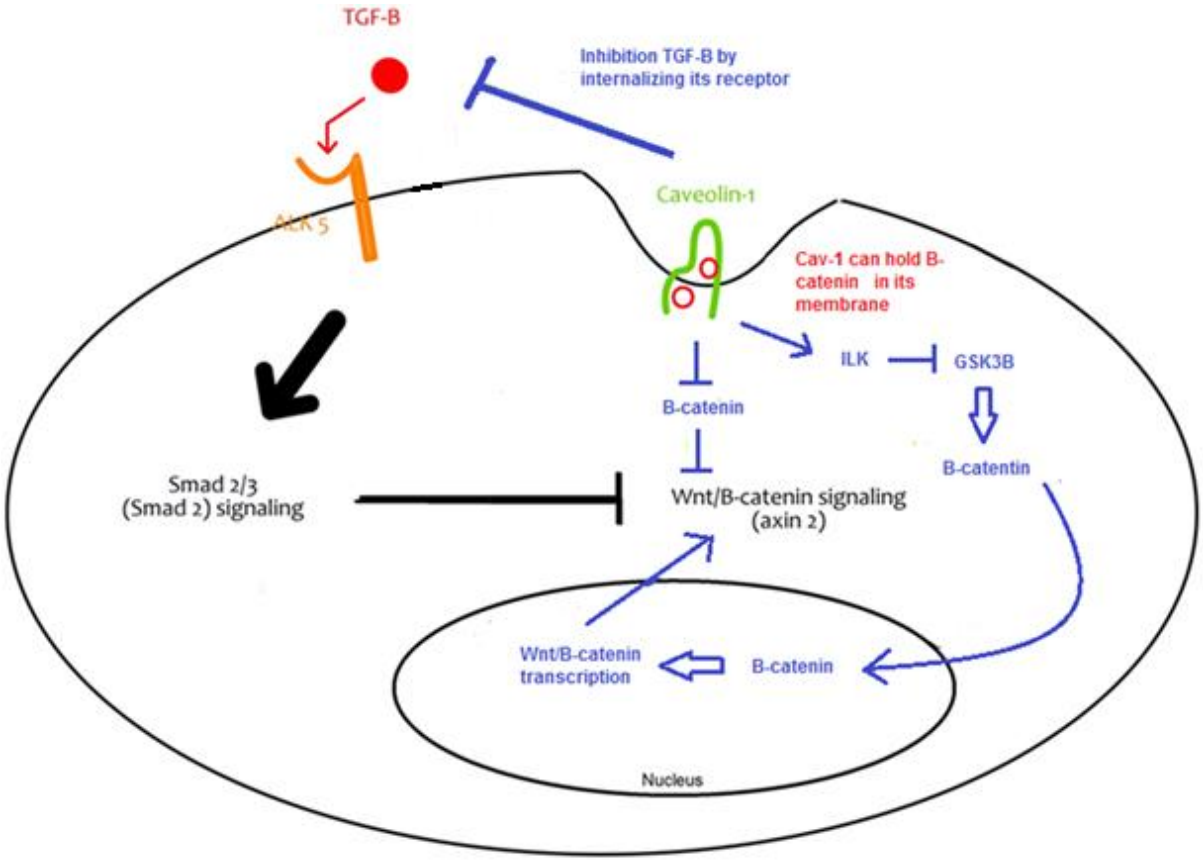

Fig. 21: The effects of caveolin-1 on Wnt signaling pathway. Caveolin-1 can internalize the ALK5 receptor, thereby inhibiting SMAD2/3 signaling and stimulating Wnt/β-catenin signaling. Another possibility is the stimulating effect on ILK, thereby inhibiting GSK3β and stimulating the translocation of β-catenin to the nucleus. The last option is that caveolin-1 can hold β-catenin in its membrane, which leads to 2 options: 1. β-catenin is no longer available for Wnt signaling or 2. β-catenin cannot be broken down and stays available for Wnt signaling (4).

The last hypothesis of the effect of caveolin-1 on Wnt signaling is the co-localisation of caveolin-1 and β-catenin, as is performed by Smolders et al (2012) (4). It is still not known in which way this co-localisation influences the gene expression of *Axin2* and thus Wnt signaling. With the co-immunifluorescence analysis is shown that the protein expressions of both caveolin-1 and β-catenin peaked in at the cell membrane. This may indicate that caveolin-1 can recruit β-catenin in its membrane, which means β-catenin cannot be broken down by GSK3β, so β-catenin stays available for Wnt signaling. However, when caveolin-1 holds β-catenin in its membrane, is could also be that β-catenin cannot move out and in this way cannot be translocated to the nucleus, and in this way inhibits Wnt signaling (4). Further research is necessary to be certain about the role caveolin-1 plays in Wnt signaling in NPCs.

Cell senescence

As already mentioned, caveolin-1 can stimulate Wnt/β-catenin signaling, which leads to a higher expression of MMPs and in this way to cell senescence and IVD degeneration (24). Senescent cells remain viable and metabolically active for a long period of time, despite their inability to proliferate (26). However, in this study no cell senescence is observed by adding caveolin-1 to the pellets and MMP13 was even downregulated even when caveolin-1 was introduced to the environment. This suggests that even in cell senescence, the role of caveolin-1 is uncertain.

Study limitations

Because only 5 donors in were used, the power of the experiment was low (n = 5). When more donors were used, the power of the experiment can be increased and more donors could have showed the effect of caveolin-1 on GAG content in the pellets just like in donor A. Then, an explanation could be easier to find.

It is also possible that the concentration of caveolin-1 used in this study is too low to see an additive effect of caveolin-1 on NPCs. This study could be repeated with higher concentrations of caveolin-1, to see if an additive effect on all NPCs can be accomplished.

Also, for this experiment only chondrodystrophic dogs were used. It is not for sure that non-chondrodystrophic dogs will give the same results we found here in this study. All dogs were younger than 3 years of age, so age may also play a prominent role in the effect of caveolin-1.

Suggestions for future

To figure out if the results we found also accounts for non-chondrodystrophic dogs and humans, the experiment can be repeated with cells of humans and non-chondrodystrophic dogs. In this way, a comparison can be made between the results we found in this experiment and the results we will find in the next. Also, older dogs can be used for the same experiment, because young dogs have another ratio in ALK5/ALK1 receptors (more ALK5) as old dogs (ratio near 1) (13).

To make sure if caveolin-1 can hold β-catenin in its membrane and where β-catenin is localised in the NP cells, a co-fluorescence can be performed. In this way, an answer can be obtained on the question what influence caveolin-1 has on the Wnt signaling.

At last, it would also be interested to silence caveolin-1, to see what happens when caveolin-1 is not available in NP cells.

Conclusion

Caveolin-1 does not have an additive effect on TGF-β1 treatment on degenerated nucleus pulposus cells. However, when donors are observed independently, donor A shows a significant increase in GAG content in the pellets when conditions with caveolin-1 are compared with a treatment of TGF-β1 alone. This suggests there is a donor-dependent effect of caveolin-1. After 7 days, inhibition of Wnt signaling by TGF-β treatment is less when caveolin-1 is added to the medium. This suggests caveolin-1 influences Wnt signaling in NP cells. Further experiments are necessary to find out the exact pathways of Wnt signaling and how caveolin-1 can regulates them.

References

(1) Bergknut N, Smolders LA, Grinwis GCM, Hagman R, Lagerstedt AS, Hazewinkel HAW, Tryfonidou MA, Meij BP. Intervertebral disc degeneration in the dog. Part 1: Anatomy and physiology of the intervertebral disc and characteristics of intervertebral disc degeneration. *The Veterinary Journal;* Vol. 195 (2013) 282–291

(2) Bergknut N, Smolders LA, Grinwis GCM, Hagman R, Lagerstedt AS, Hazewinkel HAW, Tryfonidou MA, Meij BP. Intervertebral disc degeneration in the dog. Part 2: Chondrodystrophic and non-chondrodystrophic breeds. *The Veterinary Journal;* Vol. 195 (2013) 292–299

(3) Heathfield SK, Le Maitre CL, Hoyland JA. Caveolin-1 expression and stress-induced premature senescence in human intervertebral disc degeneration. *Arthritis Research & Therapy* 2008, 10:R87

(4) Smolders LA, Meij BP, Onis D, Riemers FM, Bergknut N, Wubbolts R, Grinwis GCM, Houweling M, Groot Koerkamp MJA, van Leenen D, Holstege FCP, Hazewinkel HAW, Creemers LB, Penning LC, Tryfonidou MA. Gene expression profiling of early intervertebral disc degeneration reveals a down-regulation of canonical Wnt signaling and caveolin-1 expression: implications for development of regenerative strategies. *Arthritis Research & Therapy* 2013, 15:R23

(5) Gvaramia D, Blaauboer ME, Hanemaaijer R, Everts V. Role of caveolin-1 in fibrotic diseases. *Matrix Biology* 32 (2013) 307–315.

(6) Bach FC, Willems N, Penning LC, Ito K, Meij BP, Tryfonidou MA. Potential regenerative treatment strategies for intervertebral disc degeneration in dogs. *BMC Veterinary Research* 2014, 10:3

(7) Baker N, Tuan RS. The less-often-traveled surface of stem cells: caveolin-1 and caveolae in stem cells, tissue repair and regeneration. Baker and Tuan *Stem Cell Research & Therapy* 2013, 4:90

(8) Smolders LA, Meij BP, Riemers FM, Wubbolts R, Heuve D, Grinwis GCM, Vernooij HCM, Hazewinkel HAW, Penning LC, Tryfonidou MA. Canonical Wnt Signaling in the Notochordal Cell is Upregulated in Early Intervertebral Disk Degeneration. *Journey of Orthopaedic Research*, June 2012

(9) Bergknut N, Rutges JPHJ, Kranenburg HJC, Smolders LA, Hagman R, Smidt HJ, Lagerstedt AS, Penning LC, Voorhout G, Hazewinkel HAW, Grinwis GCM, Creemers LB, Meij BP, Dhert WJA. The Dog as an Animal Model for Intervertebral Disc Degeneration?. *Spine* 2012; Vol. 37: Number 5, pp 351–358

(10) Bergknut N, Meij BP, Hagman R, de Nies KS, Rutges JP, Smolders LA, Creemers LB, Lagerstedt AS, Hazewinkel HAW, Grinwis GCM. A New Histological Grading Scheme for Classification of Intervertebral Disc Degeneration in Dogs. *The Veterinary Journal* Vol. 195, Issue 2, February 2013, Pages 156–163

(11) Photo at http://dogtime.com/dog-breeds/beagle

(12) Photo at http://dogtime.com/dog-breeds/german-shepherd-dog

(13) Blaney Davidson EN, Remst DFG, Vitters EL, van Beuningen HM, Blom AB, Goumans MJ, van den Berg WB, van der Kraan PM. Increase in ALK1/ALK5 Ratio as a Cause for Elevated MMP-13 Expression in Osteoarthritis in Humans and Mice. *The Journal of Immunology* 2009; 182: 7937–7945

(14) Di Guglielmo GM, Le Roy C, Goodfellow AF, Wrana JL. Distinct endocytic pathways regulate TGF-β receptor signalling and turnover. *Nature Cell Biology*, Vol. 5, May 2003

(15) Young-Joon Kwon MD, Je-Wook Lee, Eun-Joong Moon, Yeon Gu Chung MD, Ok-Soon Kim BA, Hae-Jin Kim. Anabolic Effects of Peniel 2000, a Peptide That Regulates TGF-β1 Signaling on Intervertebral Disc Degeneration. *Spine* 2013; Vol. 38: pp E49–E58

(16) Zou H, Stoppani E, Volonte D, Galbiati F. Caveolin-1, cellular senescence and age-related diseases. *Mech Ageing Dev.* 2011 November ; 132(11 – 12): 533–542

(17) Pfaffl MW. 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 29:e45.

(18) Li TF, Chen D, Wu Q, Chen M, Sheu T, Schwarz EM, Drissi H, Zuscik M, O'Keefe RJ. Transforming Growth Factor-β Stimulates Cyclin D1 Expression through Activation of β-Catenin Signaling in Chondrocytes. *J. Biol. Chem.* 2006 July 28; 281(30): 21296–21304

(19) Dao DY, Yang X, Chen D, Zuscik M, O'Keefe RJ. Axin1 and Axin2 Are Regulated by TGF-β and Mediate Cross-talk between TGF-β and Wnt Signaling Pathways. *Ann N Y Acad Sci.* 2007 November; 1116: 82–99

(20) Hiyama A, Sakai D, Tanaka M, Arai F, Nakajima D, Abe K, Mochida J. The Relationship Between the Wnt/β-Catenin and TGF-β/BMP Signals in the Intervertebral Disc Cell. *Journal of Cellular Physiology* 2011, 26: 1139–1148

(21) Akiyama H, Lyons JP, Mori-Akiyama Y, Yang X, Zhang R, Zhang Z, Deng JM, Taketo MM, Nakamura T, Behringer RR, McCrea PD, de Crombrugghe B. Interactions between Sox9 and β-catenin control chondrocyte differentiation. *Genes & Development* 2004, 18: 1072–1087

(22) Massagué J, Chen JM. Controlling TGF-β signaling. *Genes dev.* 2000, 14: 627–644

(23) Van der Kraan PM, Goumans MJ, Davidson EB, ten Dijke P. Age-dependent alteration of TGF-β signalling in osteoarthritis. *Cell Tissue Res* (2012) 347:257–265

(24) Hiyama A, Sakai D, Risbud MV, Tanaka M, Arai F, Abe K, Mochida J. Enhancement of Intervertebral Disc Cell Senescence by Wnt/β-Catenin Signaling-Induced Matrix Metalloproteinase Expression. *Arthritis Reum.* 2010 October ; 62(10): 3036–3047

(25) Chun J, Hyun S, Kwon T, Lee EJ, Hong SK, Kang SS. The subcellular localization control of integrin linked kinase 1 through its protein-protein interaction with caveolin-1. *Cellular signaliing.* 17 (2005) 751 – 760

(26) Huafei Z, Stoppani E, Volonte D, Galbiati F. Caveolin-1, cellular senescence and age-related diseases. *Mech Ageing Dev.* 2011 November; 132 (11-12): 533 – 542