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The regenerative effects of caveolin-1 and TGF-β on nucleus pulposus cells

Honours Program project

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Summary

Introduction: Intervertebral disc (IVD) degeneration is a common, painful disorder in dogs and humans that is also an important risk factor for spinal diseases. Current treatment options are not optimal, as they do not restore the health and function of the IVD. Hence, new regenerative treatment strategies are needed. Early IVD degeneration is characterized by the loss of notochordal cells and the subsequent replacement by nucleus pulposus cells (NPCs). Microarray analysis has shown a decrease in the expression of caveolin-1 within NPCs of canines suffering from early IVD degeneration. In addition, the nucleus pulposi of wild type mice were found to be rich in viable notochordal cells, whereas the nucleus pulposi of caveolin-1 knockout mice contained chondroid matrix with relatively few cells that were mainly apoptotic. Moreover, previous research has shown that caveolin-1 inhibits TGF-β signalling. Despite its anabolic effects, TGF-β signalling mainly results in ongoing matrix degradation in the degenerating *in vivo* IVD. These findings suggest that caveolin-1 may play an important role in maintaining a healthy notochordal cell-rich nucleus pulposus. We attempted to gain more insight into the regenerative effects of caveolin-1 on NPCs in order to assess the potential use of caveolin-1 as a therapeutic target in regenerative medicine. In this study, a caveolin-1 scaffolding domain (CSD) peptide was used to supplement NPCs *in vitro*. This peptide has been reported to mimic the function of caveolin-1 *in vitro* and *in vivo*. We hypothesized that CSD can be used to facilitate caveolin-1 signalling *in vitro*.

Material and methods: Culture experiments were performed in which caveolin-1 scaffolding domain (CSD) (alone or in combination with TGF-β) was supplemented to pellets containing articular chondrocytes (ACs) and NPCs for 7 or 28 days. First, the optimal dose and culture conditions with respect to CSD treatment were determined in a toxicity pilot and a dose optimization pilot (chapter 2). The additional regenerative effects of CSD upon TGF-β1 treatment in NPCs from chondrodystrophic dogs (chapter 3), non-chondrodystrophic dogs (chapter 4) and human donors (chapter 4) were investigated. Cell proliferation and extracellular matrix production were specified as parameters for regeneration. The DNA content of the cell cultures was determined to measure cell proliferation. The extracellular matrix production was quantified by measuring the glycosaminoglycan (GAG) content and GAG release in the culture medium using a DMMB assay. Additionally, cell cultures were subjected to a Safranin O/Fast Green staining in order to visualize the GAG production. The expression levels of relevant genes (i.e. markers concerning cell proliferation, matrix production/remodelling, chondrogenesis, TGF-β and Wnt/β-catenin signalling and apoptosis) were determined using RT-qPCR.

Results: Treatment with only CSD did not induce regenerative effects. On the contrary, supplemented in concentrations of 5 μM or higher, CSD inhibited DNA synthesis and extracellular matrix production in canine ACs. TGF-β1 treatment increased extracellular matrix production in canine and human NPCs. Also a proliferative effect on canine NPCs was observed. CSD exerted no additional regenerative effects upon TGF-β1 treatment of canine and human NPCs.

Conclusion: CSD (alone or in combination with TGF-β1) did not elicit regenerative effects on canine or human NPCs. Hence, the scaffolding domain of caveolin-1 did not demonstrate to be a promising target for regenerative medicine in the current (anabolic) culture experimental set-ups. On the other hand, in 3 individual donor cases CSD exerted additional regenerative effects upon TGF-β1 treatment, indicating that CSD's effects could be donor-dependent. Given that the CSD may also interfere with the intrinsic caveolin-1 function, we cannot exclude that the CSD may have functioned as an antagonist by interfering with the caveolin-1 dependent signalling. Furthermore, considering that caveolin-1 has anti-inflammatory properties, it cannot be excluded that under inflammatory and catabolic culture conditions, CSD treatment may exert beneficial effects. However, it can also not be excluded that CSD antagonized caveolin-1 function by interfering with caveolin-1 dependent signalling, and that caveolin-1 itself does have regenerative effects upon NPC cells.

1. General introduction

1.1. Clinical importance

Intervertebral disc (IVD) degeneration commonly causes low back pain in dogs and humans. It is considered the most important cause of spinal diseases such as IVD herniation, cervical spondylomyelopathy and degenerative lumbosacral stenosis [1,2]. Current treatments such as physiotherapy, anti-inflammatory/analgesic medication and surgery are not optimal as they focus on the end stage of the disease and do not restore the physiological function of the IVD. Moreover, surgical interventions such as partial discectomy, spinal fusion or artificial IVD replacement are invasive, expensive and associated with various complications. These disadvantages have led to an increasing interest in developing regenerative treatments that aim to restore the health and function of the IVD [1]. In this respect it is most obvious to focus on IVD degeneration at an early stage, in which there is still a chance to stop the degeneration and possibly turn it into a regenerative process.

1.2. The healthy intervertebral disc

The healthy intervertebral discs consist of the nucleus pulposus, the annulus fibrosus and the cartilaginous endplates (**figure 1**) [2,3,4]. The nucleus pulposus is a gelatinous structure that forms the inner part of the intervertebral disc. It contains a dense population of notochordal cells that is embedded in matrix, rich in proteoglycans (containing high levels of negatively charged side-chain glycosaminoglycans (GAGs)) and collagen type II fibers. The proteoglycans create a strong osmotic gradient that attracts water into the nucleus pulposus. The hydrated matrix provides stability for spinal segments by absorbing levels of stress that are considered physiological for the healthy IVD. The nucleus pulposus is surrounded by the annulus fibrosus, which is characterized by a dense network of several concentric fibrous lamellae. It is populated by a mix of fibrocyte- and chondrocyte-like cells [2]. The cranial and caudal borders of the IVD are formed by the cartilaginous endplates. The endplates consists of cranio-caudally oriented layers of matrix and chondrocyte-like cells [2]. The annulus fibrosus and the cartilaginous endplates protect the nucleus pulposus from being sheared by compressive forces and its own internal swelling pressure. Furthermore, both are responsible for supplying nutrients to the nucleus pulposus, since there are no blood vessels present in the nucleus pulposus [2].

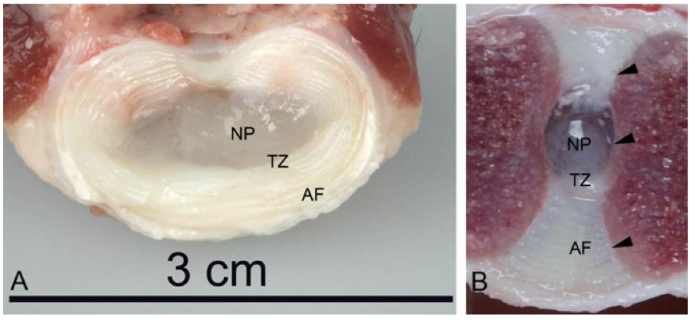


Figure 1. Macroscopic photos of a transverse (A) and a sagittal (B) section through L5–L6 of a healthy canine intervertebral disc. The nucleus pulposus (NP), which is located at the centre, is surrounded by the annulus fibrosus (AF) and the endplates (arrowheads). The transition zone (TZ) interconnects the NP and AF (Bergknut *et al.* (2013)).[2]

1.3. Intervertebral disc degeneration

The early process of IVD degeneration is characterized by cellular changes in the nucleus pulposus (**figure 2**). The dense population of notochordal cells in a healthy nucleus pulposus is replaced by single or clustered chondrocyte-like cells (henceforth named nucleus pulposus cells) [1,2,3,4]. The mechanisms through which this transition in cell type occurs is poorly understood. It is hypothesized that notochordal cells possess the ability to differentiate into a chondrocyte-like cell phenotype as the IVD matures. Another explanation could be that the notochordal cells undergo apoptosis and are replaced by chondrocyte-like cells that originate from the annulus fibrosus or endplates [3].

These cellular changes are accompanied by changes in extracellular matrix. More specifically, the content of proteoglycans and collagen II decreases, while the content of collagen I increases [2]. The loss of proteoglycans in the matrix is accompanied by a decrease in water content. As a result, the matrix of the nucleus pulposus becomes rigid and the nucleus pulposus loses its properties to function as a shock absorber. Eventually, the intervertebral disc will no longer be able to offer stability and mobility to the spinal segments. The degenerative process may ultimately lead to cleft formation, collapse and/or herniation of the nucleus pulposus [2].

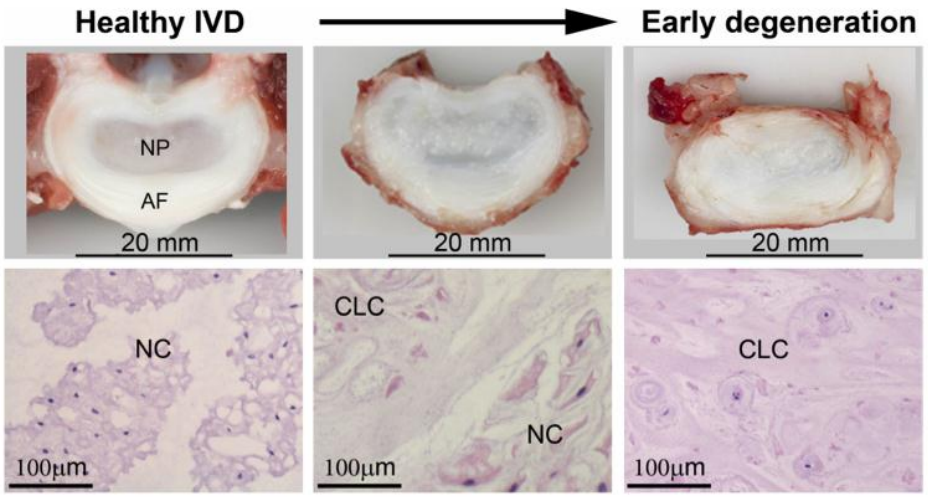


Figure 2. Macroscopic and microscopic photos showing the transition from a healthy to an early degenerated intervertebral disc. The healthy nucleus pulposus has a jelly-like consistency (top left) and notochordal cells (NC) are the main cell type (lower left). During the process of degeneration, the distinction between the nucleus pulposus and the annulus fibrosus becomes less defined (upper middle and top) and the NCs are replaced by chondrocyte-like cells (CLC) (lower middle and right) [3].

1.4. The dog as animal model: chondrodystrophic and non-chondrodystrophic dogs

Dog breeds are classified into two groups based on the predisposition to chondrodystrophy: chondrodystrophic breeds and non-chondrodystrophic breeds [5]. In contrast to non-chondrodystrophic breeds, the chondrodystrophic breeds, i.e. Beagles, (miniature) Dachshunds and Basset Hounds, have disproportionally short limbs due to disturbed endochondral ossification. Moreover, these two dog subspecies differ regarding the age of onset, frequency and spinal location of IVD degeneration. IVD degeneration is more common in chondrodystrophic breeds in which chondrocyte-like cells already start to invade the nucleus pulposus at 3 months of age. Complete replacement of the notochordal cells by chondrocyte-like cells takes place before 1 year of age, with a concurrent onset of IVD degeneration of mainly the cervical and/or thoracolumbar spine. Spinal diseases typically develop at 3-7 years of age. In NCD dog breeds such as the German Shepherd or mixed breeds, the NCs remain the predominant cell type of the NP until middle or old age (5 years) and IVD degeneration generally occurs at older age (6-8 years), mainly at lumbosacral spinal levels [6]. Research has shown that these two dog subspecies reflect a suitable animal model for studying the (patho)physiology of human IVD degeneration, since both share similar pathophysiological processes with respect to human IVD degeneration [1,5]. Smolders *et al.* (2013)[1] used chondrodystrophic and non-chondrodystrophic dogs to determine the gene expression profiles of nucleus pulposus tissue during different stages of IVD degeneration. They found that a protein called caveolin-1 is downregulated in early intervertebral disc degeneration. They used caveolin-1 knockout mice to further investigate the physiological role of caveolin-1. The IVDs of these mice contained cells that lacked the typical vacuolar appearance of notochordal cells. Moreover, the nucleus pulposus of the knockout mice contained a large amount of chondroid-like extracellular matrix and apoptotic cells. These findings suggest a crucial role for caveolin-1 in maintaining a healthy nucleus pulposus.

1.5. Caveolin-1 protein

Caveolin-1 is an integral membrane protein that is largely present in the caveolae, which are flask-shaped invaginations of the cell membrane [7,8]. From this location, caveolin-1 regulates several cellular processes including cell cycle regulation, cell senescence, endocytosis, vesicle-transport, cell-adhesion and signalling pathways [7,9,10,11].
Previous research has indicated a role for caveolin-1 in tissue degeneration and regeneration [11]. However, literature is not unambiguous regarding the effects of caveolin-1. This is because caveolin-1 acts differently depending on cell type, cellular context and stage of degeneration [1,11]. Depending on the type of cell or tissue, it may either positively or negatively influence the process of tissue repair. Caveolin-1 negatively influences the cell proliferation and differentiation of mesenchymal stem cells and progenitor cells in muscle tissue, corneal epithelium and neural tissue. In these tissues a downregulation of caveolin-1 expression is necessary for inducing tissue regeneration [11].

In contrast to mesenchymal stem cells, embryonic stem cells need caveolin-1 expression to induce cell proliferation and cell renewal [11]. This means that caveolin-1 could act as an inhibitor as well as a stimulator of cell proliferation. Caveolin-1 exerted additional effects on the cardiovascular system, liver and kidney [11]. First of all, caveolin-1 is required for the formation of new blood vessels, which makes caveolin-1 expression important in the repair of the cardiovascular system. Secondly, caveolin-1 has positive effects on liver tissue as it demonstrated to have an essential role in lipid droplet formation, an important step in the proliferative response of liver cells during regeneration. Lastly, caveolin-1 is highly expressed in regenerating proximal tubules, suggesting that it is also important in the regeneration of kidney tissue.

Regarding IVD degeneration, caveolin-1 expression has been associated with cellular senescence in degenerated IVDs [10,12]. In fact, upregulation of caveolin-1 expression is sufficient to induce premature senescence in intervertebral disc cells [12]. This suggests that caveolin-1-mediated cellular senescence plays a role in the pathogenesis of IVD degeneration. These findings are in contrast to the results of a study by Smolders *et al.* (2013)[1] who investigated the expression of caveolin-1 in early IVD degeneration. As previously mentioned, their study demonstrated that caveolin-1 is important in maintaining a healthy nucleus pulposus since its expression decreases during early IVD degeneration and because caveolin-1 knockout mice were unable to develop healthy IVDs [1].

Regarding its function in regulating signalling pathways, each caveolin-1 protein possesses a domain which is critical for its signalling function: the caveolin-1 scaffolding domain. This domain is located at the N-terminal region (oriented towards the cytosol). Through this domain, caveolin-1 is capable of interacting with many intracellular signalling molecules, i.e. eNOS, epidermal growth factor receptor and TGF-β receptors [9,13]. Consequently, caveolin-1 can regulate many signalling pathways. This report focuses on only two of these pathways: the Wnt/β-catenin and the TGF-β signalling pathway, since these pathways are considered to play important roles in IVD degeneration and regeneration [9,14,15,16].

1.5.1. Wnt/β-catenin signalling

The Wnt/β-catenin signalling pathway is involved in embryogenesis and adult tissue regeneration and degeneration [17,18]. More specifically, sustained Wnt/β-catenin signalling is required for differentiation of progenitor cells to notochordal cells during embryogenesis [19]. Futhermore, it regulates stem cell renewal and apoptosis [1]. Previous research found that Wnt/β-catenin signalling is decreased in early IVD degeneration [1]. Given its function in stem cells, it was suggested that the diminished Wnt/β-catenin signalling resulted in increased apoptosis and decreased self-renewal of notochordal cells or nucleus pulposus' stem cells and ultimately in chondroid metaplasia of the nucleus pulposus.

Above-mentioned research suggests that Wnt/β-catenin signalling is required for maintaining a healthy IVD. However, the effect of Wnt/β-catenin signalling does not appear as one-sided, as researchers also mention its contribution to the pathogenesis of IVD degeneration. More specifically, it was shown that β-catenin suppresses IVD cell proliferation by targeting and

suppressing cell proliferation markers *CyclinD1* and *c-myc* [15,16]. This cytostatic activity may lead to a loss of cells, which is an important factor in IVD degeneration [20]. Moreover, Wnt/β-catenin signalling induces notochordal cell senescence and apoptosis [21], and also stimulates the expression and activation of matrix metalloproteinases (MMPs). The latter leads to the breakdown of the nucleus pulposus' extracellular matrix and the progression of the IVD degeneration process [21].

Caveolin-1 regulates the Wnt/β-catenin signalling in several ways. It recruits β-catenin from the cytosol to the caveolar membranes. As a result, β-catenin becomes compartmentalized in restricted domains of the plasma membrane, which could lead to the inhibition of the Wnt/β-catenin signalling [14]. However, it may also lead to activation of the Wnt/β-catenin signalling since the breakdown of β-catenin by glycogen synthase kinase-3b becomes impossible [14]. Furthermore, caveolin-1 may activate Wnt/β-catenin signalling by internalization and subsequent activation of the Wnt receptor [15] and by activation of integrin-linked kinases [16]. These kinases inhibit glycogen synthase kinase-3b, thereby preventing the breakdown of β-catenin.

1.5.2. TGF-β pathway

Activation of the TGF-β pathway requires the binding of TGF-β to the TGF-β type 2 receptor [22]. Subsequently, the TGF-β type 1 receptor is recruited into the binding complex and it is activated upon phosphorylation by the kinase domains of the TGF-β type 2 receptor. The activated TGF-β type 1 receptor relays the signalling by phosphorylating effector proteins, i.e. the Smad proteins. Thus, the type 1 receptor acts downstream of TGF-β type 2 receptors in TGF-β mediated responses. Consequently, the type 1 receptor determines the specificity of the intracellular signals [22].

TGF-β is known to stimulate cell proliferation, differentiation and extracellular matrix production [23]. *In vitro* culture experiments have shown that IVD cells respond to TGF-β treatment with increased cell proliferation and extracellular matrix protein synthesis [24,25–27,28]. Despite these anabolic effects, TGF-β overexpression has harmful effects on cartilage and IVD tissue due to its induced upregulation of matrix proteinases (MMPs and aggrecanases) [23]. It has been postulated that these effects are the result of TGF-β's ability to signal through two different TGF-β type 1 receptors, i.e. ALK5 and ALK1. Activation of the ALK5 receptor results in the phosphorylation of effector proteins Smad2 and Smad3, whereas activation of the ALK1 receptor results in the phosphorylation of Smad1, Smad5, and Smad8 [23,29,30]. Upon phosphorylation, these Smad proteins associate with Smad4 and enter the cell nucleus to modulate the expression of target genes [22,29]. The ALK5 (Smad2/3) signalling pathway leads to extracellular matrix component synthesis [23,30], whereas the ALK1 (Smad1/5/8) signalling pathway leads to progression of IVD degeneration by upregulation of matrix metalloproteinase 13 (MMP13) [23,30]. The increased level of TGF-β signalling during IVD degeneration allows activation of both the Smad2/3 and the Smad1/5/8 pathway. However, since activation of the Smad1/5/8 pathway inhibits the Smad2/3 signalling pathway and the subsequent favourable production of extracellular matrix components[29], the increased TGF-β levels will only result in further disc degeneration [23] (**figure 3**).

Caveolin-1 is known to inhibit TGF-β signalling [9]. Caveolin-1 has been reported to downregulate gene expression of TGF-β type 2 receptors [31]. Furthermore, caveolin-1 interacts directly with the TGF-β type 1 receptors through its scaffolding domain. This interaction leads to sequestration of these receptors, resulting in the inability of TGF-β to activate its type 1 receptors and to initiate baseline Smad2 phosphorylation [9]. Previous work has shown that caveolin-1 is able to interact with both TGF-β type I receptors (ALK5 and ALK1) [9,32–35]. Since nucleus pulposus cells express higher levels of the ALK5 receptor than the ALK1 receptor, caveolin-1 can completely inhibit the low amount of ALK1 receptors present, while still leaving a sufficient amount of ALK5 receptors left to be activated by TGF-β [23]. In other words, we hypothesize that the inhibitory effect of caveolin-1 may be higher on the ALK1 receptor than on the ALK5 receptor. This results into higher Smad2/3 signalling relative to Smad1/5/8 signalling, which ultimately leads to more extracellular matrix production rather than a progression of IVD degeneration (**figure 3**). However, further research is necessary to confirm this hypothesis.

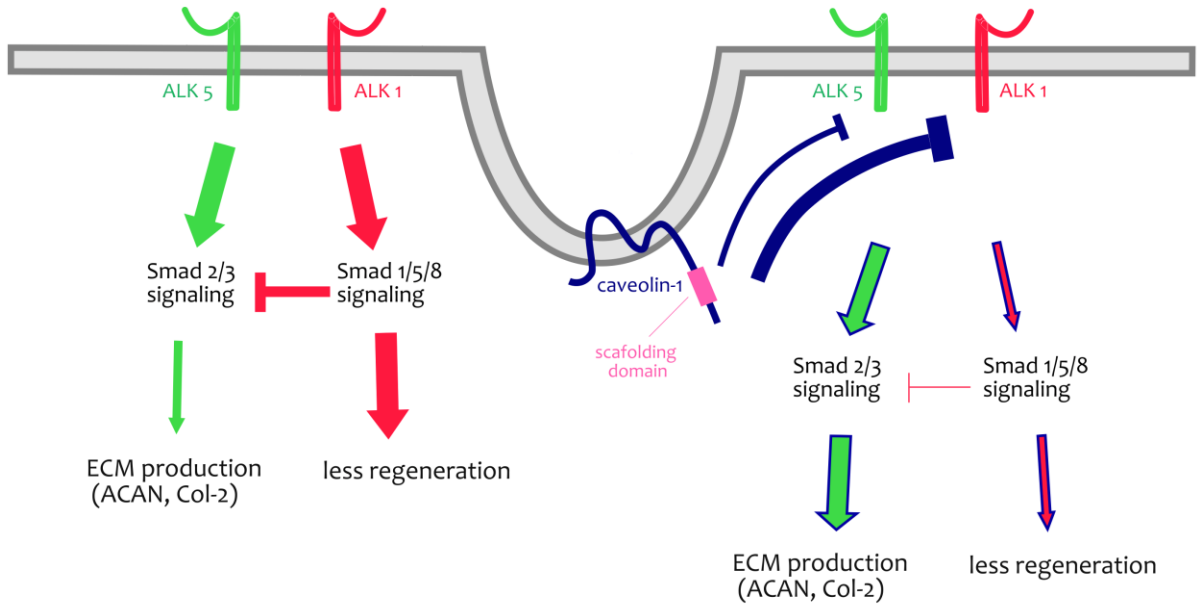


Figure 3. Schematic overview of TGF-β signalling and the hypothetical effects of caveolin-1. During IVD degeneration, TGF-β will mainly stimulate the unfavourable ALK1 (Smad1/5/8) pathway. Caveolin-1's inhibitory effect on TGF-β signalling may be higher on the ALK1 (Smad1/5/8) signalling pathway due to differences in basal expression levels of the different ALK receptors. Inhibition of ALK1 (Smad1/5/8) signalling leads to higher ALK5 (Smad2/3) signalling and ultimately to more extracellular matrix proteins, i.e. proteoglycan Aggrecan (ACAN) and Collagen II (Col-2), rather than a progression of IVD degeneration. TGF-β type 2 receptors are not shown in this image. It should be noted that Smad signalling involves the phosphorylation of independent Smad molecules (i.e. Smad1, Smad2, Smad3, etc.).

1.6. Cell permeable caveolin-1 scaffolding domain (CSD)

In the *in vitro* experiments that have been performed within this project, not the whole caveolin-1 protein, but solely the caveolin-1 scaffolding domain (CSD) was used to treat the NPCs. Enzo

Life Sciences, the manufacturer of the CSD peptide, based this product on a study performed by Bucci *et al.* (2000)[13]. They were the first to manufacture this cell permeable CSD peptide. This CSD peptide was fused to a cell permeable antennapedia (AP) internalization sequence that enables the CSD to be taken up by cultured cells [13]. The amino acid sequence of this AP-CSD fusion peptide (Enzo Life Sciences, ALX-153-064) is:

H- **rqikiwfqnrrmkwkkdgiwkasfttftvtkywfyr** –OH

The green part indicates the AP internalization sequence, whereas the blue part indicates the CSD sequence. The CSD sequence is coded by amino acid 82-101 of the true caveolin-1 protein (NCBI, Protein Database):

1 msggkyvdse ghlytvpire qgniykpnnk amaeemsekq vydahtkeid lvnrdpkhln
61 ddvvkidfed viaepegths f**dgiwkasft tftvtkywfy r**llsalfgip maliwgiyfa
121 ilsflhiwav vpciksflie iqcisrvysi yvhtfcdpff eavgkifsni rinmqket

Previous studies reported the use of this CSD peptide to agonize/upregulate the function of caveolin-1. Bucci *et al.* (2000)[13] and Bernatchez *et al.* (2005)[36] investigated the role of caveolin-1 in eNOS-mediated NO release and found that CSD inhibits eNOS, thereby regulating NO production and vascular function (similar as the caveolin-1 protein). Besides, Bucci *et al.* (2000)[13] reported that CSD also exerted effects *in vivo*, i.e inhibition of vascular leakage and edema formation in mice. Since caveolin-1 is considered an anti-fibrotic agent[9], CSD has been used to investigate the role of caveolin-1 in fibrosis. Tourkina *et al.* (2008)[37] investigated the involvement of caveolin-1 in extracellular matrix production in lung fibrosis. They found that CSD inhibits collagen and tenascin-C production (similar as the caveolin-1 protein) by negatively regulating MEK/ERK activation in healthy lung fibroblasts. They reported that the effect of CSD (5 µM) was rapid and powerful, since it inhibited collagen synthesis with more than 95% after 5 hours of treatment. Zhang *et al.* (2011)[38] investigated the role of caveolin-1 in dermal fibrosis by treating keloid fibroblasts with CSD in the presence of TGF-β. They found that CSD inhibited TGF-β type I receptor levels, thereby reducing Smad2/3 phosphorylation (similar as the caveolin-1 protein). Miyasato *et al.* (2011)[39] used CSD to confirm the protective role of caveolin-1 in the development and progression of myocardial fibrosis. Their results show that CSD treatment reduced interstitial fibrosis and development of perivascular fibrosis as caveolin-1 protein does. Additionally, CSD treatment reduced the activation of canonical TGF-β1 signalling (Smad2 dependent) and non-canonical ERK1 signalling. They concluded that activation of these TGF-β1 dependent pathways is caveolin-1 dependent. Since caveolin-1 is known to inhibit several growth factor receptors, including the TGF-β1 receptor, EGFR, and PDGFR, Schmitz *et al.* (2011)[40] investigated the effect of CSD on the nerve growth factor receptor TrkA. In line with caveolin-1's inhibiting effects on growth factor receptors, CSD inhibited the activation of TrkA and the activity of downstream components of the nerve growth factor signalling pathway (i.e. MAPK Erk1 and 2) [40].

Based on these studies, we hypothesized that the CSD mimics/upregulates caveolin-1's function. However, it should be noted that we cannot exclude that the CSD peptide may

function as a caveolin-1 antagonist rather than an agonist. It is possible that CSD interferes with caveolin-1 dependent signalling by binding to the cytosolic ligands (signalling molecules) of caveolin-1, thereby blocking their interaction with caveolae. CSD can, in this way, possibly interfere with, and hence inhibit, the action of caveolin-1 on signalling molecules that require endogenous caveolin for their function. However, since all above mentioned studies demonstrated that the cell permeable CSD fusion protein mimics the action of caveolin-1 (by influencing signalling pathways and cellular processes in the same way as endogenous caveolin-1 does), we hypothesized that the cell permeable CSD is a suitable tool to mimic caveolin-1 function *in vitro* in nucleus pulposus cells.

1.7. Aim of the project

The main aim of this Honours program project is to gain insight into the regenerative effects of caveolin-1 on nucleus pulposus cells in order to clarify the potential of caveolin-1 as a target for developing regenerative treatments for IVD degeneration. In total, five cell culture experiments were performed. The first two were pilot experiments that aimed at optimizing the dosage and culture conditions of NPCs receiving caveolin-1 scaffolding domain peptide. The subsequent three experiments were main experiments that aimed at determining the effects of caveolin-1 scaffolding domain peptide on nucleus pulposus cells from chondrodystrophic dogs, non-chondrodystrophic dogs and humans respectively.

1.8. Embedding in faculty program

This project is part of the faculty's "Tissue repair" research program and was funded by a grant from the AO Spine Research Network (in collaboration with the dept. of Biomedical Bioengineering of the Technical University Eindhoven, PI. Prof.dr. Keita Ito). The strategic goal of this research program is to develop new methods for stimulating regeneration and tumor prevention by delivery of essential cells and/or signals and/or biomaterials and to provide preventive strategies for degenerative diseases.

2. Pilot experiments toxicity study and dose optimization

2.1. Abstract

Introduction. Caveolin-1 is expressed in many cell types and may have opposing functions depending on cellular context. To our knowledge, caveolin-1 scaffolding domain peptide (CSD) has never been supplemented to nucleus pulposus cells (NPCs). In order to determine CSD's optimal dose and culture conditions for NPCs, two pilot experiments were performed. The aim of the first pilot was to estimate the dose in which CSD should be supplemented to NPCs. The aim of the second pilot was to optimize the dose and culture condition.

Material and methods. *Toxicity study*: articular chondrocytes (ACs) were cultured in monolayers and pellets (200.000 cells) for 7 days in basal chondrogenic medium. Cells were treated with CSD (1 μM, 5 μM and 10 μM) or TGF-β1 (10 ng/mL). The DNA content of the pellets was measured using a Qubit® dsDNA High Sensitivity Assay. Gene expression levels of relevant genes were determined by RT-qPCR. Histology was performed using a Safranin O/Fast Green staining. *Dose optimization study*: pellets containing 200.000 ACs or NPCs were cultured for 28 days in basal chondrogenic medium. The pellets were treated with CSD (0.5 μM, 1 μM and 2 μM), TGF-β1 (10 ng/mL), FCS (10%), TGF-β1 in combination with CSD or FCS in combination with CSD. Glycosaminoglycan (GAG) content and the GAG release of these NPC pellets were determined using a DMMB assay in order to quantify matrix production. In addition, pellets were stained with Safranin O/Fast green to visualize the GAG production in the pellets. Cell proliferation was quantified by measuring the DNA content of the pellets. RT-qPCR was performed to determine the gene expression level of relevant genes.

Results. Treatment with only CSD did not stimulate matrix production and cell proliferation. Moreover, in concentrations higher than 2 μM, CSD exerted negative effects on these parameters. The pellets that were treated with TGF-β1 showed increased cell proliferation and matrix production compared with pellets cultured in basal chondrogenic medium. Addition of CSD to the culture medium increased TGF-β1's effect on GAG production. A concentration of 1 μM CSD established the highest additional increase in GAG content.

Conclusion. CSD demonstrated to have additive regenerative effects upon TGF-β1 treatment of NPCs. The optimal concentration of CSD treatment was 1 μM and hence this concentration will be included in the follow up studies. Explanations for the decreased ECM production and DNA content of the cells treated with high concentrations of CSD (>1 μM) could be A) the high DMSO concentrations in which CSD was dissolved, B) the possibility that caveolin-1 and CSD have no regenerative effects at high concentrations (assuming that CSD indeed mimicked caveolin-1 function), or C) the possibility that CSD worked as a caveolin-1 antagonist instead of an agonist, which would indicate that caveolin-1 is needed for NPC/AC cell proliferation and ECM production.

2.2. Introduction

Caveolin-1 is expressed in a wide range of cell types, but is most prevalent in differentiated cell types including adipocytes, fibroblasts, endothelial cells, smooth muscle cells, type 1 alveolar epithelial cells, and nucleus pulposus cells (NPCs) [41,42,43,1]. The caveolin-1 scaffolding domain peptide (CSD) has been subjected to several cell culture experiments, i.e. to treat cancer derived cell lines, endothelial cells and fibroblasts [44,37,45]. But, to our knowledge it has never been supplemented to NPCs. Thus information regarding optimal dose and culture conditions for treating nucleus pulposus cells is lacking. Therefore in a pilot toxicity study we aimed to roughly determine the dose of CSD in which it can be supplemented to NPCs. Based on the results of the toxicity pilot, a follow-up pilot study was set up to optimize the dose of CSD. Since caveolin-1's effects are highly dependent on cellular context [46], CSD's responsiveness in different culture conditions was also investigated.

2.3. Material and methods

In total, cells of 5 canine donors were used. For the toxicity study, articular chondrocytes (ACs) from donor 1 were isolated. For the dose optimization study, ACs and NPCs were isolated from 4 and 2 donors respectively. **Table 1** provides an overview of the donor characteristics.

	Donor number	Breed	Age	Gender	Cell type
Toxicity study	233	Boerboel	9 months	Female	ACs
Dose optimization study	233	Boerboel	9 months	Female	ACs
	405	Labrador	4 months	Female	ACs
	156	Beagle	21 months	Male	ACs and NPCs
	496	Beagle	22 months	Female	ACs
	662	Mixed breed	15 months	Male	NPCs

Table 1. Donor characeristics and used celltypes. ACs = articular chondrocytes, NPCs = nucleus pulposus cells.

In the Biobank of the Orthopedics group new NPC donors are hard to obtain and very valuable and therefore mainly used in final experiments. For this purpose, mainly ACs were used in the current pilot studies as they closely resemble the NPCs [47].

2.3.1. Isolation NPCs and ACs

Nucleus pulposus tissue was collected by a precise separation from the annulus fibrosus and cartilaginous endplates of the IVDs. Articular cartilage was collected by carefully scraping the layer of cartilage from the stifle and/or shoulder joints. Tissue was washed with

hgDMEM+Glutamax (Invitrogen, 31966) + 1% P/S (PAA Laboratories, P11-010) and digested with 0.15% pronase for 45 minutes at 37 °C. Afterwards, the digested tissue was washed to remove the pronase. Next, tissues were subjected to an overnight (16 hr) digestion with 0.15% collagenase II at 37 °C. The cells were filtered over a 70-µm filter and then washed to remove the collagenase. Finally, cells were centrifuged and re-suspended in freeze media (hgDMEM + Glutamax + 20% DMSO and hgDMEM + Glutamax + 20% fetal calf serum (FCS) (High performance, Gibco, 16000-044) (1:1)). Cells were counted and, before the start of the experiment, stored in aliquots in liquid nitrogen.

2.3.2. Cell culture

For both pilot experiments, cells were expanded in expansion medium (**table 2**) in 175 cm^2 cell culture flasks (Greiner bio-one, Cellstar, 660175) to acquire an adequate amount of cells. At passage 2, cells were counted and pelleted in a 96 wells plate (Corning Costar 7007) at a density of 200.000 cells/well by centrifugation at 185 g for 8 minutes.

Toxicity study: The cell pellets for the toxicity study were cultured in chondrogenic medium (**table 2**) (200 µl/well with 200.000 cells) for 7 days. The control pellets received plain chondrogenic medium (**table 2**). The other pellets were treated with either caveolin-1 scaffolding domain peptide (CSD) (Enzo Life Sciences, ALX-153-064) in 3 different concentrations (1 µM, 5 µM and 10 µM) or recombinant human TGF-β1 (10 ng/mL) (R&D Systems, 240-B-010) (**table 3**).

Expansion medium	Final concentration
DMEM, high glucose, GlutaMAX(TM), pyruvate (Invitrogen, 31966)	
FCS (High performance, Gibco, 16000-044)	10%
Penicillin/Streptomycin (PAA Laboratories, P11-010)	1%
Ascorbic acid 2-phosphate (Sigma, A8960)	0,1 mM
Dexamethasone (Sigma, D1756)	0,001 µM (10-9 M)
bFGF (AbD Serotec, PHP105)	1 ng/mL
Fungizone (15290-018, Invitrogen)	
Chondrogenic medium	
DMEM, high glucose, GlutaMAX(TM), pyruvate (Invitrogen, 31966)	
ITS+ premix (Corning, 354352)	1%
L-Proline (Sigma, P5607)	0,04 mg/ml
Penicillin/Streptomycin (PAA Laboratories, P11-010)	1%
Fungizone (15290-018, Invitrogen)	5 µL/mL
Dexamethasone (Sigma, D1756)	0,0001 mM
Ascorbic acid 2-phosphate (Sigma, A8960)	0,1 mM
Bovine Serum Albumin (Sigma, A9418)	1,25 mg/mL

Table 2. Compounds expansion medium and basal chondrogenic medium.

Since previous research cultured with CSD in concentrations ranging from 2 to 5 µM [44,37,45], it was decided to culture with CSD concentrations ranging from 1 to 10 µM. TGF-β1 served as the

positive control as it is a known stimulator of chondrogenesis, matrix production and cell proliferation [23].

Culture group	Time in culture	Number of monolayers per donor			Number of pellets per donor		
		RT-qPCR	DNA	Histology	RT-qPCR	DNA	Histology
Control	7 days	2	2	1	2	2	1
CSD (1 µM)	7 days	2	2	1	2	2	1
CSD (5 µM)	7 days	2	2	1	2	2	1
CSD (10 µM)	7 days	2	2	1	2	2	1
TGF-β1 (10 ng/mL)	7 days	2	2	1	2	2	1

Table 3. Overview of the study design of the toxicity pilot. AC pellets and monolayers were cultured with control medium (plain chondrogenic medium), caveolin-1 scaffolding domain peptide (CSD) in 3 different concentrations (1, 5 and 10 µM) and TGF-β1 (10 ng/mL). After a culture period of 7 days, cells were collected for RNA-isolation (and subsequent RT-qPCR), DNA measurements and histology.

Dose optimization study: The cell pellets for the dose optimization study were cultured in chondrogenic medium for 28 days. The control pellets received plain chondrogenic medium. The other pellets were treated with either CSD in 3 different concentrations (0.5 µM, 1 µM and 2 µM), TGF-β1 (10 ng/mL), 10% FCS (High performance, Gibco, 16000-044) or CSD in combination with TGF-β1 and FCS respectively (**table 4**). Like the toxicity study, the culture media were changed twice a week. But in this pilot, culture medium from each condition was collected at the end of the culture period to assess the GAG release. The CSD concentrations were chosen based on the results of the toxicity study. The FCS condition was included to examine the effect of CSD in a more *in vivo* setting.

Culture group	Time in culture	Number of pellets per donor		
		RT-qPCR	GAG/DNA	Histology
Control	28 days	2	2	1
CSD (0.5 µM)	28 days	2	2	1
CSD (1 µM)	28 days	2	2	1
CSD (2 µM)	28 days	2	2	1
TGF-β1 (10 ng/mL)	28 days	2	2	1
TGF-β1 + CSD (0.5 µM)	28 days	2	2	1
TGF-β1 + CSD (1 µM)	28 days	2	2	1
TGF-β1 + CSD (2 µM)	28 days	2	2	1
FCS (10%)	28 days	2	2	1
FCS + CSD (0.5 µM)	28 days	2	2	1
FCS + CSD (1 µM)	28 days	2	2	1
FCS + CSD (2 µM)	28 days	2	2	1

Table 4. Overview of the study design of the toxicity pilot. AC and NPC pellets were cultured with control medium (plain chondrogenic medium), caveolin-1 scaffolding domain peptide (CSD) in 3 different concentrations (1, 5 and 10 µM), TGF-β1 (10 ng/mL), FCS (10%), TGF-β1 in combination with CSD or FCS in combination with CSD. After a culture period of 28 days, cells were harvested for RNA-isolation (and subsequent RT-qPCR), GAG and DNA measurements and histology.

2.3.3. Outcome parameters

Cell proliferation and matrix production were used as the main outcome parameters to measure the regenerative effects of CSD. In order to visualize the matrix production, glycosaminoglycans (GAGs) were detected through a Safranin O/Fast Green staining. A Di-Methyl Methylene Blue (DMMB) assay was performed in the dose optimization study to quantify the pellets' GAG content and GAG release. DNA content was used as a measure for cell proliferation and RT-qPCR was performed to assess gene expression of relevant markers (**tables 3 and 4**).

2.3.3.1. DNA content

Two pellets per donor per condition were cultured for DNA analysis, enabling dual measurements. After 28 days of culture, the pellets were washed with Hanks Balanced Salt Solution (HBSS, Gibco) to remove any residual medium. Papain digestion solution was prepared (per mL solution: 1,57 mg cysteine HCL (Sigma, C7880) and 250 µg papain (Sigma, P3125)) and 200 µL was added to each pellet in order to release the DNA from the cell pellets. The samples were incubated overnight at 60 °C. They were vortexed the next day and incubated for an additional hour. DNA content was measured using the Qubit® dsDNA High Sensitivity Assay Kit (Invitrogen, Q32851) according to the manufacturer's instructions. For the measurement of each sample, 5 µL lysate was added to 195 µL Working Solution.

2.3.3.2. GAG content and GAG release

A DMMB assay was performed to quantify the GAGs in the collected culture medium and digested pellets that were initially used for DNA analysis. All digested samples were diluted 10 times with PBS-EDTA and standard line dilutions were prepared with Chondroitin Sulphate (Sigma, C4384). The standard line dilutions, the diluted lysed samples and the collected culture medium were pipetted into 96 wellsplates (Greiner Bio-One, 655191) and 200 µL DMMB solution (Sigma, 341088) was added to each well. Immediately after adding the DMMB solution, the extinction was measured at 540 and 595 using a microplate reader. The measurements of the standard dilution were used to generate a trendline with polynomic properties. The amounts of GAG in the samples were calculated according to the formula of this trendline.

2.3.3.3. RNA isolation

Per culture condition, 2 pellets of each donor were cultured for RNA-isolation, enabling dual measurements (**table 4**). Pellets were frozen on dry ice and crushed using pestles (Argos, 9551-901) in order to release the RNA. The freezing and crushing were repeated until the pellets were macroscopically lysed. RLT buffer (RNeasy® Micro kit (Qiagen, 74004)) was added to each sample after which the sample was vortexed. The released RNA was extracted using the RNeasy® Micro kit (Qiagen, 74004) according to the manufacturer's instructions. Finally, the obtained RNA was quantified using the Nanodrop®-1000 spectrophotometer (Thermo Scientific).

2.3.3.4. cDNA

cDNA was synthesized using the iScript™ cDNA Synthesis Kit (Bio-Rad, Veenendaal, the Netherlands, 170-8891), in accordance with the manufacturer's instructions. An RNA input of 50 ng was used for each sample. To this amount, RNAse free water was added to create a total volume of 15 µL. Subsequently, 4 µl 5x iScript reaction mix and 1 µl iScript reverse transcriptase was added to each sample to create a total reaction volume of 20 µL. Finally, samples were incubated to initiate the cDNA synthesis reactions.

2.3.5.5. RT-qPCR

The cDNA samples were 10 times diluted with MiliQ. From each sample, 4 µl was pooled to make standard dilutions (S1 – S7). The remaining cDNA was diluted again to create a total dilution of 50 times. Into each well of the 384 well plate, 5 µL iQ™ SYBR Green Supermix (Bio-rad, Veenendaal, the Netherlands), 0.04 µL forward primer, 0.04 µL reverse primer and 0.92 µL MiliQ were pipetted. Into this reaction mix, 4 µL of the diluted cDNA samples was added. The well plate was centrifuged at 1900 rpm for 2 minutes, after which it was placed into the MyiQ™ single color Real-Time PCR Detection System (Bio-Rad, Veenendaal, the Netherlands) to initiate the RT-qPCR-reactions. A set of 4 reference genes were used: *GAPDH*, *HPRT*, *RPS19* and *SDHA*. The following target genes were tested: *Aggrecan*, *Collagen II*, *CyclinD1* and *Collagen I* (**table 5**). A 2-step protocol was carried out for every gene, starting with a 2-minute incubation period at 95 °C in order to initiate denaturation and enzyme activation. This was followed by 40 cycles of 10 seconds incubation at 95 °C and 30 seconds incubation at 55 °C. Finally, temperature was increased again to 95 °C with 0.5 °C increments in order to generate a melt curve. Relative gene

expression was calculated using the Pfaffl method [48]. This method allow the calculation of the expression ratio between the sample and the calibrator using the following formula: ratio = $(E_{target})^{\Delta_{CT,\ target\ (calibrator\ -\ test)}}/(E_{ref})^{\Delta_{CT,\ ref\ (calibrator\ -\ test)}}$. E_{target} and E_{ref} are the amplification efficiencies of the target and reference genes respectively. ΔCT, target (calibrator – test) is the CT value of the target gene of the calibrator minus the CT value of the target gene in the test sample. Similarly, ΔCT, ref (calibrator – test) is the CT value of the reference gene of the calibrator minus the CT value of the reference gene in the test sample. The CT values of the control pellets were used as calibrators.

Primername	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'	
Aggrecan	5' GGACACTCCTTGCAATTTGAG 3'	61 – 62
	5' GTCATTCCACTCTCCCTTCTC 3'	
Collagen IIa	5' GCAGCAAGAGCAAGGAC 3'	60.5 – 65
	5' TTCTGAGAGCCCTCGGT 3'	
CyclinD1	5' GCCTCGAAGATGAAGGAGAC 3'	60
	5' CAGTTTGTTCACCAGGAGCA 3'	
Collagen I	5' GTGTGTACAGAACGGCCTCA 3'	61
	5' TCGCAAATCACGTCATCG 3'	

Table 5. Specific information of the primers used in the toxicity pilot and the dose optimization pilot.

2.3.3.6. Histology

To fix the pellets, 4% neutral buffered formaldehyde (Boom BV, Memmel), supplemented with eosin 1% (Merck 115935) was added to each pellet. The next day, the pellets were embedded in alginate and stored in casettes in 70% ethanol. After they were subjected to an alcohol program for dehydration, pellets were embedded into paraffin blocks. The blocks were cut into slides with a thickness of 5 µm using a microtome.

In order to prepare for the Safranin O/Fast Green staining, slides were deparaffinised by subjection to a series of xylene (2x), 96% ethanol, 80% ethanol, 70% ethanol and 60% ethanol for 5 minutes each. Subsequently, slides were placed in citrate-solution (pH=6) for 15 minutes to remove the alginate. Next, slides were rinsed in demi-water to remove any residual citrate solution. Slides were dipped into Mayers haematoxylin (J.T. Baker, 3870) (diluted 1:1 with MiliQ) and rinsed in demi-water for a second time to remove all residual haematoxylin. Slides were in turn plunged in filtered 0.4% aqueous Fast Green (Sigma, F7252) for 4 minutes, 1% acetic acid for 3 minutes and in new clean 1% acetic acid for 2 minutes. Next, slides were stained in

0.125% aqueous Safranin O (Sigma, 58884) for 5 minutes. To finish the staining process, the slides were subjected to a series of 96% ethanol (2x), 100% ethanol and xylene (2x) for 3 minutes each. To prevent the pellets from detaching from the slides, they were covered with coverglasses (VWR International, 631-0146) using a droplet of Vectamount (Vector Laboratories, H5000). The covered slides were left to dry for 2 days. Images were acquired using an Olympus BX60 microscope with colorview III digital camera and cellimaging software (Olympus, Zoeterwoude, the Netherlands).

2.3.3.7. Statistics

As these were only pilot experiments, the relatively small datasets lacked power to perform proper statistics. Moreover, R Studio version 3.0.2 was unable to converge statistical models that fit these small datasets.

2.4. Results & Discussion

This section describes the results of the two pilot studies that were performed: a toxicity study and a dose optimization study. The aim of these pilot studies was to determine the optimal concentration and culture conditions for AC/NPC pellets and monolayers treated with CSD.

2.4.1. Toxicity study

The toxicity study was performed to estimate the concentration in which CSD should be supplemented to AC pellets and monolayers. AC pellets and monolayers (200,000 cells/well) were cultured with different CSD concentrations ranging from 1 to 10 µM. The potential regenerative effects of the different CSD concentrations were determined using two outcome parameters, including cell proliferation and extracellular matrix (GAG) production. Cell proliferation was quantified by measuring the DNA content of the AC pellets and monolayers. Matrix production was visualized by detecting glycosaminoglycans (GAGs) through a Safranin O/Fast Green staining. Additionally, RT-qPCR was performed to assess gene expression of relevant markers. The results of the RT-qPCR were used to investigate the signalling pathways underlying the effects on DNA content and the GAG staining.

2.4.1.1. Cell proliferation

The DNA content of the AC control pellets decreased over time indicating that cells had died during the pellet culture (**figure 1**).
TGF-β1 treatment did not exert a convincing proliferative effect. Compared with the AC control pellets, TGF-β1 caused a minute increase in DNA content (figure 1). A similar minor effect on cell proliferation was reported in AF cells that were treated for 5 days with TGF-β1 [26].

CSD exerted restraining effects regarding the DNA content since the CSD treated pellets showed a considerable lower DNA content compared with controls (**figure 1**). No convincing dose-dependent effect of CSD treatment of AC pellets was observed.

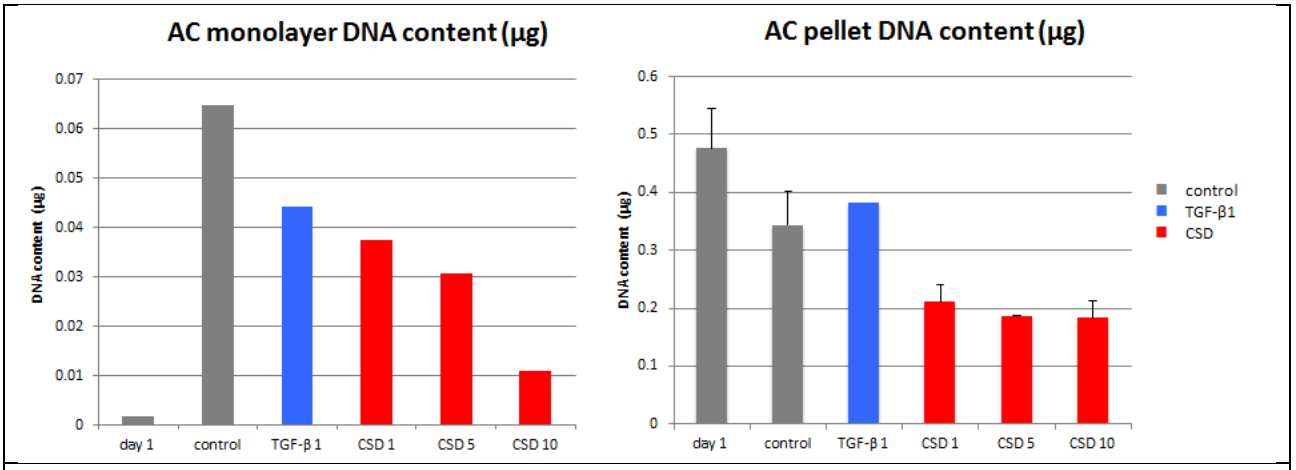


Figure 1. The DNA content of the AC pellets (left) and AC monolayers (right) receiving no treatment (grey), TGF-β1 (blue) or caveolin-1 scaffolding domain peptide (CSD) (red) for 7 days. All cultures received basal chondrogenic medium. TGF-β1 did not have the expected stimulating effects on DNA: it slightly increased cell proliferation of the AC pellets and negatively affected the cells cultured in monolayers compared to controls. In both culture systems, CSD exerted a negative effect on the cell proliferation.

As opposed to the effects in AC control pellets, the DNA content of the AC control monolayers increased over time (**figure 1**). Contrary to expectation, TGF-β1 treatment decreased the DNA content of the AC monolayers. Similar to the AC pellets, CSD treated monolayers had a lower DNA content compared with the controls. Moreover, a dose-dependent effect was demonstrated, in which treatment with a higher CSD concentration resulted into a lower DNA content. This inhibiting effect of CSD could be explained by its ability to induce premature cell senescence in IVD cells [10]. Furthermore, the CSD peptides were dissolved in DMSO prior to supplementation, which could have maltreated the cells. The application of higher CSD concentrations coincides with higher concentrations of DMSO (1 µM CSD in 0.2% DMSO an 10 µM CSD in 2% DMSO, respectively). These higher concentrations of DMSO could have established cytotoxic effects upon the cells in culture [49].

Also, RT-qPCR results demonstrated that gene expression of the cell proliferation marker *CyclinD1* corresponded to the DNA content results: TGF-β1 only caused a slight upregulation of *CyclinD1* expression in the AC pellets while it downregulated *CyclinD1* expression in the AC monolayers (**figure 2**). Treatment with CSD exerted a dose-dependent negative effect on *CyclinD1* expression. In the AC pellets, the effect of 1 µM CSD was comparable with the effects of control and TGF-β1 treatment, yet in its highest concentration (10 µM) CSD exerted an anti-proliferative effect. In AC monolayers, all CSD concentrations downregulated *CyclinD1* expression, but in the presence of higher CSD concentrations (**figure 2**) CSD exerted a more intense effect. Thus, CSD treatment dose-dependently decreased cell proliferation of ACs.

2.4.1.2. Extracellular matrix production

Gene expression levels of matrix proteins *Aggrecan* (the main proteoglycan in the healthy nucleus pulposus) and *Collagen II* were additionally measured to monitor extracellular matrix production. The TGF-β1 treated AC pellets upregulated the expression of both *Aggrecan* and *Collagen II* (**figure 2**). However, the TGF-β1 treated AC monolayers upregulated *Collagen II* only.

Again, CSD exerted a dose-dependent effect on the expression of the matrix proteins. The effects of 1 µM CSD were comparable with controls, and higher CSD concentrations (5 µM and 10 µM) exerted negative effects on the expression of these matrix proteins (**figure 2**).

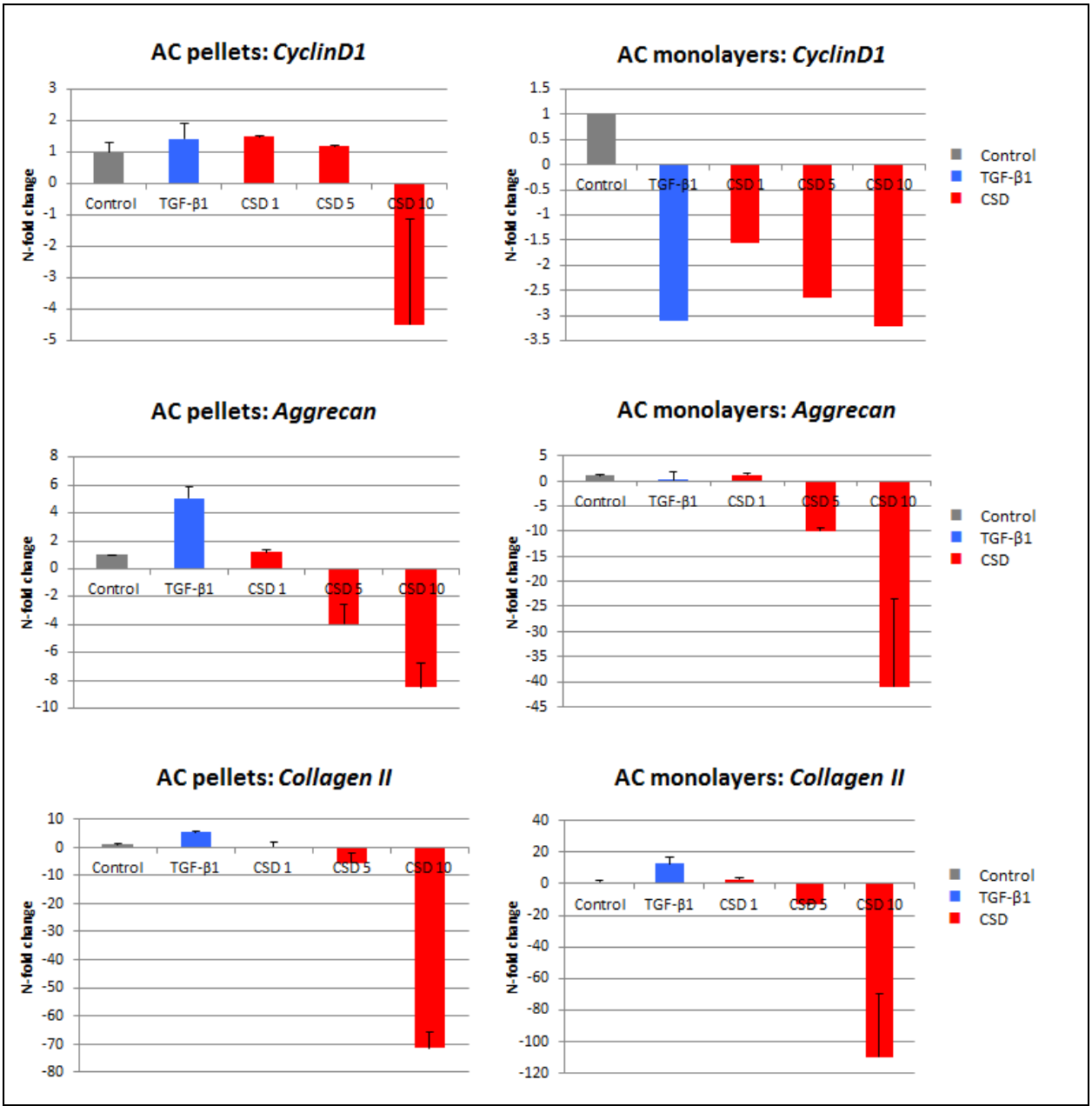


Figure 2. Relative gene expression of cell proliferation marker *CyclinD1* and matrix proteins *Aggrecan* and *Collagen II*. The left graphs show the results of the AC pellets. The right graphs show the results of the AC monolayers. Pellets and monolayers were cultured for 7 days in basal medium and treated with TGF-β1 (blue), caveolin-1 scaffolding domain peptide (CSD) (red) or were left untreated (grey). Gene expression analysis of *CyclinD1* confirms the results of the DNA content: TGF-β1 did not induce the desired up-regulation of *CyclinD1* and CSD downregulated this marker with a dose-dependent effect. Gene expression analysis of *Aggrecan* and *Collagen II* shows that TGF-β1 increased the expression of these matrix proteins while caveolin-1 decreased the expression of these matrix proteins dose-dependently.

Consistent with the upregulated gene expression of *Aggrecan*, all TGF-β1 treated pellets obtained a saturated positive (red) staining, indicating the presence of many GAGs (**figure 3**). In addition, the dose-dependent inhibiting effect of CSD encountered in the gene expression levels of *Aggrecan,* was also observed in the staining. The control and the 1 µM caveolin-1 treated pellets presented equal red histological pictures, in which the red staining drifted off in the pellets treated with higher CSD concentrations (**figure 3**). Thus, CSD treatment decreased extracellular matrix production in ACs dose-dependently as demonstrated by gene expression profiles of *Aggrecan* and *Collagen II* and by the performed Safranin O/Fast Green staining of the AC pellets.

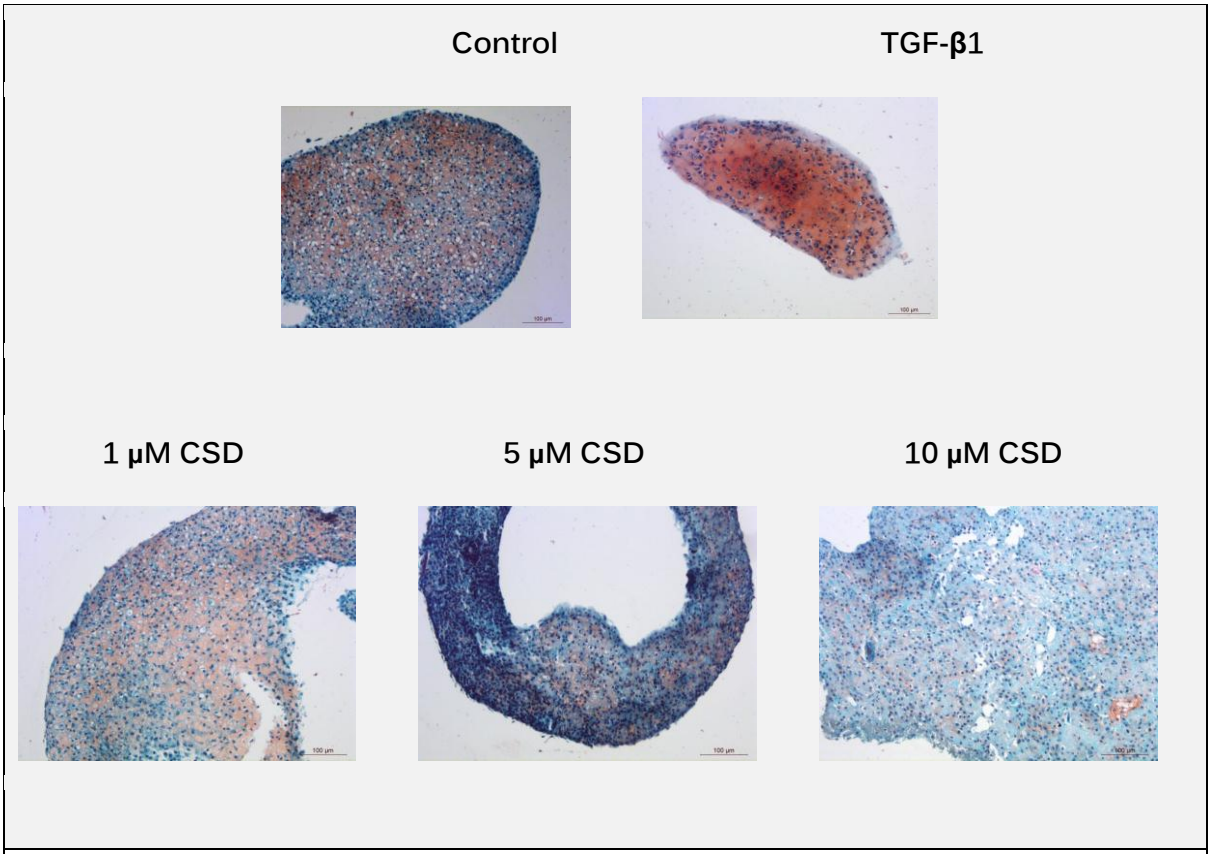


Figure 3. Microscopic images of the AC pellets of the toxicity pilot. Pellets were stained with Safranin O/Fast Green. A red color indicates the presence of GAGs. TGF-β1 treated pellets has more red staining compared to control. A dose-effect of caveolin-1 scaffolding domain (CSD) is noticeable: the higher the CSD concentration, the lower the amount of redness.

2.4.1.3. **Summary results toxicity study**

When summarizing the results of the toxicity study, TGF-β1, used as a positive control, only showed regenerative effects on matrix production as was shown by the upregulated gene expression of *Aggrecan* and *Collagen II*, and positive Safranin O/Fast Green staining. However, TGF-β1 failed to stimulate cell proliferation. A possible explanation for this could be the relative short culture period of 7 days. This explanation is strengthened by the results of other studies that used TGF-β1 to treat NPCs: a proliferative effect was not visible untill 14 days of treatment [28]. Moreover, the regenerative effects on matrix production in the current pilot study were only present in the AC pellets and not in the monolayers. For this reason, and because of the fact

that NPCs have a tendency to dedifferentiate in monolayer culture, NPCs will be cultured in pellet form instead of monolayers in future experiments within this project [25].

The lowest CSD concentration (1 µM) failed to show an effect on cellproliferation and extracellular matrix production as gene expression levels of *CyclinD1*, *Aggrecan, Collagen II* and Safranin O staining intensity was comparable with controls. However, a decreased extracellular matrix (GAG) production and DNA content was shown when cells were treated with high concentrations of CSD (5 and 10 µM). Yet, this can be explained by cytotoxic effects of the high DMSO concentrations in which the CSD peptide was dissolved (1% and 2%, respectively). This toxicity study should therefore be repeated with a higher concentration of the stock solution of CSD peptide in DMSO, thereby resulting into lower final concentrations of DMSO in the culture media. Furthermore, for future studies within the scope of this project, CSD peptides will be dissolved in lower DMSO concentrations (1 µM CSD in 0.02% DMSO) to avoid potential cytotoxic side effects. Another explanation for the decreased GAG and DNA content, observed in cells treated with 5 and 10 µM CSD, is that caveolin-1 and sole CSD treatment have negative or degenerative effects rather than regenerative effects on ACs/NPCs at high concentrations (assuming that CSD indeed mimicked caveolin-1 function). In this case, concentrations below or around 1 µM should be investigated. However, we cannot exclude that CSD functioned as a caveolin-1 antagonist instead of an agonist (by interfering with caveolin-1 dependent signalling), which would indicate that caveolin-1 is needed for AC/NPC cell proliferation and the production of extracellular matrix.

2.4.2. Dose optimization study

Assuming that CSD mimicked the effects of caveolin-1 and taking the abovementioned negative effects of CSD in higher concentrations (5 and 10 µM) into consideration, the toxicity study was continued with a dose optimization study using CSD concentrations around 1 µM, employing both ACs and NPCs. In this pilot study ACs and NPCs were only cultured in pellet form, since TGF-β1 previously demonstrated more pronounced regenerative effects upon cells in pellet culture rather than in monolayer culture. Besides this, NPCs are preferably cultured in pellets or alginate since they have the tendency to dedifferentiate in monolayer culture [25]. Similar to the toxicity study, cell proliferation and matrix production were used as outcome parameters and were determined by measuring the DNA content, gene expression of relevant markers and by performing a Safranin O/Fast Green staining. In this pilot, a DMMB assay was additionally performed to quantify matrix (GAG) production.

2.4.2.1. Cell proliferation

Whereas TGF-β1 in the toxicity study did not elicit a proliferative effect on ACs, this pilot demonstrated that TGF-β1 did have a stimulating effect on the cell proliferation of NPC pellets after 28 days of culture (**figure 4**). The proliferative effect of TGF-β1 was also seen in a study by Tilwani *et al.* (2012)[28] in which NPCs were treated with TGF-β1 for 14 days. An explanation for the absence of a proliferative effect of TGF-β1 in the toxicity study could be the short culture period as mentioned before. A treatment period of 7 days could be insufficient for TGF-β1 to exert a proliferative effect on ACs. It could also be that ACs are less responsive to TGF-β1 treatment than NPCs.

The DNA content of the CSD treated NPC pellets was comparable with the DNA content of the control pellets irrespective of the CSD concentration (0.5 µM, 1 µM or 2 µM). The addition of CSD (all concentrations) to TGF-β1 showed a slight additive effect in donor NPC 156. A concentration of 1 µM seems to be the optimal concentration, since it caused the highest additional increase in DNA content (+27%), compared with +24% (0.5 µM) and +6% (2 µM) (**figure 4**). There was no additive effect of CSD present in the NPC pellets of donor NPC 662.

Fetal calf serum (FCS) did not have an effect on DNA content compared with controls and the addition of CSD did not result in an additive effect on sole FCS treatment.

Data of the AC pellets showed a similar trend across all culture conditions and hence data are not shown.

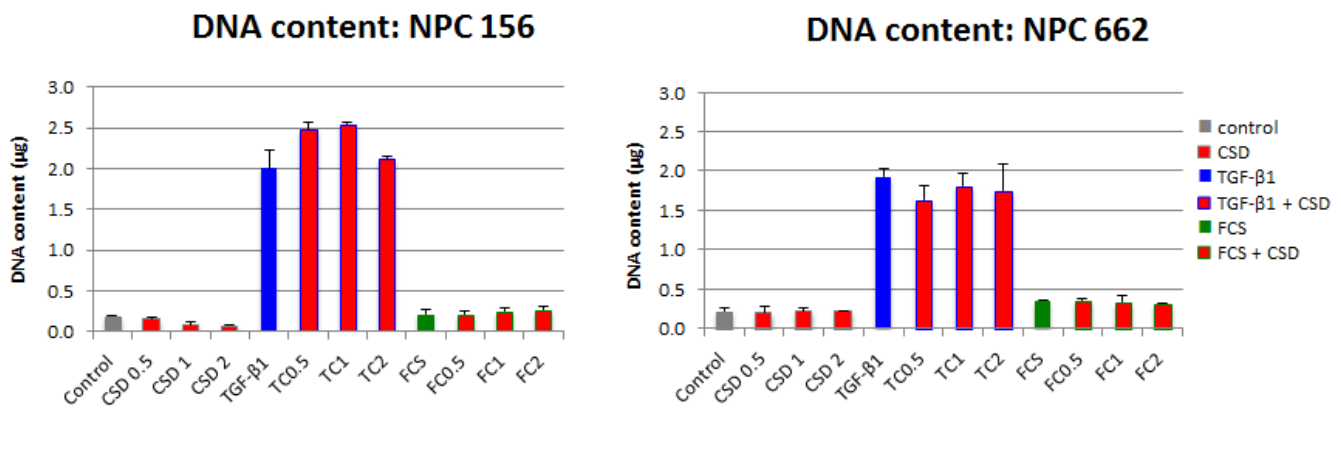


Figure 4. DNA content of the pellets from donors NPC 156 (left) and NPC 662 (right). Pellets were cultured for 28 days in basal medium and treated with TGF-β1 (solid blue), caveolin-1 scaffolding domain peptide (CSD) (solid red), TGF-β1 in combination with CSD (red/blue), FCS (solid green), FCS in combination with CSD (red/green) or left untreated (grey). TGF-β1 had a strong stimulating effect on DNA synthesis in both donors. In donor NPC 156, CSD slightly enhanced TGF-β1's effect. FCS treatment (with and without CSD) and sole CSD treatment did not affect DNA content.

2.4.2.2. Extracellular matrix production

Beside its proliferative effect on NPC and AC pellets, TGF-β1 had a stimulating effect on the GAG production as it increased GAG content and GAG release with at least 2.3-fold and 3-fold compared with control pellets, respectively (**figure 5,** data AC pellets not shown). This stimulating effect of TGF-β1 on the GAG production is confirmed by other studies that treated NPCs with TGF-β1 [28,27].

CSD treatment (0.5 µM, 1 µM and 2 µM) did not have a positive effect on the GAG production. In fact, it decreased the GAG content and GAG release of the NPC pellets compared with controls. On the other hand, the combination of CSD and TGF-β1 was able to increase the GAG content of the NPC pellets of the two NPC donors (**figure 5**). Similar to its additive effect on DNA synthesis, 1 µM CSD caused the highest additional increase of GAGs in the NPC pellets (+35%,

compared with -26% (0.5 µM CSD) and +21% (2 µM CSD)). Moreover, the combination of TGF-β1 and CSD led to a decreased GAG release into the culture medium. This suggests that CSD was responsible for capturing the newly produced GAGs in the pellets, which could be a favourable effect since a healthy nucleus pulposus requires an internal environment rich in GAGs. The capturing of GAGs prevents release and subsequent breakdown and loss of these essential proteins. This effect of CSD can be explained by its important role in modulating cell interactions to extracellular matrix [50]. Unfortunately, the additive effect of CSD on the GAG production was not convincingly present in the AC pellets (data not shown). This could have been caused by differences in cell type. Unlike the NPCs, ACs exert a higher expression of *Collagen II* compared with proteoglycans, whereas NPCs produce matrix that is higher in proteoglycans compared with collagen II [51]. Due to this biochemical difference, the TGF-β1 and CSD treatment could have led to a higher increase in GAG content in the NPC pellets than in the AC pellets.

FCS decreased the GAG content of the NPC pellets (**figure 5**). This is in contrast to what was found by Thompson *et al.* (1991)[27], who cultured nucleus pulposus tissues with 20% FCS: a 1.5 fold increase in GAG production was found compared with 0% FCS after 4 days of treatment. The discrepant effect of FCS could be explained by the differences in batch or supplier of FCS, since our research group experienced different effects with different batches of FCS.

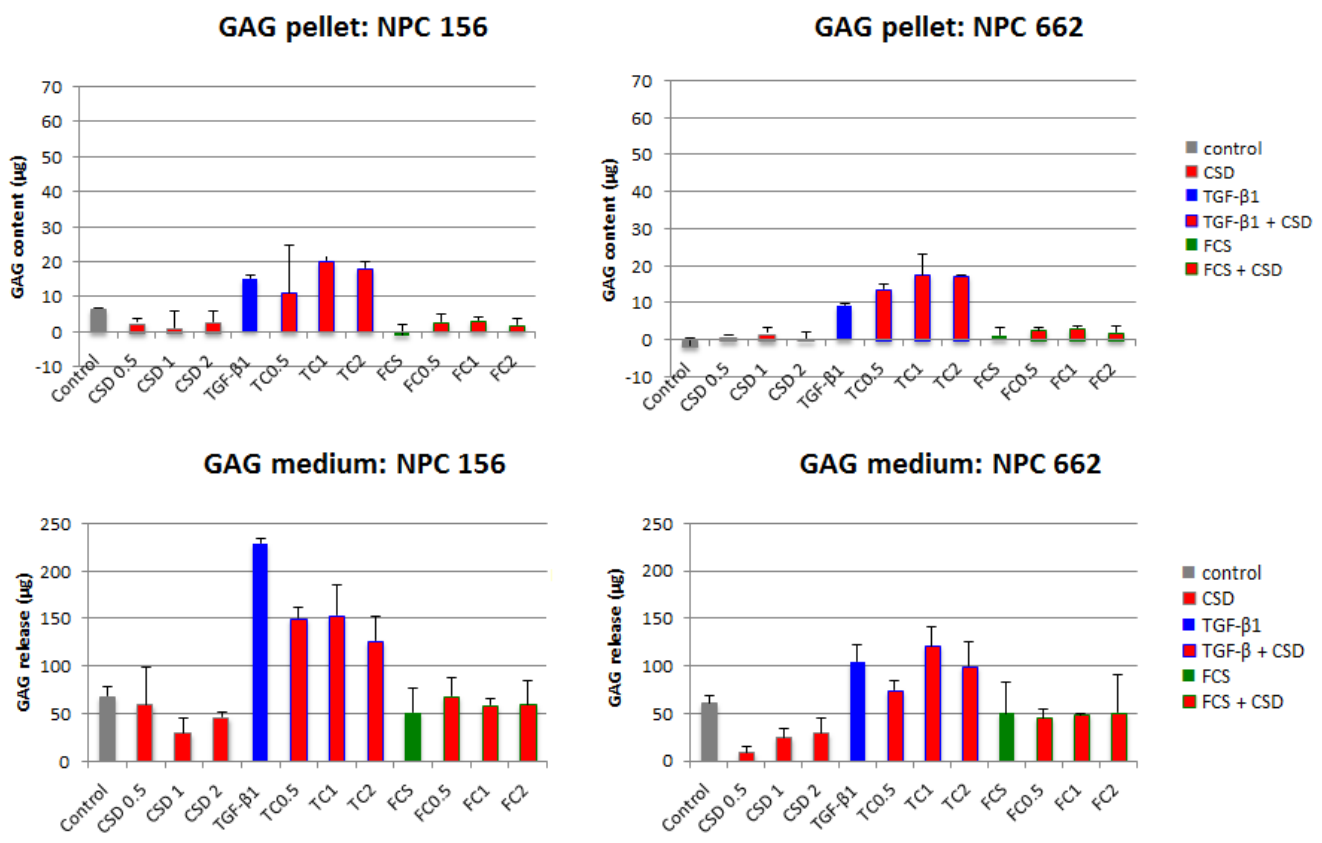


Figure 5. Results of the DMMB assay of the 2 NPC donors: NPC 156 (left) and NPC 662 (right). The top graphs show the GAG content of the NPC pellets, the bottom graphs show the GAG release of the NPC pellets. Pellets were cultured for 28 days in basal medium and treated with TGF-β1 (solid blue), caveolin-1 scaffolding domain peptide (CSD) (solid red), TGF-β1 in combination with CSD (red/blue), FCS (solid green), FCS in combination with CSD (red/green) or left untreated (grey). TGF-β1 had a strong stimulating effect on the GAG production which was visible in the pellets' GAG content and GAG release. Although CSD alone did not have a positive effect on the GAG production, it had an additive effect on the GAG content.

In line with the results of the DMMB assay, all control and sole CSD treated pellets stained negative (green) and all TGF-β1 treated pellets stained positive (red) for the presence of GAGs (**figure 6**). Despite the fact that the DMMB assay showed an additive effect of CSD (0.5 µM, 1 µM and 2 µM) on the TGF-β1 treated pellets, there was no additive effect of CSD noticeable in this staining. This could be explained by the fact that the Safranin O/Fast Green staining was not sensitive enough to detect an additive effect. Another explanation could be that TGF-β1 treatment already caused red staining to such an extent, that the detection of any additive effect of CDS had become impossible.

The FCS treated NPC pellets showed a dedifferentiated appearance, indicating that FCS does not provide a good environment for the chondrogenic differentiation of AC and NPC pellets (**figure 6**).

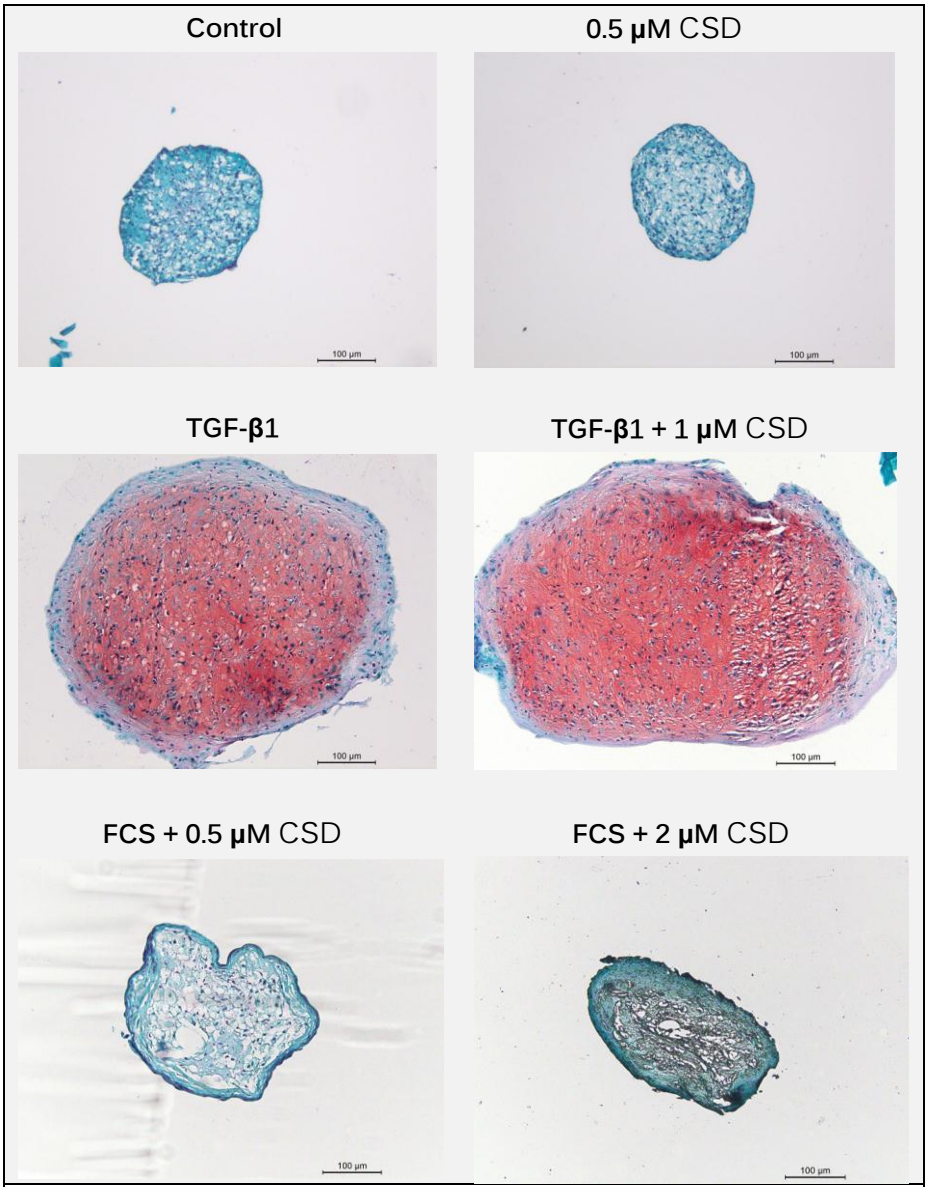


Figure 6. Microscopic images of NPC 156 pellets stained with Safranin O/Fast Green. Control and caveolin-1 scaffolding domain peptide (CSD) treated pellets were negative for this GAG staining. TGF-β1 treated pellets not only stained positive for the Safranin O, but also appeared larger compared with the control pellets. FCS treated pellets were negative for the staining and appeared dedifferentiated.

2.4.2.3. RT-qPCR

Several limitations were experienced in obtaining the RT-qPCR results. The NPC and AC pellets that were collected after 28 days of culture appeared smaller in size compared with those collected after 7 days, indicating that cells had died over time. This resulted in lower cell quantities. The small size of the pellets and low amounts of cells resulted into low RNA yields. The desired RNA input of 50 ng, for initiating cDNA reactions, could not be reached repeatedly. As a consequence, many samples failed to reach the required RT-qPCR-threshold and therefore a lot of samples could not be detected. Furthermore, RT-qPCR data were not in agreement with the DNA and GAG measurements. For instance, there was no upregulation of

cell proliferation marker *CyclinD1* noticeable, TGF-β1 caused a downregulation of this marker (**figure 7**) while TGF-β1 clearly stimulated DNA synthesis (**figure 4**). The same discrepancy applies to the gene expression of *Aggrecan* (**figure 7**). Although both DMMB assay and histology showed that the TGF-β1 treated pellets produced many GAGs, there were no clear signs of upregulated gene expression of *Aggrecan*. This discrepancy could be explained by the possibility that at the time of harvesting RNA samples (at day 28), increased gene expression of markers for cell proliferation and matrix production had already taken place, meaning that cells were at a resting phase. This explanation becomes more valid, since a recent study performed by Tilwani *et al.* (2012)[29] demonstrated an upregulation of *Aggrecan* expression already after 48 hours of TGF-β1 treatment.

Despite the above mentioned limitations, important conclusions concerning FCS treatment may still be drawn from the qPCR data. Besides a downregulation of *Aggrecan* expression, FCS treatment caused a prominent upregulation (ranging from 13 to 540 fold) of dedifferentiation marker *Collagen I* (**figure 7**). The upregulation of this dedifferentiation marker confirms the dedifferentiated appearance of the NPC pellets that were stained with Safranin O/Fast Green (**figure 6**). Thus, as demonstrated by the Safranin O/Fast Green staining and gene expression profile of *Collagen I*, FCS does not provide a good environment for the chondrogenic differentiation of AC and NPC pellets. Hence, FCS will not be used for further culture experiments within this project.

2.4.2.4. Summary results dose optimization study

TGF-β1, used as positive control, stimulated cell proliferation and matrixproduction as it increased DNA content, GAG content and GAG release of the NPC and AC pellets compared with control pellets. Furthermore, TGF-β1 treated AC and NPC pellets showed a positive Safranin O staining whereas control pellets did not.
In contrast, sole CSD treatment (0.5 µM, 1 µM and 2 µM) did not demonstrate any of these regenerative effects. However, supplementing CSD (0.5 µM, 1 µM and 2 µM) to sole TGF-β1 treatment resulted in an additive effect concerning the GAG content of the NPC pellets.
FCS treatment did not stimulate cell proliferation and matrix production in the NPC and AC pellets, yet caused dedifferentiation of these pellets. This is demonstrated by the gene expression profile of dedifferentiation marker *Collagen I* and the Safranin O/Fast Green staining (**figures 6 and 7**).

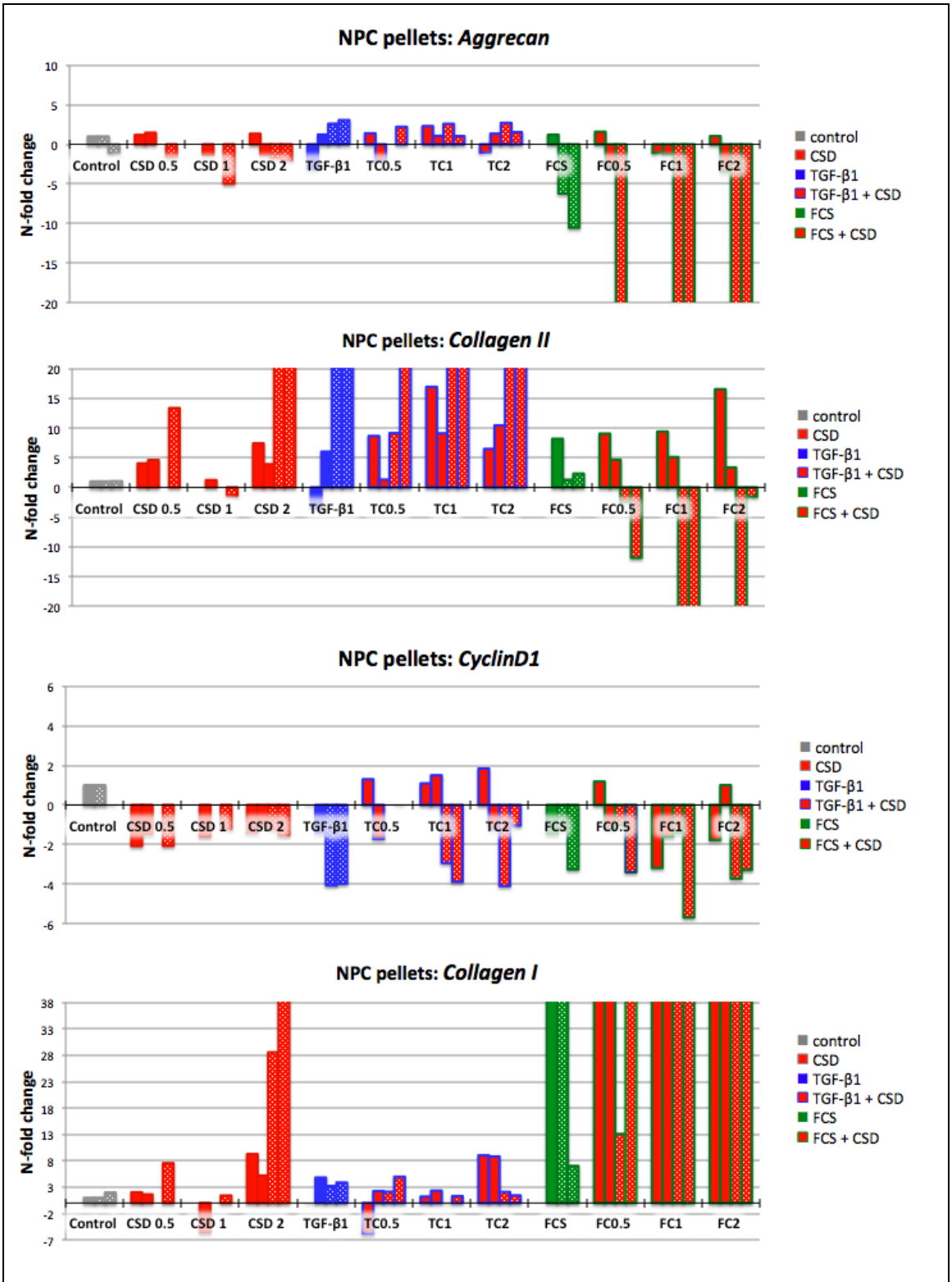


Figure 7. Relative gene expression of *Aggrecan, Collagen II, CyclinD1* and *Collagen I* of the NPC pellets that were treated with TGF-β1 (solid blue), caveolin-1 scaffolding domain peptide (CSD) (solid red), TGF-β1 in combination with CSD (red/blue), FCS (solid green), FCS in combination with CSD (red/green) or left untreated (grey). The solid bars represent the measurements of donor NPC 156, whereas the dotted bars represent the measurements of donor NPC 662.

2.5. Conclusions

Based on the results of the DNA measurements, DMMB assay (in the dose optimization study) and the Safranin O/Fast Green stainings, TGF-β1 treatment demonstrated to be a good positive control for ACs and NPCs, stimulating both cell proliferation and matrix production. Its effects were more pronounced after a 28-day culture compared with a 7-day culture. However, with respect to the gene expression profiles, it is desirable to determine gene expression levels at day 7 rather than at day 28, since changes in gene expression already take place before protein/GAG levels are affected.

Sole CSD treatment did not have a positive effect on cell proliferation nor on matrix production in NPCs and ACs. In fact, high CSD concentrations (5 µM and 10 µM) had negative effects on these parameters. The negative effects of high CSD concentrations could be explained by
a) the high DMSO concentrations in which CSD was dissolved (1 and 2% for 5 and 10 µM CSD, respectively), or
b) the possibility that caveolin-1 has no regenerative effect at these high concentrations (assuming that CSD indeed mimicked caveolin-1 function), or
c) the possibility that CSD functioned as a caveolin-1 antagonist instead of an agonist, which would indicate that caveolin-1 is needed for NPC/AC cell proliferation and matrix (GAG) production.

The supplementation of CSD (0.5 µM, 1 µM, 2 µM) resulted in an additive effect on TGF-β1 treatment concerning GAG content of the NPC pellets of the two donors tested in the dose optimization pilot. Moreover, under influence of CSD, fewer GAGs were released into the medium and more GAGs were kept in the NPC pellets. The highest additive effect of CSD was reached with a concentration of 1 µM, indicating that the optimal *in vitro* CSD concentration should be around 1 µM. Lastly, FCS caused dedifferentiation of the NPC and AC pellets and it will therefore no longer be used in further NPC and AC pellet cultures within this project.

3. Additive effect of caveolin-1 scaffolding domain on TGF-β1 treatment of nucleus puposus cells of chondrodystrophic dogs

3.1. Abstract

Introduction. Preliminary results (chapter 2) indicate that caveolin-1 scaffolding domain (CSD) is capable of inducing anabolic, regenerative effects upon TGF-β1 treatment of pelleted canine nucleus pulposus cells (NPCs). Given its role in TGF-β signalling, CSD is hypothesized to preferentially block ALK1 signalling compared with ALK5 signalling. Therefore, CSD's effect on TGF-β signalling was investigated *in vitro* using chondrodystrophic canine NPCs.

Material and methods. Pellets containing pooled early degenerated NPCs from different canine donors were cultured for 0, 1, 7 and 28 days in basal chondrogenic medium. Pellets were treated with CSD (1 μM, 0.1 μM and 0.01 μM), TGF-β1 (10 ng/mL and 2 ng/mL) or TGF-β1 in combination with CSD. Although the performed dose optimization pilot suggested an optimal CSD concentration of around 1 μM, it is possible that the optimal concentration is actually lower. Hence, lower CSD concentrations (0.1 μM and 0.01 μM) were included in the present study. A suboptimal concentration of TGF-β1 (2 ng/mL) was included as additional effects of CSD might be better detectable upon suboptimal effects of TGF-β1. In order to quantify the matrix production, the glycosaminoglycan (GAG) content and the GAG release of the pellets were determined using a DMMB assay. In addition, pellets were stained with Safranin O/Fast Green to visualize the production of GAGs in the pellets. Cell proliferation was quantified by measuring the DNA content of the pellets and RT-qPCR was performed to determine the gene expression level of relevant genes.

Results. TGF-β1 treatment stimulated matrix production and cell proliferation of NPC pellets. Despite the fact that 2 ng/mL TGF-β1 caused a desired suboptimal effect, CSD was unable to establish additional regenerative effects upon TGF-β1 treatment. However, one individual donor group demonstrated a slight additive effect of CSD upon TGF-β1 induced GAG production. Yet this donor-dependent additive effect was not statistically significant.

Conclusion. CSD did not elicit additional regenerative effects upon TGF-β1 treatment and as such the scaffolding domain of caveolin-1 is not a suitable target for developing regenerative treatments in the present study set up.

3.2. Introduction

In the *in vivo* degenerating IVD, TGF-β1 mainly signals through the ALK1 receptor [23]. Activation of the ALK1 receptor and the subsequent Smad1/5/8 signalling results in harmful, catabolic effects and the progression of IVD degeneration [23]. Consistent with its role in regulating TGF-β signalling, the previous pilot studies showed that caveolin-1 scaffolding domain peptide (CSD) is capable of inducing anabolic effects upon TGF-β1 treatment of pelleted canine nucleus

pulposus cells (NPCs). Possibly, CSD caused a preferential blockade of ALK1 signalling in proportion to the anabolic ALK5 signalling. The aim of this study was to explore the potential additive effect of CSD on TGF-β1 treatment of NPCs originating from young chondrodystrophic dogs (1-2 years) specifically. Longitudinal studies that investigate the process of IVD degeneration, like preclinical studies of interventional treatments aiming to prevent, stop or slow the course of degeneration, are best performed with chondrodystrohpic breeds, which develop IVD degeneration relatively early in life [6]. The young dogs used in this study showed early stages of IVD degeneration, with IVDs containing nucleus pulposus cell-rich nucleus pulposi. This allowed determination of CSD's effects on early IVD degeneration.

3.3. Material and methods

Cells of 12 canine donors were used in the present study. Since the donors did not provide a sufficient amount of cells to use them separately for pellet generation, cells of the different donors were pooled. Subsequently, five donor groups (A – E) were formed (**table 1**).

Donor group	Donor name	% Cells	Age (months)	Mean age (months)	Breed	Gender
A	NPC 509	75	19	20	Beagle	Female
	NPC 151	25	23		Beagle	Male
B	NPC 510	70	19	19.3	Beagle	Female
	NPC 505	30	20		Beagle	Female
C	NPC 499	70	20	20	Beagle	Female
	NPC 505	30	20		Beagle	Female
D	NPC 500	25	20	22.5	Beagle	Female
	NPC 153	25	28		Beagle	Male
	NPC 496	50	21		Beagle	Female
E	NPC 679	20	32	24.4	Beagle	Male
	NPC 150	55	23		Beagle	Male
	NPC 155	10	22		Beagle	Male
	NPC 498	15	21		Beagle	Female

Table 1. Characteristics of the donor groups (A-E).

3.3.1. Cell culture

Early degenerated NPCs were collected and isolated as described in chapter 2. Cells were grown in expansion medium (**chapter 2, table 2**) in 175 cm^2 cell culture flasks (Cellstar, Greiner bio-one) until passage 2 to acquire an adequate amount of cells. Next, cells were counted and pipetted in a 96 wells plate (Corning Costar 7007) with a density of 200.000 cells/well. Cells were pelleted by centrifugation at 185 g for 8 minutes and cultured for 0, 1, 7 or 28 days in several culture conditions. Two pellets were immediately stored for GAG and DNA measurements. The other pellets were cultured in plain chondrogenic medium. Control pellets were not treated with TGF-β1 and/or CSD. The other pellets were treated with either recombinant human TGF-β1 (10 ng/mL) (R&D Systems, 240-B-010) (10ng/mL) or TGF-β1 in combination with CSD (Enzo Life Sciences, ALX-153-064) (0.01, 0.1 and 1 μM) (**table 2**).

Although the pilot experiments showed an optimal concentration of CSD of around 1 μM, it is still possible that the actual optimal dose sets below this value. It is not possible to make statements upon this, since concentrations below 0.5 μM were not tested in the previous pilot experiments. Therefore concentrations of 0.1 μM and 0.01 μM were included in this follow up experiment. To increase the capacity of detecting a potential additive effect of CSD, a suboptimal dose of TGF-β1 (2 ng/mL) was included (**table 2**). The culture medium was refreshed two times a week. The culture medium was collected to determine the weekly glycosaminoglycan (GAG) release.

Culture condition	Time in culture	Number of pellets per donor group		
		PCR	GAG/DNA	Histology
Control	day 0	-	2	-
	day 1	-	2	-
	day 7	2	2	-
	day 28	2	2	2
TGF-β1 (10ng/mL)	day 1	-	2	-
	day 7	2	2	-
	day 28	2	2	2
TGF-β1 (10 ng/mL) + CSD (0.01 µM)	day 1	-	2	-
	day 7	2	2	-
	day 28	2	2	2
TGF-β1 (10 ng/mL) + CSD (0.1 µM)	day 1	-	2	-
	day 7	2	2	-
	day 28	2	2	2
TGF-β1 (10 ng/mL) + CSD (1 µM)	day 1	-	2	-
	day 7	2	2	-
	day 28	2	2	2
TGF-β1 (2ng/ml)	day 1	-	2	-
	day 7	2	2	-
	day 28	2	2	2
TGF-β1 (2ng/ml) + CSD (0.01 µM)	day 1	-	2	-
	day 7	2	2	-
	day 28	2	2	2
TGF-β1 (2ng/ml) + CSD (0.1 µM)	day 1	-	2	-
	day 7	2	2	-
	day 28	2	2	2
TGF-β1 (2ng/ml) + CSD (1 µM)	day 1	-	2	-
	day 7	2	2	-
	day 28	2	2	2

Table 2. Schematic overview of the study design.

3.3.2. Outcome parameters

Cell proliferation and extracellular matrix production were used as the main outcome parameters to measure the regenerative effects of CSD. The weekly GAG release and GAG content of the pellets at day 0, 1, 7 and 28 were measured with a Di-Methyl Methylene Blue (DMMB) assay to quantify matrix production. In addition, pellets at day 28 were subjected to a Safranin O/Fast Green staining. The pellets' DNA content was measured at day 0, 1, 7 and 28 of the culture period to quantify cell proliferation. Furthermore, pellets were collected at day 7 and

day 28 and pooled separately for RNA isolation to assess gene expression of relevant markers (**table 2**).

3.3.2.1. DNA content

As previously described (chapter 2, p.19), pellets were lysed using papain digestion solution (per mL solution: 1,57 mg cysteine HCL (Sigma, C7880) and 250 µg papain (Sigma, P3125)) and DNA content was measured using the Qubit® dsDNA High Sensitivity Assay Kit (Invitrogen, Q32851) according to the manufacturer's instructions.

3.3.2.2. GAG content and GAG release

A DMMB assay was performed on the collected culture medium and the digested pellets that were initially used for DNA analysis (as described previously in chapter 2, p.20).

3.3.2.3. RNA isolation

Per condition, 2 pellets of each donor group were cultured for RNA isolation. As previously described (chapter 2, p.20), pellets were crushed on dry ice in order to release the RNA. The lysed samples of the same donor group within every culture condition were pooled so that single measurements were performed. The released RNA was extracted using the RNeasy® Micro kit (Qiagen, 74004) according to the manufacturer's instructions. The obtained RNA was quantified using the Nanodrop®-1000 spectrophotometer (Thermo Scientific).

3.3.2.4. cDNA

As previously described (chapter 2, p.20), cDNA was synthesized using the iScript™ cDNA Synthesis Kit (Bio-Rad, Veenendaal, the Netherlands, 170-8891), in accordance with the manufacturer's instructions. Due to higher yields of RNA, in the present study an RNA input of 100 ng could be used for each sample.

3.3.2.5. RT-qPCR

As previously described (chapter 2, p.20), RT-qPCR was performed using the iQ™ SYBR Green Supermix Kit (Bio-rad, Veenendaal, the Netherlands) and MyiQ™ single color Real-Time PCR Detection System (Bio-Rad, Veenendaal, the Netherlands). Relative gene expression was calculated using the Pfaffl method as described in chapter 2. A set of 4 reference genes were used: *GAPDH*, *HPRT*, *RPS19* and *SDHA*. The following target genes were tested: *Aggrecan*, *Versican*, *Collagen II*, *ADAMTS5*, *TIMP-1*, *MMP13*, *CyclinD1*, *Collagen I*, *Axin2*, *PAI1*, *BCL2*, *Caspase3*, *ALK1*, *ALK5*, *ID-1*, *BAX* and *ILK*. Specific information with regard to the primers is listed in **Table 3**.

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'	
Aggrecan	5' GGACACTCCTTGCAATTTGAG 3'	61 - 62
	5' GTCATTCCACTCTCCCTTCTC 3'	
Versican	5' TCTCACAAGCATCCTGTCTCAC 3'	65
	5' CCATCGGTCCAACGGAAGTC 3'	
Collagen II	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'	
MMP13	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'	
PAI1	5' AAACCTGGCGGACTTCTC 3'	61.5
	5' ACTGTGCCACTCTCATTCAC 3'	
BCL2	5' GGATGACTGAGTACCTGAACC 3'	61.5 - 63
	5' CGTACAGTTCCACAAAGGC 3'	
TGF-β1	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'	
ID1	5' CTCAACGGCGAGATCAG 3'	59.5
	5' GAGCACGGGTTCTTCTC 3'	
BAX	5' CCTTTTGCTTCAGGGTTTCA 3'	58 - 59

	5' CTCAGCTTCTTGGTGGATGC 3'	
ILK	5' AAAGCAGGGACTTCAATGAGGA 3'	64
	5' ACTTCACAGCTTGGCTCTGG 3'	

Figure 3. Specific information of the primers.

3.3.2.6. Histology

Two pellets per donor per condition were cultured for histology. After 28 days, pellets were isolated and prepared for histology as previously described (chapter 2, p.21). Histologic slides were stained with Safranin O/Fast Green using the same protocol described in (chapter 2, p.21).

3.3.2.7. Statistics

The statistical analyses were performed using Rstudio version 3.0.2. RT-qPCR data were analyzed with a cox regression model. 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. Every cox regression model was checked for meeting the proportional hazard assumption. 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 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.
As the data of the DMMB assay was not normally distributed, it was analysed using the non-parametric Mann Whitney test in SPSS for Windows version 20 (SPSS Inc.). P values were corrected for multiple comparisons using the Benjamini Hochbergs' False Discovery Rate procedure.

3.4. Results

This section describes the effects of CSD (0.01 µM, 0.1 µM and 1 µM) and TGF-β1 (2 ng/mL and 10 ng/mL) treatment on outcome parameters cell proliferation and extracellular matrix production on NPC pellets (200,000 cells) originating from chondrodystrophic dogs. With respect to the cell proliferation, DNA content of the NPC pellets was measured. The extracellular matrix production was investigated by performing a DMMB assay and a Safranin O/Fast Green staining. The mechanisms (signalling pathways) that underlie the effects of CSD and TGF-β1 treatment were investigated using RT-qPCR.

3.4.1. Cell proliferation

Cell proliferation was quantified by monitoring the DNA content of the NPC pellets on day 0, 1, 7, and 28 of the culture period.

3.4.1.1. DNA content

The DNA content of the NPC pellets significantly decreased over time in all culture conditions ($p<0.05$) (**figure 1**). From day 7 onwards, all TGF-β1 treated pellets had a significantly higher DNA content than the control pellets ($p<0.01$). CSD did not have an additive effect on TGF-β1 treatment regarding DNA content of the NPC pellets.

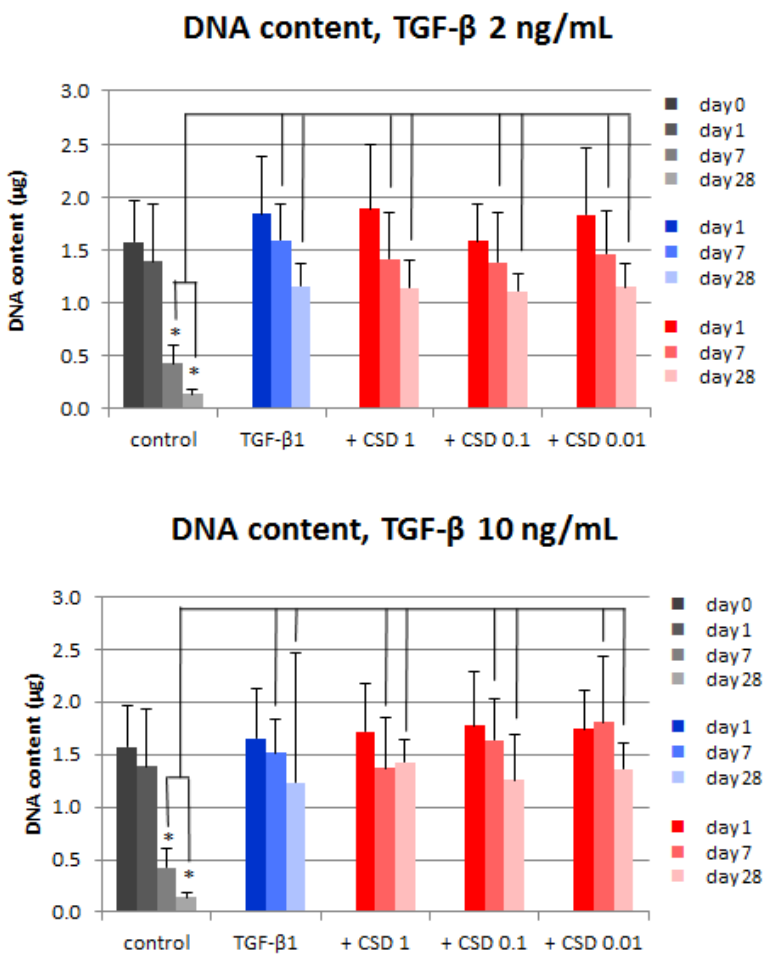


Figure 1. DNA content of the NPC pellets that were cultured in basal chondrogenic medium for 28 days and treated with TGF-β1 (blue), TGF-β1 in combination with caveolin-1 scaffolding domain peptide (CSD) (red) or untreated (grey). The top graph shows the results of the DNA content of the pellets treated with the suboptimal concentration of TGF-β1 (2 ng/mL). The bottom graph shows the results of the DNA content of the pellets treated with the optimal concentration of TGF-β1 (10 ng/mL). TGF-β1 increased DNA-content of the pellets compared with control pellets. CSD did have an additive effect on the TGF-β1 treatment regarding DNA content. * = $p<0.01$.

3.4.2. Extracellular matrix production

In addition to cell proliferation, extracellular matrix (GAG) production was used as an outcome parameter to measure the potential additive regenerative effects of CSD supplementation to TGF-β1 treatment. This parameter was quantified by monitoring the GAG content on day 0, 1, 7, and 28 of the culture period and by measuring the weekly GAG release of the NPC pellets in the culture medium. To support the results of the DMMB assay, extracellular matrix (GAG) content of the NPC pellets was visualized by carrying out Safranin O/Fast Green staining.

3.4.2.1. DMMB assay

Control pellets showed a significant decrease in GAG content over time ($p < 0.001$) (**figure 2**), whereas all TGF-β1 treated pellets showed a significant increase in GAG content over time ($p<0.001$). From day 7 onwards, TGF-β1 treated pellets had a significantly higher GAG content than the control pellets ($p<0.001$). A significant dose-dependent effect of TGF-β1 treatment was observed regarding the GAG content of the pellets ($p<0.001$): 10 ng/mL TGF-β1 caused a higher increase in GAG content compared with 2 ng/mL TGF-β1. Irrespective of its concentration, CSD did not elicit an additive effect on 2 ng/ml and 10 ng/ml TGF- β1 treated pellets regarding the GAG content.

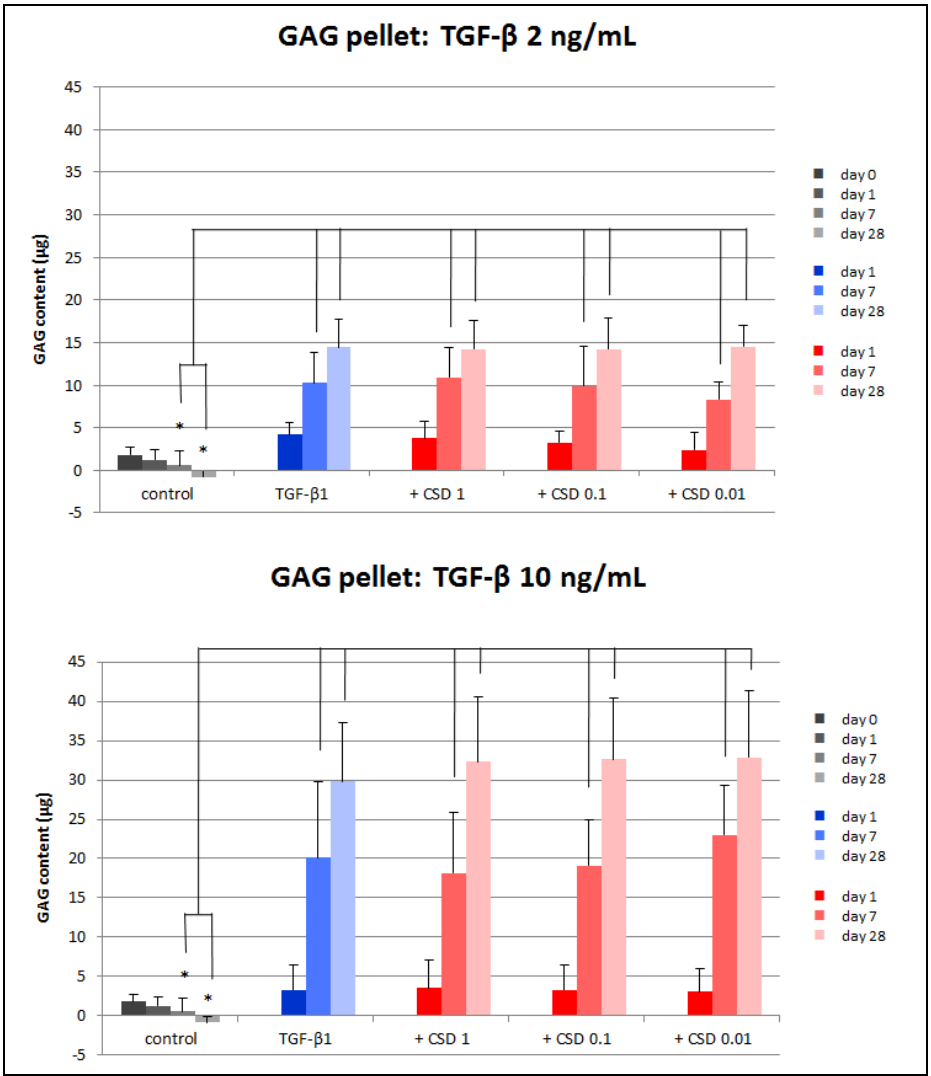


Figure 2. Mean GAG content of the NPC pellets that were cultured in basal chondrogenic medium for 28 days and treated with TGF-β1 (blue), TGF-β1 in combination with caveolin-1 scaffolding domain peptide (CSD) (red) or were left untreated (grey). The top graph shows the results of the GAG content of the pellets treated with the suboptimal concentration of TGF-β1 (2 ng/mL). The bottom graph shows the results of the GAG content of the pellets treated with the optimal concentration of TGF-β1 (10 ng/mL). * = $p<0.001$.

Beside the GAG content of the NPC pellets, the GAG release into the culture medium was measured every week of the total culture period (4 weeks). A significant decrease of GAG release was measured during the second week compared with the first week of the culture period with respect to the control pellets ($p<0.001$) and the pellets treated with TGF-β1 10 ng/ml ($p<0.01$), TGF-β1 2 ng/ml ($p<0.05$), TGF-β1 2 ng/ml + CSD 1 µM ($p<0.01$), TGF-β1 2 ng/ml + CSD 0.1 µM ($p<0.05$) and TGF-β1 2 ng/ml + CSD 0.01 µM ($p<0.05$) (**figure 3**). After the second week, GAG release remained constant over time. Significantly more GAGs were released into the culture medium by all TGF-β1 treated pellets compared with the control pellets throughout the culture period (week 1 to week 4) ($p<001$). A significantly higher GAG release in the culture medium was found when TGF-β1 was supplemented in a concentration of 10 ng/mL compared

with a concentration of 2 ng/mL (p=0.01). In the presence of either TGF-β1 concentration, there was no additive effect of CSD on GAG release into the culture medium.

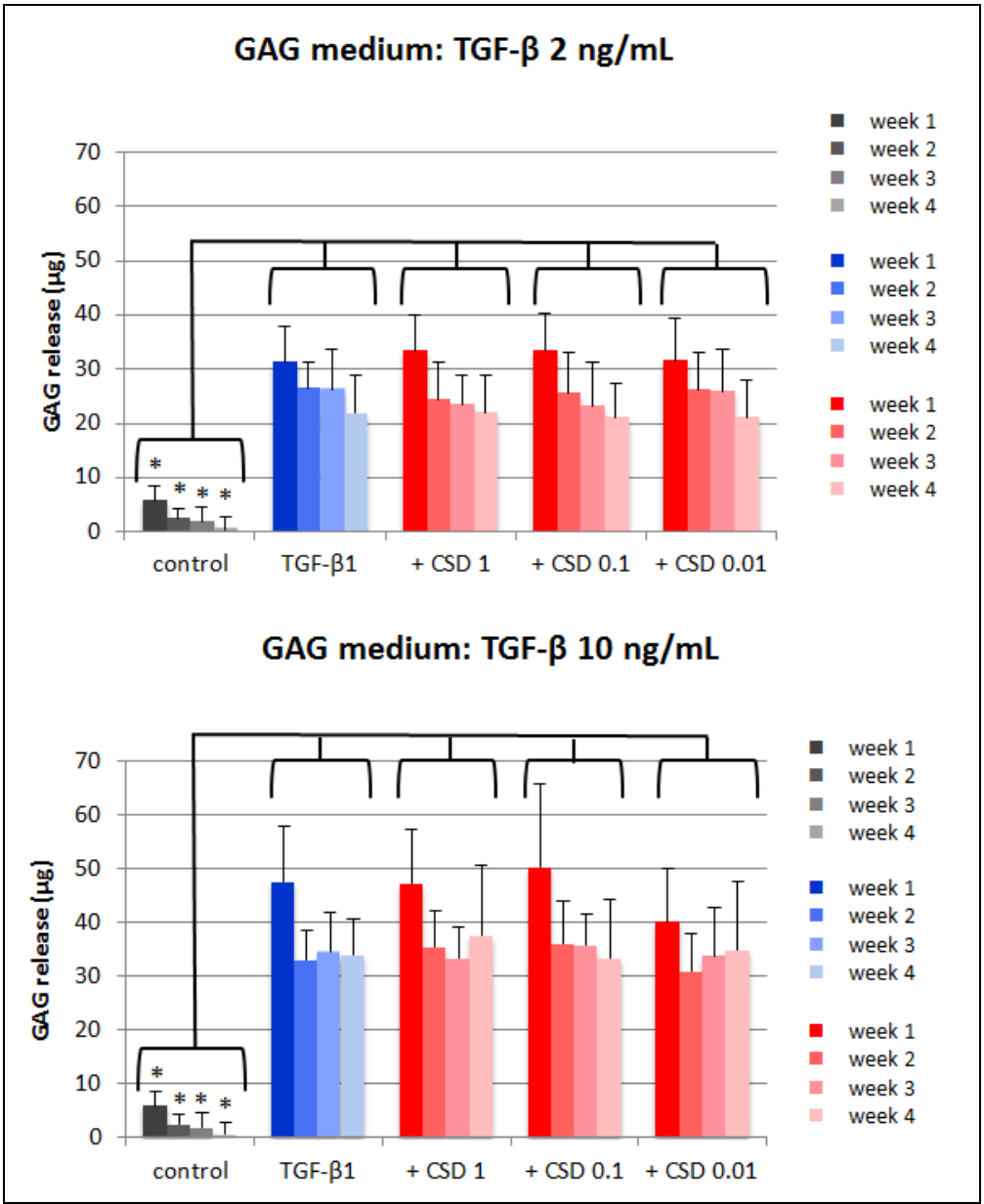


Figure 3. Mean GAG release of the NPC pellets that were cultured in basal chondrogenic medium for 28 days and treated with TGF-β1 (blue), TGF-β1 in combination with caveolin-1 scaffolding domain peptide (CSD) (red) or were left untreated (grey). The top graph shows the results of the GAG release of the pellets treated with the suboptimal concentration of TGF-β1 (2 ng/mL). The bottom graph shows the results of the GAG release of the pellets treated with the optimal concentration of TGF-β1 (10 ng/mL). * = p<0.001.

The GAG content of the NPC pellets was corrected for differences in cell numbers between pellets by dividing the GAG content by the DNA content of each pellet (µg/µg). The corrected results yielded a similar pattern as the uncorrected results (**figures 2 and 4**). Specifically, the GAG/DNA ratio of the control pellets decreased over time (p<0.001), while the GAG/DNA ratio of the TGF-β1 treated pellets increased over time (p<0.001). Significantly more GAGs per DNA

were found in all TGF-β1 treated pellets compared with control pellets ($p<0.05$). Treatment with 10 ng/mL TGF-β1 caused a significantly higher increase in GAG/DNA ratio compared with 2 ng/mL TGF-β1 ($p<0.001$). Despite, the suboptimal effect of 2 ng/mL TGF-β1, CSD (0.01 µM, 0.1 µM and 1 µM) was unable to exert an additive effect on the GAG/DNA ratio of the pellets.

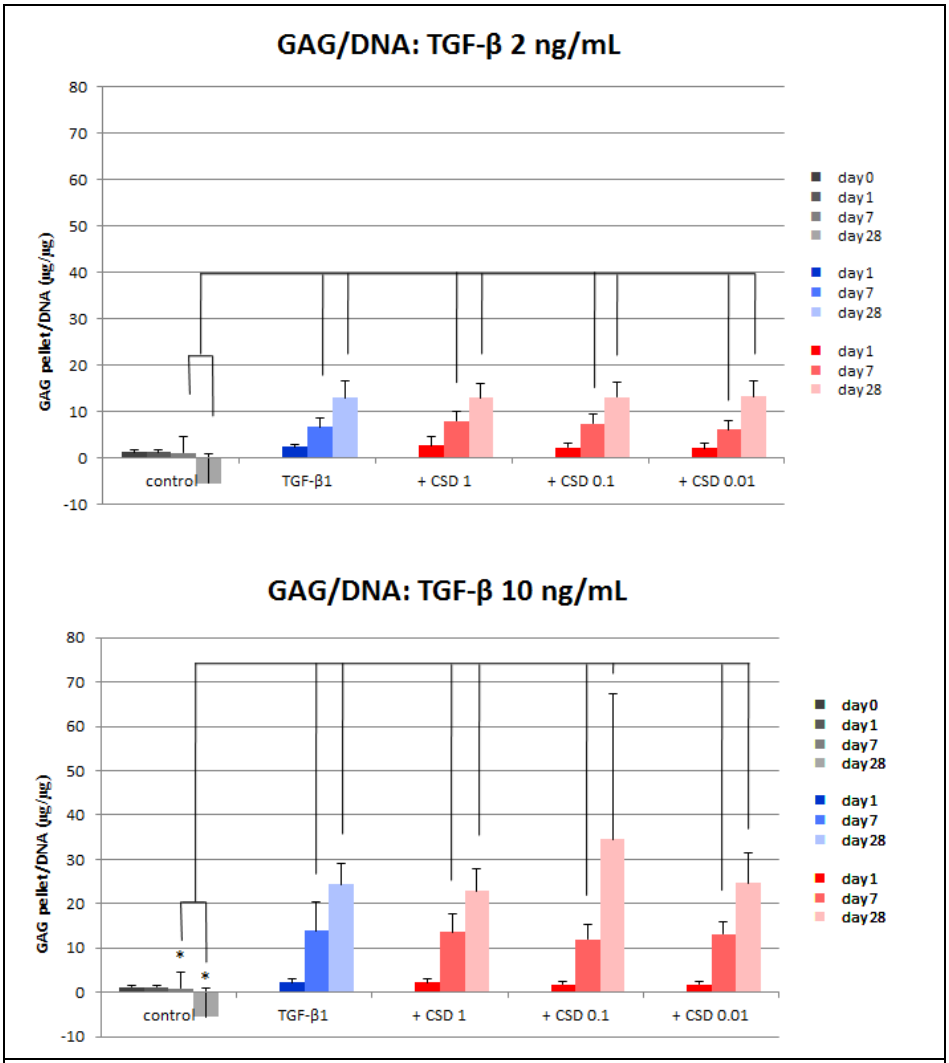


Figure 4. Mean GAG/DNA (µg/µg) of the NPC pellets that were cultured in basal chondrogenic medium for 28 days and treated with TGF-β1 (blue) or TGF-β1 in combination with caveolin-1 scaffolding domain peptide (CSD) (red) or were left untreated (grey). The top graph shows the results of the GAG/DNA of the pellets treated with the suboptimal concentration of TGF-β1 (2 ng/mL). The bottom graph shows the results of the GAG/DNA of the pellets treated with the optimal concentration of TGF-β1 (10 ng/mL). * = $p<0.001$.

3.4.2.2. Histology

In order to support the results of the DMMB assay, pellets were subjected to a Safranin O/Fast Green staining to visualize GAG production. Microscopic images of the pellets of donor C that are displayed in **figure 5** are representative for the pellets of all the other donors. In line with the results of the DMMB assay, all control pellets stained green, indicating that no GAGs were produced in these pellets. Furthermore, all TGF-β1 treated pellets showed a substantial red staining that indicates the presence of many GAGs. In contrast to the results of the DMMB

assay, no difference in red staining intensity was observed between the 10 ng/mL and 2 ng/mL treated pellets. Thus, no dose-dependent effect of TGF-β1 was noticeable. The addition of CSD (0.01 µM, 0.1 µM and 1 µM) to 2 ng/mL and 10 ng/mL TGF-β1 treatment exerted no differential effect on the red staining intensity.

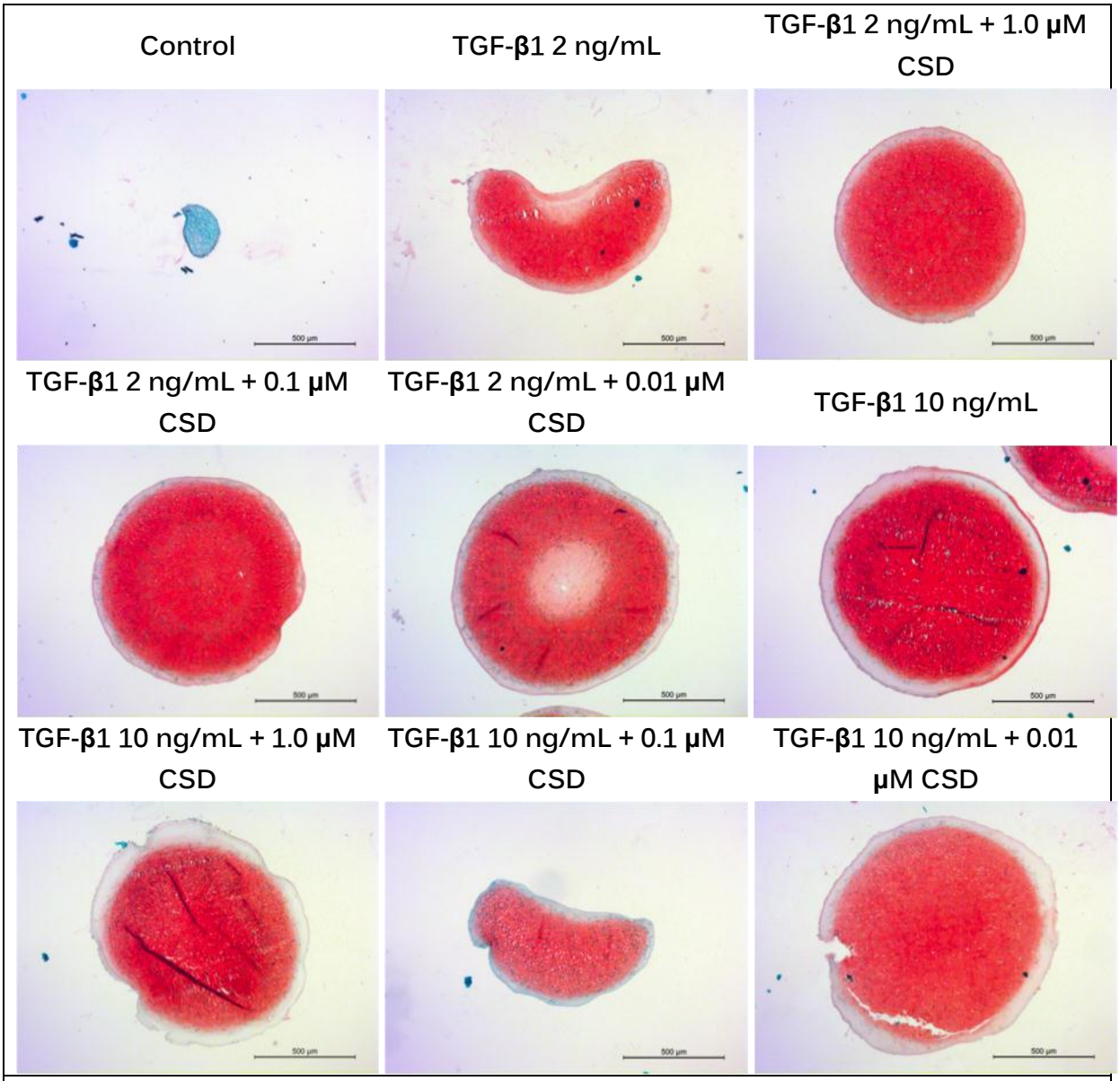


Figure 5. Microscopic images of the pellets from donor C after they were stained with Safranin O/Fast Green. A red staining indicates the presence of GAGs in the pellets.

3.4.3. RT-qPCR

To investigate the signalling pathways underlying the effects of CSD and TGF-β1 treatment on the NPC pellets' DNA content and GAG production, the relative gene expression levels of several matrix proteins (*Aggrecan, Versican, Collagen II, Collagen I*), matrix remodelling proteins (*ADAMTS5, MMP13, TIMP1*), cell proliferation marker (*CyclinD1*), markers concerning the Wnt/β-catenin signalling (*Axin2, ILK*) and TGF-β signalling (*ALK5, ALK1, PAI1, ID1*) were determined at day 7 and 28. The relative gene expression of these markers in each NPC pellet

was compared with the gene expression of the control pellets at day 7. The n-fold gene expression of the control pellets at day 7 was set at 1.

3.4.3.1. Extracellular matrix production

Proteoglycans *Aggrecan* and *Versican*, and matrix protein *Collagen II* are largely present in healthy nucleus pulposus tissue [12]. Conversely, *Collagen I* is not pliable and therefore not favorable for the shock absorbing function of the nucleus pulposus.

Aggrecan expression was significantly upregulated by TGF-β1 treatment at day 7 compared with controls (CI: 0.056, 0.8649) (**figure 6**). While a similar trend was seen at day 28, at this point TGF-β1's effect was no longer significant. TGF-β1 treatment in a concentration of 10 ng/mL caused a significantly higher increase in *Aggrecan* expression than the suboptimal concentration (2 ng/mL) (CI: 2.351e^{-2}, 0.409). CSD (0.01 μM, 0.1 μM and 1 μM) did not exert an additive effect on *Aggrecan* expression compared with sole TGF-β1 treatment.

Versican gene expression is not shown as no significant treatment effects were found.

Collagen II expression was significantly upregulated in all TGF-β1 treated pellets at both day 7 and day 28 compared with controls (CI: 17.4182,318.992) (**figure 6**). TGF-β1 10 ng/mL was significantly more effective in upregulating *Collagen II* expression compared with TGF-β1 2 ng/mL (CI: 0.0959, 0.66896). Again, CSD (0.01 μM, 0.1 μM and 1 μM) did not have an additive effect upon TGF-β1 treatment regarding *Collagen II* expression.

Collagen I expression was significantly upregulated in all treatment groups at day 7 compared with controls (CI: 2.535895, 22.55287) (**figure 6)**. This upregulation, however, became a statically significant downregulation by day 28 in all culture groups (CI: 0.001524, 0.01825). TGF-β1 did not exert a dose-dependent effect and CSD (0.01 μM, 0.1 μM and 1 μM) did not have an additive effect on 2 ng/mL and 10 ng/mL TGF-β1 treatment regarding *Collagen I* expression.

Thus, with the exception of *Versican*, TGF-β1 upregulated the gene expression of all matrix production markers. CSD did not exert any additional regenerative effects regarding the expression of these markers.

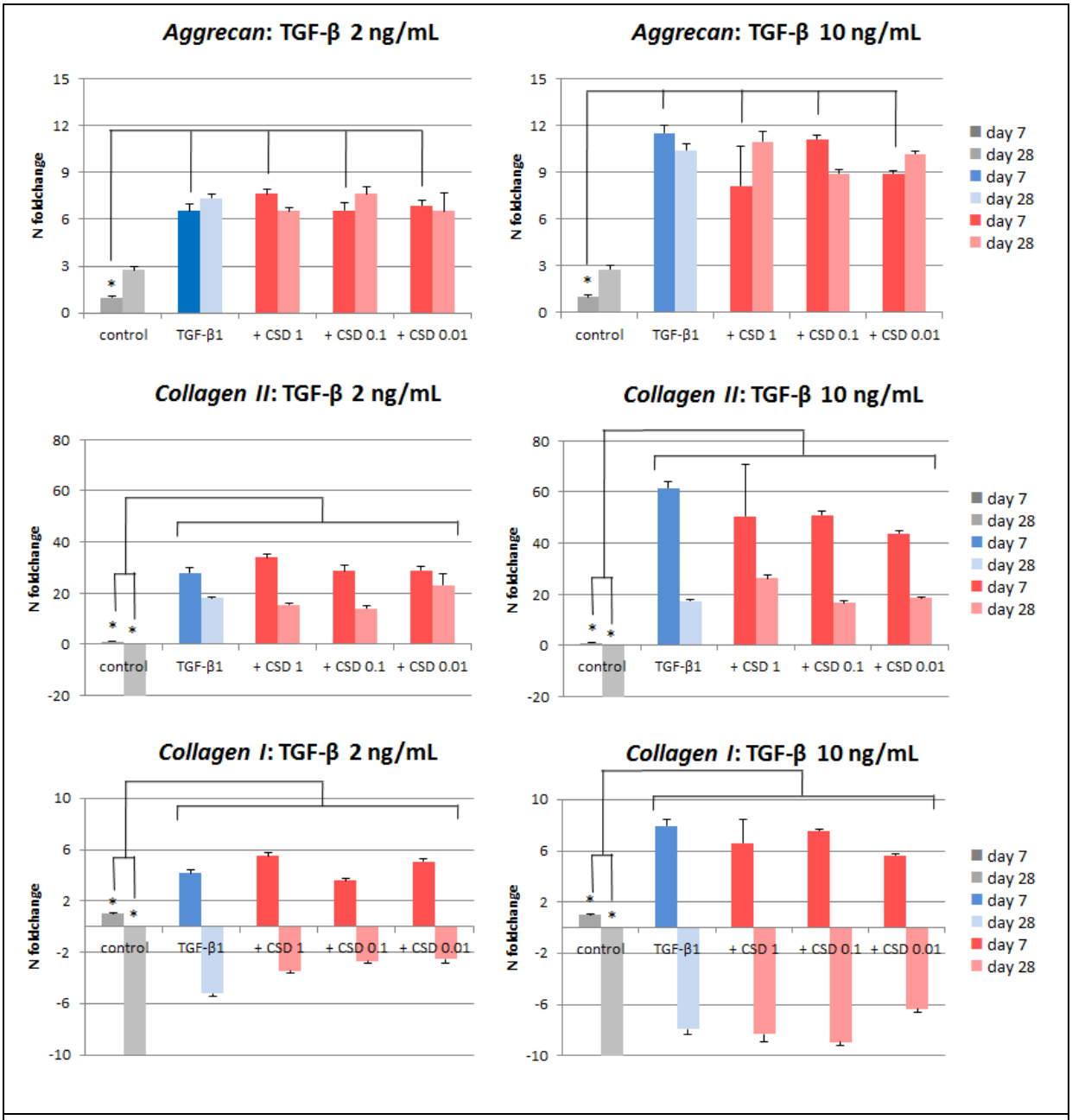


Figure 6. Relative gene expression of *Aggrecan (ACAN)*, *Collagen II (Col2a1)* and *Collagen I (Col1)* in NPC pellets cultured in basal chondrogenic medium and treated with TGF-β1 (blue), TGF-β1 in combination with caveolin-1 scaffolding domain peptide (CSD) (red) or untreated (grey) for 7 or 28 days. The left graphs represent the relative gene expression of the NPC pellets that were cultured with the suboptimal concentration of TGF-β1 (2 ng/mL). The right graphs represent the relative gene expression of the NPC pellets that were cultured with the optimal concentration of TGF-β1 (10 ng/mL). * = CI does not contain value 1.

3.4.3.2. Extracellular matrix degradation

ADAMTS5 and MMP13 are metalloproteinases, which are enzymes that degrade extracellular matrix proteins. ADAMTS5 primarly breaks down proteoglycans such as *Aggrecan*, whereas *MMP13* targets both proteoglycans and collagen fibers [52]. *TIMP-1 acts as an inhibitor of such metalloproteinases* [53].

ADAMTS5 expression was significantly downregulated at day 7 and day 28 by TGF-β1 treatment compared with controls (CI: $2.146e^{-7}$, $6.006e^{-5}$) (**figure 7**). This downregulation was significantly stronger in pellets treated with TGF-β1 10 ng/mL compared with TGF-β1 2 ng/mL on both day 7 and day 28 (CI: 7150, 79600). On day 28, downregulation of *ADAMTS5* expression was only statistically significant for the combined treatment of TGF-β1 10 ng/mL and 1 µM CSD (CI: 4.619e-02 , 8.833e-01).

MMP13 expression was significantly downregulated at day 28 in all TGF-β1 treated pellets compared with controls (CI: 0.0171, 0.2923) (**figure 7**). Optimal TGF-β1 treatment (10 ng/mL, irrespective of the combination with CSD) also significantly downregulated *MMP13* expression at day 7 (CI: 0.0053, 0.1473), but more interestingly, CSD (0.01 µM, 0.1 µM and 1 µM) had a statistically significant additive effect on the downregulation of *MMP13* expression (CI: 3.5656, 101.411).

The gene expression of *TIMP-1* on day 28 was significantly lower than on day 7 in all culture condition (CI: 0.00448, 0.03106) (**figure 7**). *TIMP-1* expression was significantly downregulated by optimal TGF-β1 treatment (10 ng/ml, irrespective of the combination with CSD) (CI: 0.0142, 0.25481). The TGF-β1 (10 ng/mL) induced downregulation of *TIMP-1* expression was inhibited at both day 7 and day 28 by CSD (0.01 µM, 0.1 µM and 1 µM). No significant differences were found regarding the 2 ng/mL TGF-β1 treated pellets.

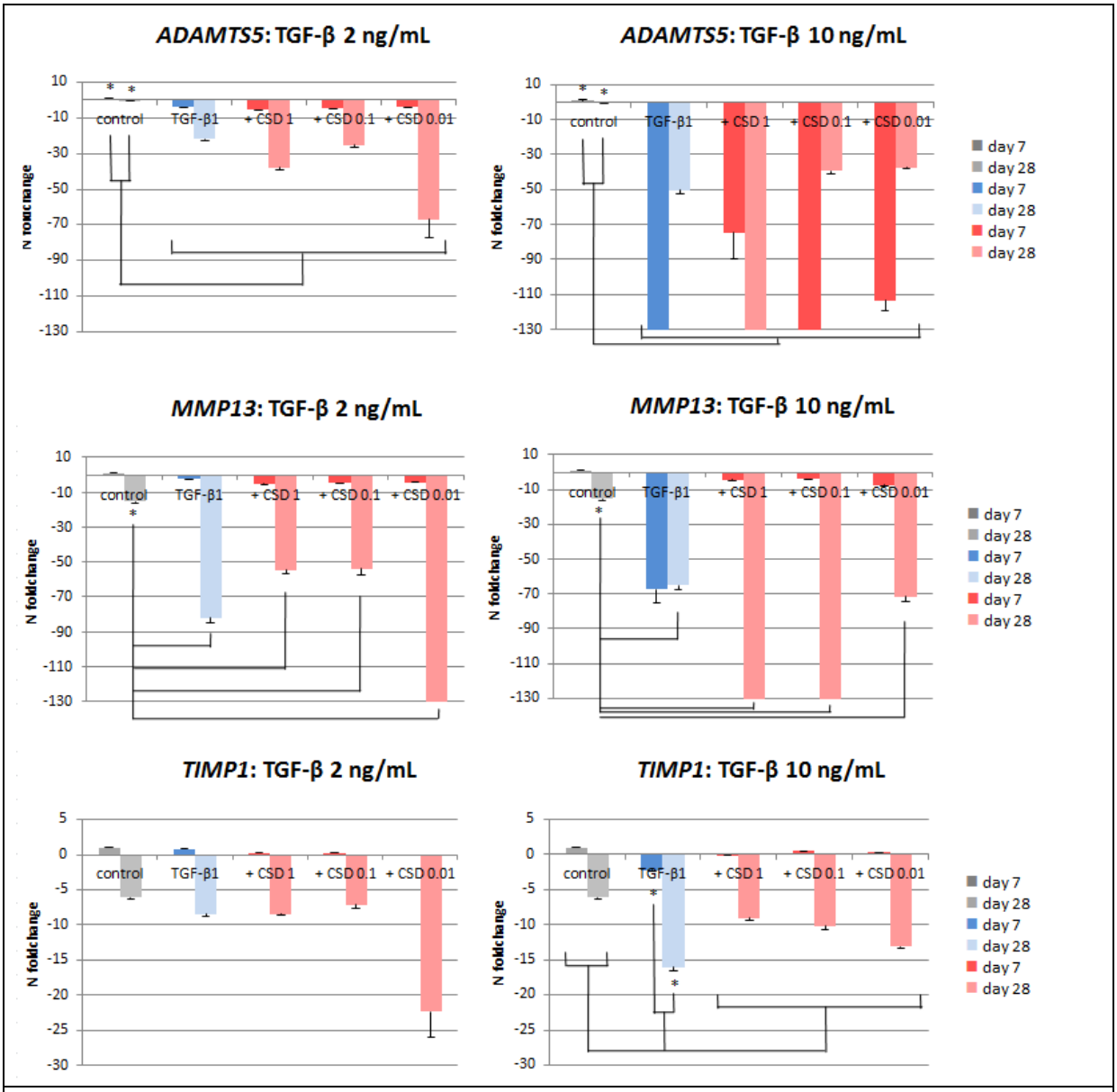


Figure 7. Relative gene expression of *ADAMTS5*, *MMP13* and *TIMP-1* in NPC pellets cultured in basal chondrogenic medium and treated with TGF-β1 (blue), TGF-β1 in combination with caveolin-1 scaffolding domain peptide (CSD) (red) or untreated (grey). The left graphs represent the relative gene expression of the NPC pellets that were cultured with the suboptimal concentration of TGF-β1 (2 ng/mL). The right graphs represent the relative gene expression of the NPC pellets that were cultured with the optimal concentration of TGF-β1 (10 ng/mL). * = CI does not contain value 1.

Thus, TGF-β1 treatment downregulated the expression of the matrix degradation markers compared with controls. Generally, CSD did not show additional effects upon TGF-β1 treatment. Exceptionally, CSD (0.01 μM, 0.1 μM and 1 μM) enhanced the 10 ng/mL TGF-β1's induced downregulation of MMP13 expression. Furthermore, CSD (0.01 μM, 0.1 μM and 1 μM) counteracted TGF-β1's induced downregulation of TIMP-1 expression.

3.4.3.3. Cell proliferation

The gene expression of *CyclinD1* was upregulated in all TGF-β1 treated pellets compared with controls. This upregulation was only statistically significant at day 7 of 2 ng/mL TGF-β1

treatment (**figure 8**). There was no significant difference in effect between 2 ng/mL and 10 ng/mL TGF-β1 treatment and CSD treatment (0.01 μM, 0.1 μM and 1 μM) did not elicit any additional effects.

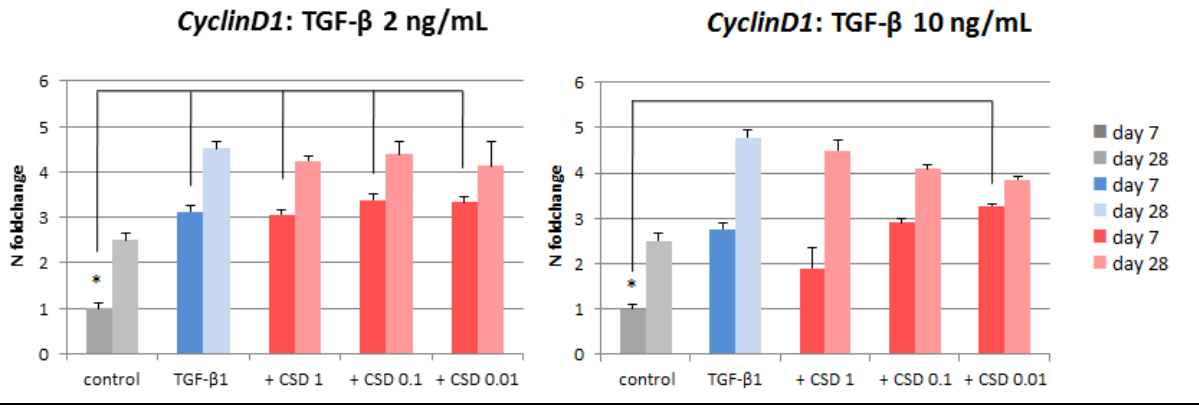


Figure 8. Relative gene expression of cell proliferation marker *CyclinD1* in NPC pellets cultured in basal chondrogenic medium and treated with TGF-β1 (blue), TGF-β1 in combination with caveolin-1 scaffolding domain peptide (CSD) (red) or untreated (grey). The left graphs represent the relative gene expression of the NPC pellets that were cultured with the suboptimal concentration of TGF-β1 (2 ng/mL). The right graphs represent the relative gene expression of the NPC pellets that were cultured with the optimal concentration of TGF-β1 (10 ng/mL). * = CI does not contain value 1.

3.4.3.4. Wnt/β-catenin signalling

To gain more insight into the effects of CSD on Wnt/β-catenin signalling with respect to IVD degeneration, the expression of markers of the Wnt/β-catenin, including *Axin2* and *ILK* were measured.

Axin2 is a specific read-out marker for active Wnt/β-catenin signalling [1]. *Axin2* expression was significantly downregulated on day 28 in all culture groups compared with controls (CI: 0.01498, 0.32220) (**figure 9**). CSD had a dose-dependent effect on TGF-β1 treatment: in the pellets treated with TGF-β1 2 ng/mL, CSD seemed to enhance TGF-β1's effects in downregulating *Axin2* expression on day 28, although this was not statistically significant. Furthermore, in NPC pellets treated with TGF-β1 in a concentration of 10 ng/mL, CSD showed an inhibiting effect both at day 7 and at day 28, but this was only statistical significant on day 7 (CI: 13.6308, 700.828). This inhibiting effect of CSD on TGF-β1 (10 ng/mL) seemed to be dose-dependent, with increasing inhibition at lower CSD concentrations. However, this dose-dependent effect was not statistically significant.

Integrin linked kinase (*ILK*) stimulates Wnt/β-catenin signalling by inhibiting glycogen synthase kinase-3b that degrades β-catenin [16]. *ILK* expression showed no significant differences in all culture groups (**figure 9**). Although TGF-β1 treatment tended to downregulate *ILK* expression, the n-fold changes were minimally affected and no significant downregulation was encountered. *ILK* expression remained constant during the culture period.

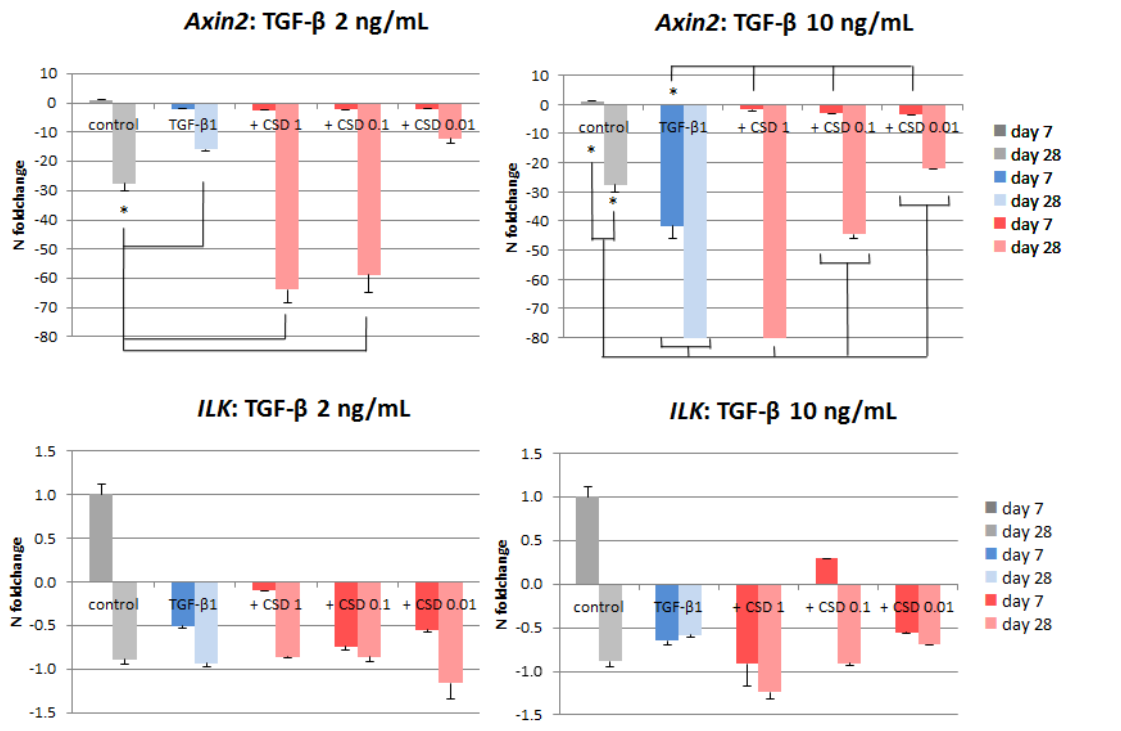


Figure 9. Relative gene expression of Wnt/β-catenin signalling molecules *Axin2* and *ILK* in NPC pellets cultured in basal chondrogenic medium and treated with TGF-β1 (blue), TGF-β1 in combination with caveolin-1 scaffolding domain (CSD) (red) or untreated (grey). The left graphs represent the relative gene expression of the NPC pellets that were cultured with the suboptimal concentration of TGF-β1 (2 ng/mL). The right graphs represent the relative gene expression of the NPC pellets that were cultured with the optimal concentration of TGF-β1 (10 ng/mL). * = CI does not contain value 1.

3.4.3.5. TGF-β signalling

As previously mentioned, we hypothesized that caveolin-1 could induce regeneration by shifting the TGF-β signalling from the catabolic ALK1 (Smad1/5/8) pathway towards to the anabolic ALK5 (Smad2/3) pathway. To investigate this, gene expression levels of TGF-β type 1 receptors *ALK5* and *ALK1* and their respective downstream pathways read out markers *PAI1* (ALK5 (Smad2/3) pathway) and *ID1* (ALK1 (Smad1/5/8) pathway) were measured (**figure 10**).

No differences in *ALK5* expression were seen across the different culture groups (**figure 10**). However, its downstream marker *PAI1* showed a significant upregulation in all TGF-β1 treated pellets at both day 7 and day 28 of the culture period compared with controls (CI: 3.4126, 31.133) (**figure 10**). No additive effect of CSD (0.01 μM, 0.1 μM and 1 μM) was observed on 2 ng/mL and 10 ng/mL TGF-β1 treatment, regarding *PAI1* expression.

No significant differences were found in *ALK1* expression across the different culture groups (**figure 10**). Although CSD (1 μM and 0.1 μM) tended to downregulate *ALK1* expression upon 2 ng/mL TGF-β1 treatment at day 28, this effect was not statistically significant. However, *ALK1's* downstream marker *ID1* was significantly downregulated in all TGF-β1 treated pellets at both day 7 and day 28. CSD supplementation (0.01 μM, 0.1 μM and 1 μM) did not have an additive effect on 2 ng/mL and 10 ng/mL TGF-β1, regarding *ID1* expression.

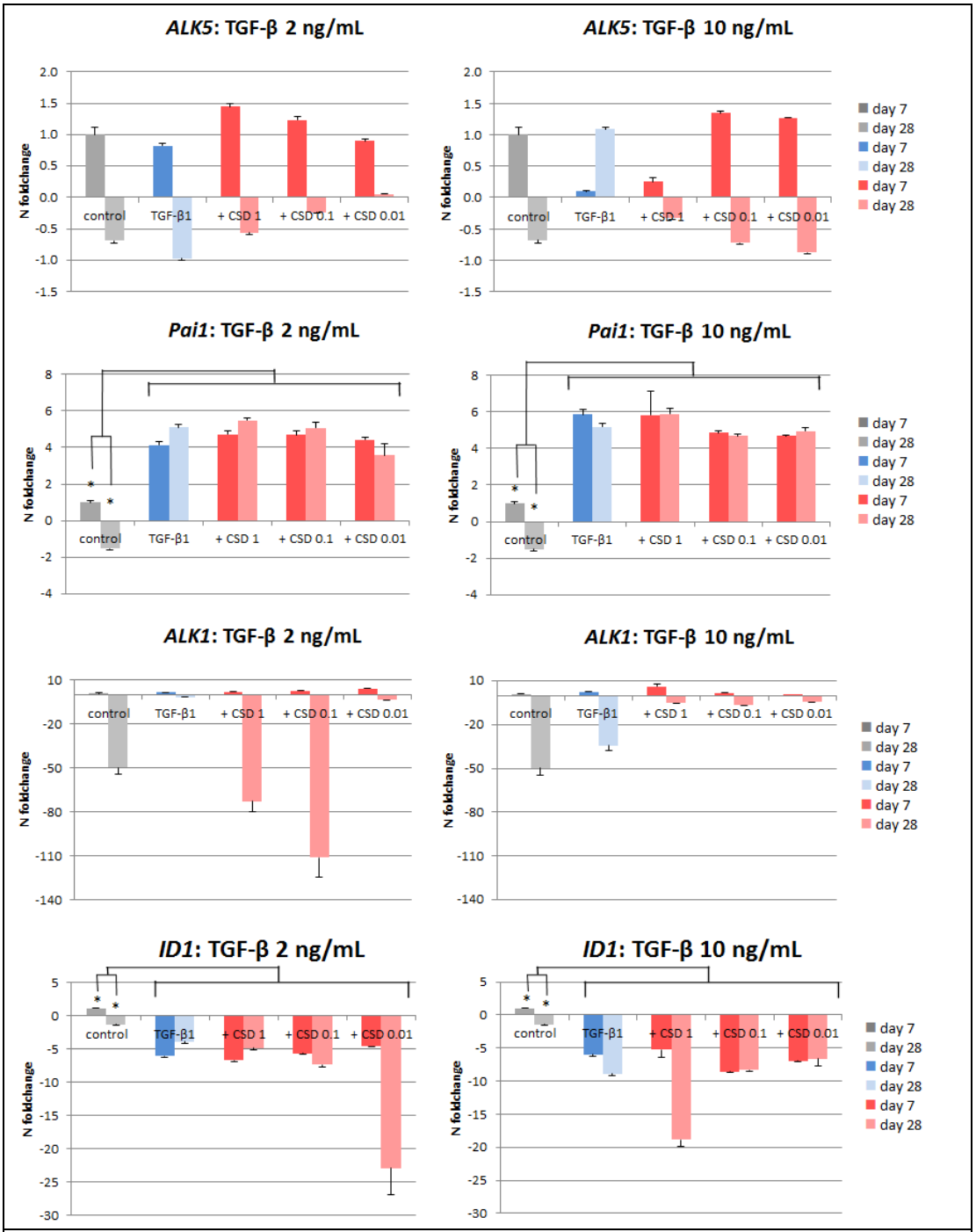


Figure 10. Relative gene expression of TGF-β signalling molecules *ALK5*, *PAI1*, *ALK1* and *ID1* in NPC pellets cultured in basal chondrogenic medium and treated with TGF-β1 (blue), TGF-β1 in combination with caveolin-1 scaffolding domain (red) or untreated (grey). The left graphs represent the relative gene expression of the NPC pellets that were cultured with the suboptimal concentration of TGF-β1 (2 ng/mL). The right graphs represent the relative gene expression of the NPC pellets that were cultured with the concentration of TGF-β1 (10 ng/mL). * = CI does not contain value 1.

ALK5/ALK1 and *PAI1/ID1* expression ratios were calculated using the CT values (**figure 11**) to investigate whether CSD differentially influenced ALK 5 (Smad2/3) and ALK 1 (Smad1/5/8) signalling. High CT values indicate low expression levels (and vice versa), hence high *ALK5/ALK1* indicate low *ALK5* expression relative to *ALK1* expression (and vice versa). The

ALK5/ALK1 ratios seem to decrease during the culture period, but this time effect was not statistically significant. No differences in *ALK5/ALK1* ratios were observed between the culture groups. However, 0.1 µM CSD had a significant additive effect on TGF-β1 (2 ng/mL) treatment in lowering the *ALK5/ALK1* ratio at day 28 ($p<0.05$). This indicates that 0.1 µM CSD increased *ALK5* expression compared with *ALK1* expression on day 28. The *PAI1/ID1* ratio decreased significantly in all TGF-β1 treated pellets ($p<0.001$), meaning that TGF-β1 increased *PAI1* expression relative to *ID-1* expression (**figure 11**). Interestingly, 0.01 µM CSD significantly decreased the *PAI1/ID1* ratio upon TGF-β1 10 ng/mL treatment at day 28 of the culture period. This indicates that 0.01 µM CSD increased *PAI1* expression relative to *ID1* expression in the NPC pellets.

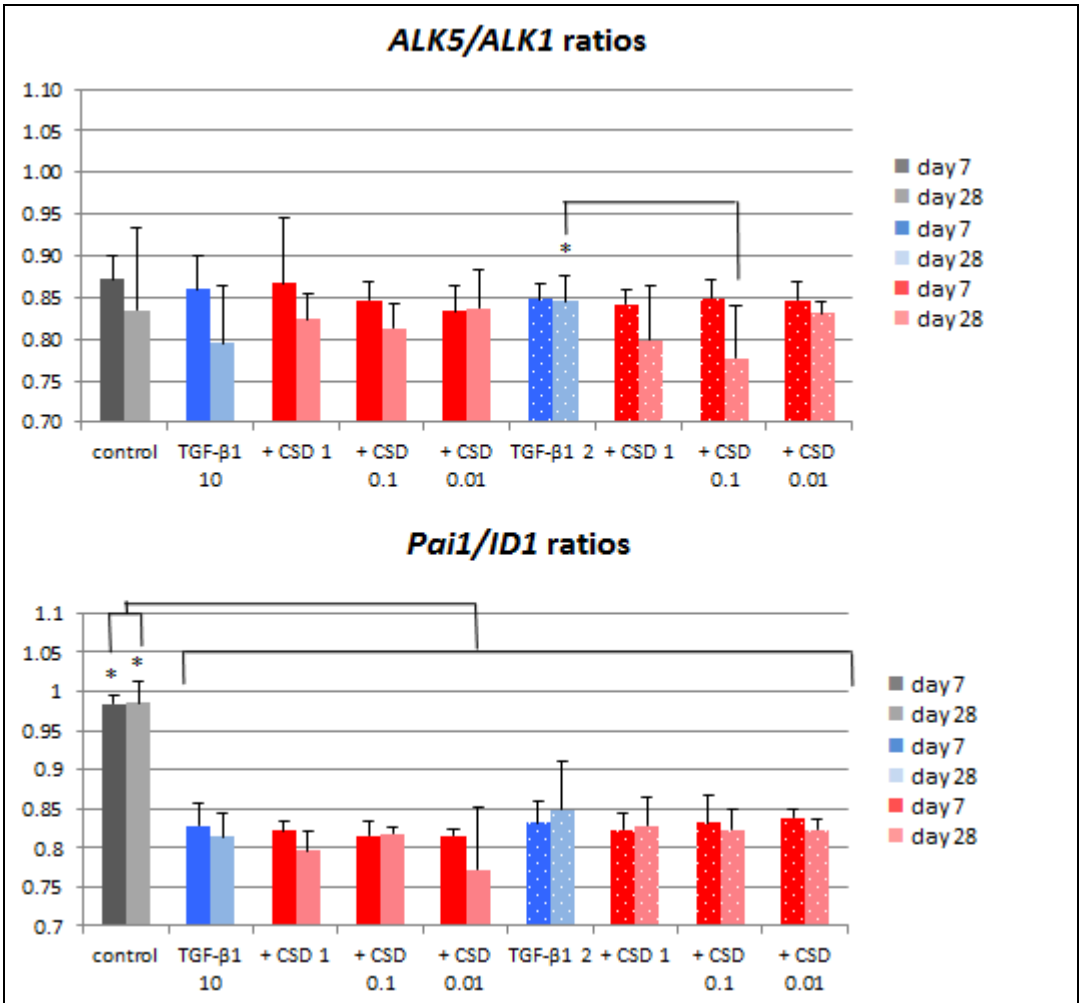


Figure 11. *ALK5/ALK1* and *PAI1/ID1* expression ratios in NPC pellets cultured in basal chondrogenic medium and treated with TGF-β1 (blue), TGF-β1 in combination with caveolin-1 scaffolding domain peptide (CSD) (red) or untreated (grey) for 7 or 28 days. The solid bars represent the *PAI/ID1* ratios of the 10 ng/mL TGF-β1 treated pellets whereas the dotted bars represent the *PAI/ID1* ratios of the 2 ng/mL TGF-β1 treated pellets. * = $p<0.05$

3.4.3.6. Apoptosis

Gene expression of apoptosis regulators *BAX* and *BCL2* were measured to determine apoptosis levels. *BAX* promotes apoptosis, while *BCL2* is an anti-apoptotic protein. *BAX* expression was

significantly downregulated in all TGF-β1 treated pellets at both days 7 and 28 compared with controls (CI: 0.0013, 0.0325) (**figure 12**). The downregulation of *BAX* expression was unaffected by CSD treatment (CSD (0.01 μM, 0.1 μM and 1 μM). *BCL2* expression significantly increased from day 7 to day 28 in all culture groups (CI: 11.521, 841.4) (**figure 12**). In accordance with the downregulation of apoptosis promotor *BAX*, anti-apoptosis molecule *BCL2* was upregulated in all TGF-β1 treated pellets at both day 7 and day 28, but with statistical significance only at day 28 (CI: 14.92, 1094). A significantly higher upregulation of *BCL2* expression was caused by TGF-β1 in a concentration of 10 ng/mL compared with TGF-β1 in a concentration of 2 ng/mL at day 28 (CI: 1.266, 2.022). However, CSD (0.01 μM, 0.1 μM and 1 μM) did not have an additive effect on TGF-β1 treatment concerning the expression of these apoptosis related genes.

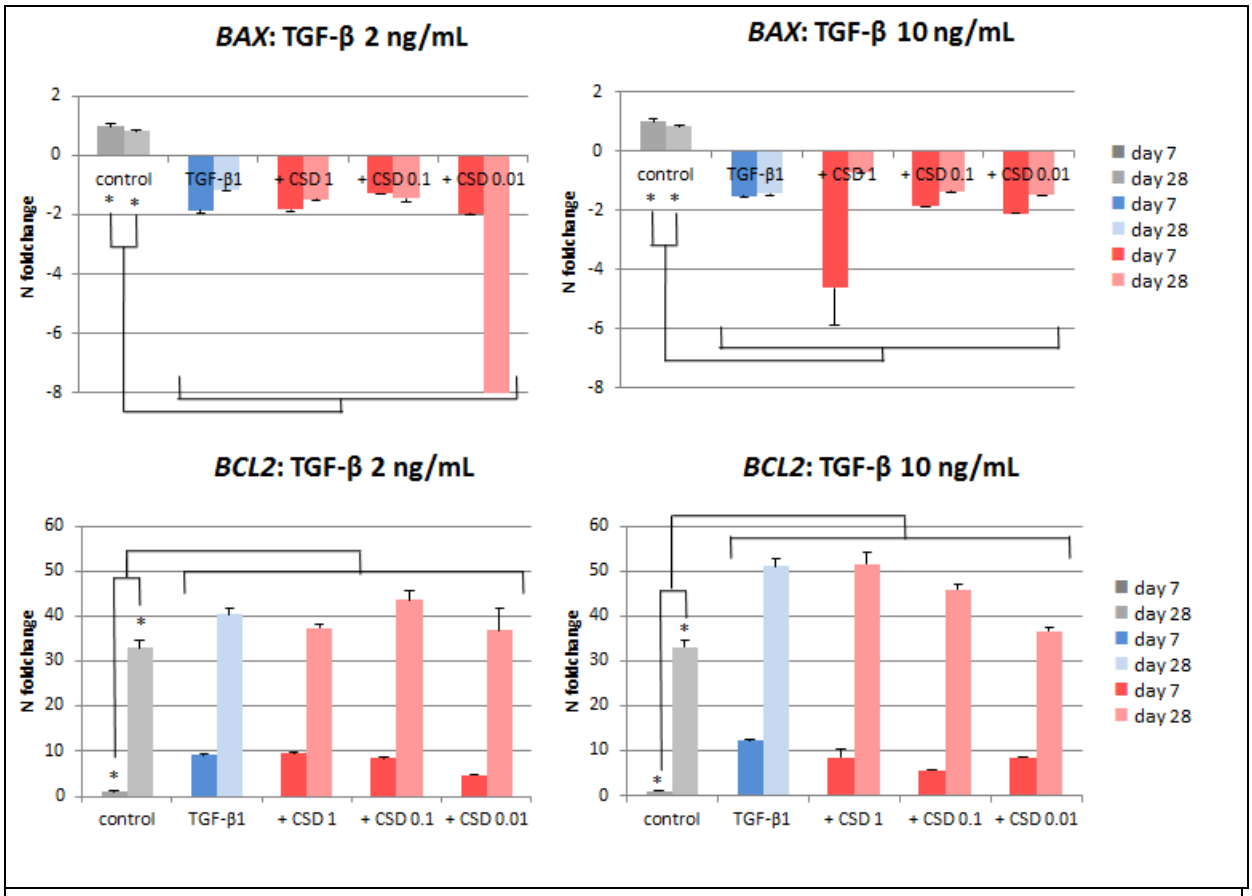


Figure 12. *BAX* and *BCL2* expression in NPC pellets cultured in basal chondrogenic medium and treated with TGF-β1 (blue), TGF-β1 in combination with caveolin-1 scaffolding domain (CSD) (red) or untreated (grey) for 7 or 28 days. The left graphs represent the relative gene expression of the NPC pellets that were cultured with the suboptimal concentration of TGF-β1 (2 ng/mL). The right graphs represent the relative gene expression of the NPC pellets that were cultured with the optimal concentration of TGF-β1 (10 ng/mL).* = CI does not contain value 1.

3.5. Discussion

3.5.1. Regenerative effects of TGF-β1 and CSD

Consistent with the previous pilot experiments, this study showed that TGF-β1 positively influenced cell proliferation and matrix production of chondrodystrophic canine NPCs. After 28 days of culture, all TGF-β1 (10 ng/mL) treated pellets presented an increase in DNA synthesis (4-fold) (**figure 1**) and GAG production (30-fold in pellet and 6-fold in medium respectively) compared with control pellets (**figures 2 and 3**). Even a suboptimal concentration of TGF-β1 (2 ng/mL) increased DNA synthesis by 4-fold and GAG production by 15-fold in pellet and 5-fold in medium respectively compared with (**figures 2 and 3**). A statistically significant dose-dependent effect of TGF-β1 treatment was present in most parameters concerning matrix production, i.e. GAG content, GAG release, GAG/DNA, and gene expression of *Aggrecan* and *Collagen II*, indicating that the concentration of 2 ng/ml TGF-β1 resulted in the desired suboptimal effect. However, both in the optimal and suboptimal concentration of TGF-β1, CSD exerted no additive effect upon TGF-β1 treatment regarding matrix production. No dose-dependent effect of TGF-β1 treatment was present concerning the DNA content of the pellets and gene expression of *CyclinD1*. This indicates that the 2 ng/mL concentration of TGF-β1 remained sufficient to exhibit an optimal effect on cell proliferation. Again, the addition of CSD exerted no additive effect upon TGF-β1 treatment regarding cell proliferation. The RT-qPCR results showed an upregulated gene expression of matrix proteins *Aggrecan* and *Collagen II* and cell proliferation marker *CyclinD1* and a downregulated gene expression of matrix proteinases *ADAMTS5* and *MMP13* in all TGF-β1 treated pellets (**figures 6, 7 and 8**). These results were confirmed by the increased GAG production and DNA synthesis caused by TGF-β1 treatment. Within the TGF-β1 10 ng/mL treatment, 1 µM CSD caused an enhanced downregulation of *ADAMTS5* expression, indicating that CSD shifts TGF-β signalling to the anabolic Smad2/3 pathway. In contrast, CSD (all concentrations) caused inhibition of TGF-β1 10 ng/mL induced downregulation of *MMP13* expression at day 7. This suggests that CSD shifts TGF-β signalling to the catabolic Smad/1/5/8 pathway. Finally, addition of CSD (all concentrations) caused an inhibition of the TGF-β1 10 ng/mL induced downregulation of *TIMP-1* expression at day 7 and day 28. Since *TIMP-1* inhibits matrix degradation, this finding also suggests that CSD shifts TGF-β signalling to the anabolic Smad2/3 signalling pathway. The effects of CSD on the gene expression of these matrix remodelling proteins were not reflected in differences in GAG content/release of the pellets (**figures 2 and 3**). The importance of the above described effect of CSD is therefore questionable.

Furthermore, the increased GAG production and cell proliferation by TGF-β1 treatment was confirmed by histology: all TGF-β1 treated pellets were considerably larger than the control pellets and showed an intense positive staining for GAGs (**figure 5**). Although CSD did not add to the intensity of the red staining, it should be noted that the staining was not sensitive enough to detect an additive effect of CSD, as sole TGF-β1 treatment (even supplemented in a suboptimal dose (2 ng/mL)) already caused a complete saturation of the red staining of the pellets.

TGF-β1 treatment inhibited apoptosis and stimulated anti-apoptosis, as demonstrated by the expression levels of *BAX* and *BCL2* (**figure 12**). This is in accordance with previous research showing that TGF-β1 has anti-apoptotic effects upon osteoarthritic human chondrocytes [54]. The anti-apoptotic effects of TGF-β1 treatment of canine NPCs were not additionally affected by CSD supplementation.

3.5.2. Effects of TGF-β1 and CSD on ALK5 (Smad2/3) and ALK1 (Smad1/5/8) signalling.

TGF-β can signal through 2 different receptors, i.e. ALK5 and the ALK1 [23,55]. Activation of the ALK5 receptor and subsequently the Smad2/3 signalling pathway results in anabolic effects (i.e. increased matrix production), while activation of the ALK1 receptor and subsequently the Smad1/5/8 signalling pathway results in catabolic effects (i.e. matrix degradation) (**General Introduction, figure 3**). The stimulating effect of TGF-β1 on matrix production in this study suggests that TGF-β1 signalled through the Smad2/3 signalling pathway, rather than the Smad1/5/8 signalling pathway. To explore this possibility, gene expression levels of *PAI1* and *ID1* were measured. *PAI1* is a readout marker for active ALK 5 (Smad2/3) signalling, while *ID1* is a readout marker for active ALK 1 (Smad1/5/8) signalling [56]. *PAI1/ID1* expression ratios confirm the expected shift to Smad2/3 signalling caused by TGF-β1 treatment: TGF-β1 pellets presented lower *PAI1/ID1* ratios than the control pellets, indicating a higher Smad2/3 signalling than Smad1/5/8 signalling in these pellets. As Smad1/5/8 signalling is associated with increased MMP13 expression [30], the downregulation of MMP13 in this study also indicates a decreased Smad1/5/8 signalling. These results demonstrate that TGF-β1 already mainly signals through the favourable Smad2/3 pathway, which is contradictive to the *in vivo* nucleus pulposus where TGF-β mainly signals through the catabolic Smad1/5/8 pathway [23]. The presence of various influencing factors, i.e. loading and influencing molecules or substances, may account for the different effects of TGF-β *in vivo*.

We hypothesized that CSD would establish a shift in ALK5 (Smad2/3) and ALK1 (Smad1/5/8) signalling by favouring ALK5 signalling over ALK1. However, our data did not support this hypothesis: CSD influenced neither *ALK5/ALK1* gene expression (with exception of 0.1 µM CSD on TGF-β1 2 ng/mL) nor *PAI1/ID1* gene expression (with the exception of 0.01 µM CSD on TGF-β1 10 ng/mL), indicative for ALK5 (Smad2/3) and ALK1 (Smad1/5/8) signalling. The relevance of these exceptions is questionable, given that the additive effects of 0.01 µM CSD on TGF-β1 10 ng/mL and of 0.1 µM CSD on TGF-β1 2 ng/mL failed to influence the gene expression of *Aggrecan*, *Collagen II* and *MMP13*.

3.5.3. Effects of TGF-β1 and CSD on Wnt/β-catenin signalling

TGF-β1 downregulated *Axin2* expression, suggesting that TGF-β1 treatment inhibits Wnt/β-catenin signalling. This is in accordance with previous research, which indicated that TGF-β1 suppressed *Axin2* expression via a Smad2/3 dependent mechanism [57]. Interestingly, CSD showed an inhibiting effect on the TGF-β1 10 ng/mL induced downregulation of the Wnt/β-catenin signalling pathway as from day 7. This could be explained by caveolin-1's previously described inhibiting effect on TGF-β receptors and TGF-β signalling (CSD is able to interact and compartmentalize with TGF-β receptors, thereby inhibiting both Smad2/3 and Smad1/5/8 signalling). However, CSD did not cause a downregulated expression of Smad2/3 read out

marker *PAI1* and hence this is not a satisfying explanation. CSD's reversing effects on TGF-β1 induced inhibition of the Wnt/β-catenin signalling could also be explained by caveolin-1's stimulating effects on Wnt/β-catenin signalling independently of the TGF-β signalling: caveolin-1 is capable of recruiting β-catenin to caveolar membranes, thereby inhibiting β-catenin's degradation. Moreover, caveolin-1 may activate Wnt/β-catenin signalling by activating the Wnt receptor and/or integrin-linked kinase [16,15]. Integrin-linked kinase prevents the breakdown of β-catenin, leading to persistent Wnt/β-catenin signalling. Although CSD did not have an effect on the expression of integrin-linked kinase (**figure 9**), CSD could still have activated ILK in terms of protein-protein interactions, thereby activating its kinase activity [16].

3.5.4. Additive effect caveolin-1 in donor A

No additive regenerative effect of CSD on TGF-β1 treatment was observed when pooling the results of all the different canine donors. However, when observing the results of the donors individually, all CSD concentrations in donor group A exerted an additive effect on TGF-β1 treatment regarding the GAG content of the pellet at day 28 (**figure 13**).

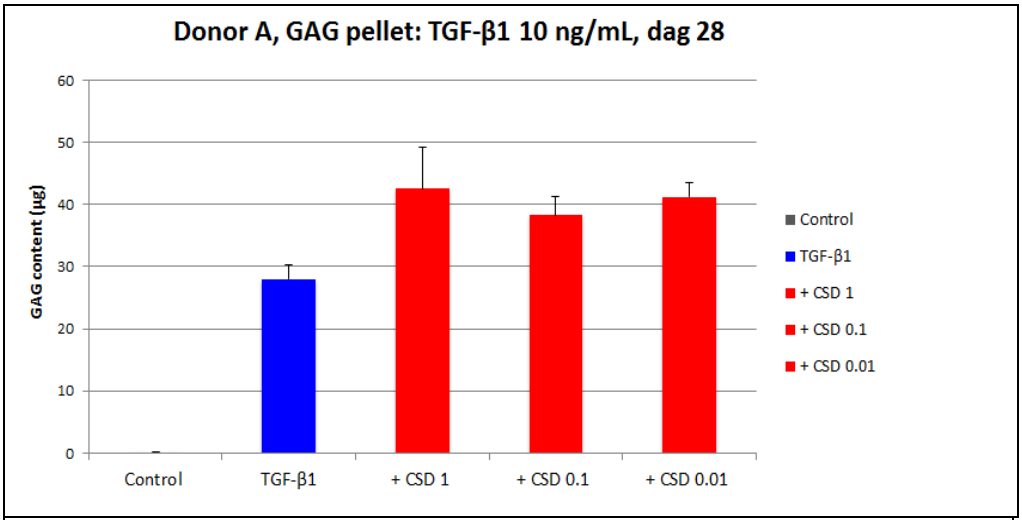


Figure 13. GAG content of the pellets of donor A. Pellets were cultured for 28 days and treated with TGF-β1 (blue), caveolin-1 scaffolding domain peptide (CSD) (red) or untreated (grey).

This donor-dependent additive effect could not be explained by differences in age, breed or gender. First of all, the mean age of donor group A was comparable with the mean age of the other donor groups (± 20 months). Secondly, all donors used in the study were Beagles. And thirdly, donor group A contained a mix of cells from a female and a male donor. This was similar to donor group C and D, which did not present an additive effect of CSD. This excludes a possible gender dependent effect.

To explain CSD's additive effect, donor group A was further investigated by comparing the *ALK5/ALK1* and *PAI1/ID1* ratios among all donor groups (**figure 14**). Donor group A did not exert a lower *ALK5/ALK1* or *PAI1/ID1* signalling compared with the other donor groups as would be expected when CSD had stimulated GAG production through TGF-β1 signalling. In fact, donor group A demonstrated higher *ALK5/ALK1* ratios at day 28 compared with the other donor

groups, which suggests even lower anabolic ALK5 (Smad2/3) signalling. Since there were no differences in basal Wnt/β-catenin signalling (*Axin2* expression), CSD's additive effect in donor group A could not be explained by differences in TGF-β or Wnt/β-catenin signalling. Hence, CSD's additive effect on donor group A remains and unsolved matter.

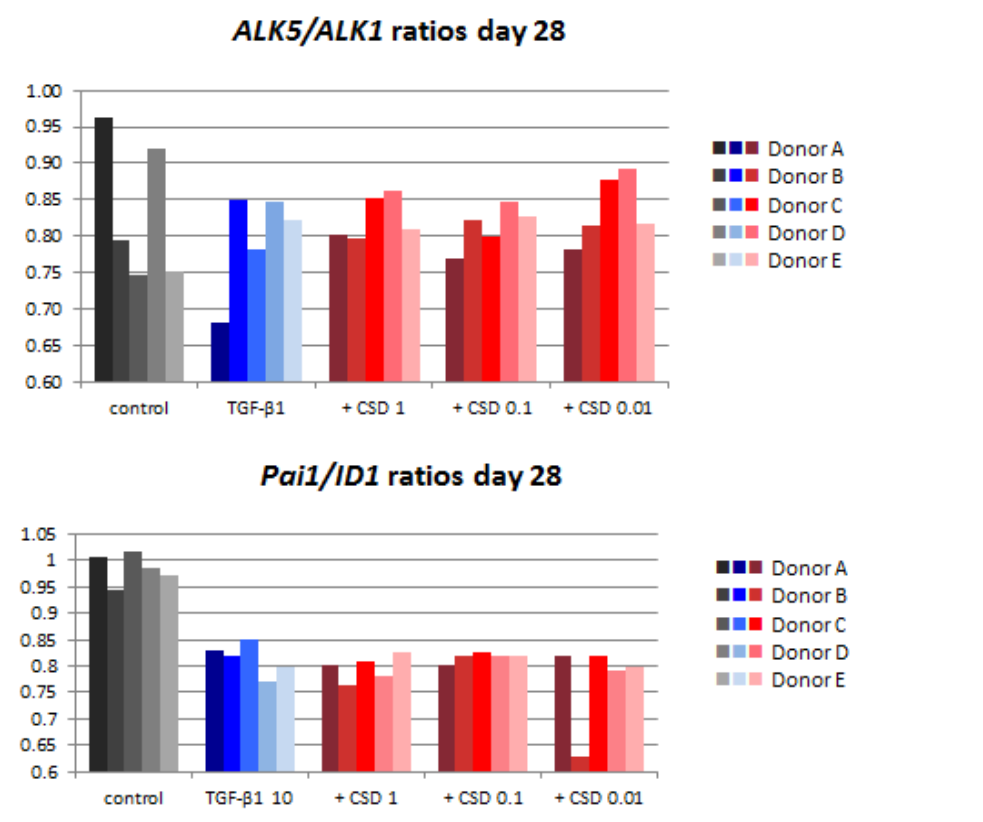


Figure 14. *ALK5/ALK1* and *PAI1/ID1* ratios of the different donors (from dark to light: A, B, C, D and E). Pellets of the different donors were cultured for 28 days and treated with TGF-β1 (blue), caveolin-1 scaffolding domain peptide (CSD) (red) or untreated (grey).

Overall, CSD (0.01, 0.1 and 1 μM) supplementation did not have an additive effect on the regenerative effects of TGF-β1 treatment (2 and 10 ng/mL) of early degenerated canine (CD) NPC pellets. One explanation could be that CSD does not have a regenerative effect on these specific young chondrodystrophic canine donors and that the specific effects may be related to the genetic background. In order to evaluate the influence of the genetic background on the effects of CSD, other donor types i.e. humans and (older) non-chondrodystrophic dogs, will be tested in a follow up experiment (chapter 4) to determine whether CSD has a regenerative effect upon their NPCs. Another explanation is that, at high concentrations, caveolin-1 and sole CSD treatment exert negative/degenerative effects upon NPCs in terms of cell proliferation and matrix production (which was indeed shown in the toxicity study, chapter 2), and that the concentration of 0.01-1 μM CSD is too low to exert any effect at all (hereby assuming that CSD indeed mimics caveolin-1 function as was hypothesized in the general introduction). However, the additive effect that was demonstrated for donor group A cannot be explained by this theory. Lastly, we cannot exclude that CSD may have functioned as a caveolin-1 antagonist instead of agonist, by interfering with caveolin-1 dependent signalling. If this is true, the donor-dependent

additive effect of CSD on TGF-β1 treatment of NPCs (donor group A) is difficult to explain, but can be caused by interference with parallel signaling pathways in this specific donor.

3.6. Conclusions

TGF-β1 treatment stimulated cell proliferation and matrix production in the NPC pellets from chondrodystrohpic dogs. Supplementation with 2 ng/ml TGF-β1 successfully resulted in the desired suboptimal stimulating effects regarding the matrix production. Despite these suboptimal effects, CSD did not have an additive regenerative effect upon TGF-β1 treatment of chondrodystrophic canine NPCs. As an exception, CSD showed a slight additive effect on optimal TGF-β1 treatment (10 ng/mL) regarding the GAG production in a single donor group at day 28 of the culture period. Apart from being statistically insignificant, this donor-dependent additive effect of CSD was unaccountable. Hence, in the present culture set-up, the scaffolding domain of caveolin-1 did not appear to be a promising target for regenerative treatment strategies.

4. Additive effect of caveolin-1 scaffolding domain on TGF-β1 treatment of nucleus pulposus cells of non-chondrodystrophic dogs and humans

4.1. Abstract

Introduction. Non-chondrodystrophic dogs and chondrodystrophic dogs differ in the onset of IVD degeneration. Non-chondrodystrohpic dogs closely resemble the onset of IVD degeneration in humans, as both dog and human patients develop IVD degeneration at an old age. Since the ALK1/ALK5 signalling ratio increases with ageing, caveolin-1 scaffolding domain peptide (CSD) may establish a more pronounced regenerative effect upon TGF-β1 treatment in these canine and human patients. **Material and methods**. Pellets containing canine or human nucleus pulposus cells (NPCs) were cultured for 0, 7 and 28 days in basal chondrogenic medium. The pellets were treated with TGF-β1 or TGF-β1 in combination with 1, 0.1 or 0.01 µM CSD. Glycosaminoglycan (GAG) content and release in the culture medium of these NPC pellets was determined using a DMMB assay in order to quantify matrix production. Cell proliferation was quantified by measuring the DNA content of the pellets. RT-qPCR was performed to determine the gene expression level of relevant genes. Histology on the cell pellets was performed using Safranin O/Fast Green staining. **Results**. TGF-β1 stimulated matrix production in canine and human NPCs. Furthermore, TGF-β1 had a proliferative effect on the canine NPCs. However, CSD did not have any additional effect on the TGF-β1 treatment. **Conclusion**. CSD did not exert a regenerative additive effect upon TGF-β1 treated NPCs originating from humans and non-chondrodystrophic dogs and hence, the scaffolding domain of caveolin-1 was not proven to be a suitable target for developing regenerative strategies against IVD degeneration in the present study set up.

4.2. Introduction

Both chondrodystrophic (CD) and non-chondrodystrophic (NCD) breeds are used as animal models with construct validity for IVD degeneration [1,65]. Despite the similarity in pathophysiological processes between these dog breeds and between dogs and humans, there are differences in etiology [6]. IVD degeneration in chondrodystrophic dogs is believed to be of genetic origin, as their IVDs show signs of degeneration from an early age. Conversely, IVD degeneration in NCD dogs is more likely to be caused by "wear and tear" or chronic IVD stress and hence IVD degeneration develops over a longer time period. As the latter resembles the human etiology of IVD degeneration, NCD breeds are thought to be a more suitable model for investigating the development of human IVD degeneration [6]. The aim of this study was to determine a possible additive effect of caveolin-1 scaffolding domain peptide (CSD) on TGF-β1 treatment of nucleus pulposus cells (NPCs) originating from middle-aged NCD dogs and humans. Since NCD dogs and humans suffer from IVD degeneration at an old age, these middle-aged donors were suitable for determining the effect of CSD on early IVD degeneration. Moreover, as the ALK1/ALK5 ratio increases with age [30], these older donors ought to have a

higher ALK1/ALK5 ratio compared with the young CD donors (described in chapter 3). This change in balance between both ALK pathways may affect the action of CSD. For instance, CSD could establish a more pronounced effect on inhibiting the ALK1 pathway and by that rebalance the ALK1/ALK5 ratio.

4.3. Material and methods

For this study, cells of 4 canine donors and 3 human donors were used (**table 1**). Donor NPC 796 suffered from Cushing's disease. Consequently, the nucleus pulposi of this donor may be more severely impaired due to the fact that high cortisol levels have negative effects upon the intervertebral disc [58].

Species	Donor name	Age (years)	Breed	Gender	Relevant details
Canine	NPC 796	5	Irish setter	Female	Cushing's disease
	NPC 994	1.5	Mixed breed	Female	
	NPC 190	2	Mixed breed	Female	
	NPC 364	2	Mixed breed	Female	
Human	S13-061	48	-	Female	Thompson grade 3
	S13-128	48	-	Male	Thompson grade 3
	S13-142	47	-	Female	Thompson grade 3

Table 1. Overview of the canine and human donor characteristics.

4.3.1. Cell culture

The moderately degenerated human NPCs were kindly provided by the UMC Utrecht. The early (mildly) degenerated canine NPCs were collected and isolated as described in chapter 2. Cells were expanded in expansion medium (**chapter 2, table 2**) in 175 cm^2 cell culture flasks (Cellstar, Greiner bio-one). The human and canine cells were cultured until passage 2 and passage 3 respectively, to acquire an adequate amount of cells to start the experiment. Next, the cells were cultured in 96 wells plates (Corning Costar 7007) with a density of 200.000 cells/well. Cells were pelleted by centrifugation at 185g for 8 minutes and cultured for 0, 7 or 28 days under several culture conditions. Two pellets were immediately stored for GAG and DNA measurements. The other pellets were cultured in plain chondrogenic medium (**chapter 2, table 2**). Control pellets were not treated, whereas the other pellets were treated with either recombinant human TGF-β1 (10 ng/mL) (R&D Systems, 240-B-010) or TGF-β1 in combination with CSD (Enzo Life Sciences, ALX-153-064) (0.01, 0.1 and 1 μM). The culture media were

refreshed two times a week. The culture medium was collected to determine the weekly GAG release.

Culture condition	Time in culture	Number of pellets per donor group		
		PCR	GAG/DNA	Histology
Control	day 0	-	2	-
	day 7	2	2	2
	day 28	-	2	2
TGF-β1 (10ng/mL)	day 7	2	2	2
	day 28	-	2	2
TGF-β1 (10 ng/mL) + CSD (0.01 µM)	day 7	2	2	2
	day 28	-	2	2
TGF-β1 (10 ng/mL) + CSD (0.1 µM)	day 7	2	2	2
	day 28	-	2	2
TGF-β1 (10 ng/mL) + CSD (1 µM)	day 7	2	2	2
	day 28	-	2	2

Table 2. Schematic overview of the study design.

4.3.2. Outcome parameters

Cell proliferation and matrix production were used as the main outcome parameters to measure the regenerative effects of CSD. The weekly GAG release and GAG content of the pellets at day 7 and day 28 were measured with a DMMB assay to quantify matrix production. In addition, pellets of day 7 and day 28 were subjected to a Safranin O/Fast Green staining. The pellets' DNA content was measured at day 0, 1, 7 and 28 to quantify cell proliferation. Furthermore pellets were collected at day 7 and pooled for RNA isolation to assess gene expression of relevant markers. An overview of the outcome parameters is given in **Table 2**.

4.3.2.1. DNA content

As previously described (chapter 2, p.19), two pellets per donor per condition were lysed using papain digestion (per mL solution: 1,57 mg cysteine HCL (Sigma, C7880) and 250 µg papain (Sigma, P3125)) and DNA content was measured using the Qubit® dsDNA High Sensitivity Assay Kit (Invitrogen, Q32851) according to the manufacturer's instructions.

4.3.2.2. GAG content and GAG release

As previously described (chapter 2, p.20), a Di-Methyl Methylene Blue (DMMB) assay was performed on the collected culture media and the digested pellets that were initially used for DNA analysis.

4.3.2.3. RNA isolation

Per culture condition, 2 pellets of each donor were cultured for RNA-isolation. As previously described (chapter 2, p.20), pellets were crushed on dry ice in order to release the RNA. The

lysed samples of the same donor within every culture condition were pooled so that single measurements could be performed. The released RNA was extracted using the RNeasy® Micro kit (Qiagen, 74004) according to the manufacturer's instructions. The obtained RNA was quantified using the Nanodrop®-1000 spectrophotometer (Thermo Scientific).

4.3.2.4. cDNA

As previously described (chapter 2, p.20), cDNA was synthesized using the iScript™ cDNA Synthesis Kit (Bio-Rad, Veenendaal, the Netherlands, 170-8891), according to the manufacturer's instructions. An RNA input of 100 ng of each sample was used to initiate cDNA reactions.

4.3.2.5. RT-qPCR

Gene expression levels were only determined on day 7, since the previous experiments (chapter 2 and chapter 3) demonstrated that measuring gene expression at day 28 has no additional benefits. As previously described (chapter 2, p.30), RT-qPCR was performed on the human and canine pellets using the iQ™ SYBR Green Supermix Kit (Bio-rad, Veenendaal, the Netherlands) and MyiQ™ single color Real-Time PCR Detection System (Bio-Rad, Veenendaal, the Netherlands). Depart from the protocol, 2-fold standard dilutions were prepared for the human samples. This was necessary to obtain decent standard curves. Relative gene expression was calculated using the Pfaffl method. A set of 6 reference genes were used for the human RT-qPCR samples: *GAPDH, HPRT, TBP, B2M, YWHAZ, SDHA,* and the following target genes were tested: *Aggrecan, Collagen II, ADAMTS5, MMP13, TIMP-1, CyclinD1, Collagen I, Axin2, BCL2* and *BAX*. Regarding the canine RT-qPCR samples, a set of 4 reference genes were used: *GAPDH*, *HPRT*, *RPS19* and *SDHA*,and the following target genes were tested: *Aggrecan*, *Versican*, *Collagen II*, *ADAMTS5*, *TIMP-1*, *MMP13*, *CyclinD1*, *Collagen I*, *Axin2*, *PAI1*, *Caspase3*, *ALK1*, *ALK5*, *ID1, BCL2* and *BAX* (**table 3**). A 3-step protocol was carried out for the human primers *SDHA, ADAMTS5, Sox9, ALK1* and *Axin2*, starting with a 2-minute incubation period at 95 °C in order to initiate denaturation and enzyme activation. This was followed by 40 cycles of 10 seconds incubation at 95 °C, 15 seconds incubation at 55°C and 30 seconds incubation at 55 °C. Finally, temperature was increased again to 95 °C with 0.5 °C increments in order to generate a melt curve. The other primers were carried out using a 2-step protocol as described (chapter 2, p.20).

Species	Gene	Sequence	Tm annealing
Canine	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'	
	Aggrecan	5' GGACACTCCTTGCAATTTGAG 3'	61 - 62
		5' GTCATTCCACTCTCCCTTCTC 3'	
	Collagen II	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'	
	PAI1	5' AAACCTGGCGGACTTCTC 3'	61.5
		5' ACTGTGCCACTCTCATTCAC 3'	
	BCL2	5' GGATGACTGAGTACCTGAACC 3'	61.5 - 63
		5' CGTACAGTTCCACAAAGGC 3'	
	ALK1	5' CCTTTGGTCTGGTGCTGTG 3'	61
		5' CGAAGCTGGGATCATTGGG 3'	
	ALK5	5' GAGGCAGAGATTTATCAGACC 3'	59.5
		5' ATGATAATCTGACACCAACCAG 3'	
	ID1	5' CTCAACGGCGAGATCAG 3'	59.5
		5' GAGCACGGGTTCTTCTC 3'	
	BAX	5' CCTTTTGCTTCAGGGTTTCA 3'	58 - 59
		5' CTCAGCTTCTTGGTGGATGC 3'	
	ILK	5' AAAGCAGGGACTTCAATGAGGA 3'	64
		5' ACTTCACAGCTTGGCTCTGG 3'	
Human	GAPDH	5' CAAGATCATCAGCAATGCCT 3'	60
		5' CAGGGATGATGTTCTGGAGAG 3'	
	TBP	5' TGCACAGGAGCCAAGAGTGAA 3'	63.5
		5' CACATCACAGCTCCCCACCA 3'	
	HPRT	5' TATTGTAATGACCAGTCAACAG 3'	60
		5' GGTCCTTTTCACCAGCAAG 3'	
	B2M	5' CTTTGTCACAGCCCAAGATAG 3'	58
		5' CAATCCAAATGCGGCATCTTC 3'	
	SDHA	5' TGGGAACAAGAGGGCATCTG 3'	58 (3-step)
		5' CCACCACTGCATCAAATTCATG 3'	
	YWHAZ	5' ACTTTTGGTACATTGTGGCTTCAA 3'	64
		5' CCGCCAGGACAAACCAGTAT 3'	
	Aggrecan	5' CAACTACCCGGCCATCC 3'	63.5
		5' GATGGCTCTGTAATGGAACAC 3'	
	Collagen II	5' AGGGCCAGGATGTCCGGCA 3'	62-63.5
		5' GGGTCCCAGGTTCTCCATCT 3'	
	ADAMTS5	5' GCCAGCGGATGTGCAAGC 3'	62.5 (3-step)
		5' ACACTTCCCCCGGACGCAGA 3'	
	Collagen I	5'TCCAACGAGATCGAGATCC 3'	58-64
		5' AAGCCGAATTCCTGGTCT 3'	
	ALK1	5'GCAACCTGCAGTGTTGCATC 3'	62.5 (3-step)
		5' CGGATCTGCTCGTCCAGCAC 3'	

	ALK5	5' GCCGTTTGACTGAAGGCTG 3'	61
		5' GGGCATCCCAAGCCTCATC 3'	
	Axin2	5' GGTAGGCATTTTCCTCCATCAC3'	57 (3-step)
		5' AGCCAAAGCGATCTACAAAAGG 3'	
	BAX	5' GGACGAACTGGACAGTAACATGG 3'	58-64
		5' GCAAAGTAGAAAAGGGCGACAAC 3'	
	BCL2	5' ATCGCCCTGTGGATGACTGAG 3'	64
		5' CAGCCAGGAGAAATCAAACAGAGG 3'	
	MMP13	5' TCCCAGGAATTGGTGATAAAGTAGA 3'	64
		5' CTGGCATGACGCGAACAATA 3'	
	TIMP-1	5' CTTCTGGCATCCTGTTGTTG 3'	56-64
		5' GGTATAAGGTGGTCTGGTTG 3'	
	CyclinD1	5' AGCTCCTGTGCTGCGAAGTGGAAAC 3'	65
		5' AGTGTTCAATGAAATCGTGCGGGGT 3'	

Table 3. Specific information of the human and canine primers.

4.3.2.6. Histology

Two pellets per donor per condition were cultured for histology. At day 7 and day 28, pellets were prepared for histology as previously described (chapter 2, p.21). Histological slides were stained with Safranin O/Fast Green using the same protocol as previously described (chapter 2, p.21). Images of the sectioned pellets are not reliable for drawing conclusions regarding pellet size, since sections that originate from the centre of a pellet are larger compared with sections that originate from the terminus of the pellet. In order to elaborate on differences in pellet size across the different culture groups, microscopic images of the pellets were obtained at day 7 and day 28 before pellets were used for histology.

4.3.2.7. Statistics

As the data of the DMMB assay and the DNA measurements were not normally distributed, it was analysed using the non-parametric Mann Whitney test in SPSS for Windows version 20. P-values were corrected for multiple comparisons using the Benjamini Hochbergs' False Discovery Rate procedure. RT-qPCR could not be analysed with a cox regression model, since R studio version 3.0.2. was not capable of converging the model that fit the small RT-qPCR dataset. Instead, statistical analyses were performed on delta-CT values using the non-parametric Mann Whitney test in SPSS for Windows version 20. These P-values were also corrected for multiple comparisons using the Benjamini Hochbergs' False Discovery Rate procedure.

4.4. Results

This section describes the effects of CSD (0.01 μM, 0.1 μM and 1 μM) and TGF-β1 treatment (10 ng/mL) on outcome parameters cell proliferation and extracellular matrix production on NPC pellets (200,000 cells) originating from humans and non-chondrodystrophic dogs. With respect to the cell proliferation, DNA content of the NPC pellets was measured. The extracellular matrix production was investigated by performing a DMMB assay and a Safranin O/Fast Green

staining. The mechanisms (signalling pathways) that underlie the effects of CSD and TGF-β1 treatment were investigated using RT-qPCR.

4.4.1. Cell proliferation

Cell proliferation was quantified by monitoring the DNA content of the human and canine NPC pellets on day 0, 7 and 28 of the culture period.

4.4.1.1. DNA content

The DNA content of the canine control pellets decreased over time, indicating that NPCs had died during culture (**figure 1**). The DNA content of the canine TGF-β1 treated pellets remained constant over time, resulting in a DNA content of the canine TGF-β1 treated pellets that was significantly higher compared with canine control pellets at the end of the culture period ($P<0.001$). The opposite is true for the human pellets: the DNA content of the control pellets increased over time and no differences were observed between control and TGF-β1 treated pellets throughout the culture period (**figure 2**). These findings indicate that TGF-β1 did not have a proliferative effect on the human NPCs and that the human NPCs were capable of proliferating without the presence of TGF-β1. CSD (0.01 µM, 0.1 µM and 1 µM) did not have an additive effect on the TGF-β1 treated canine or human pellets concerning DNA synthesis.

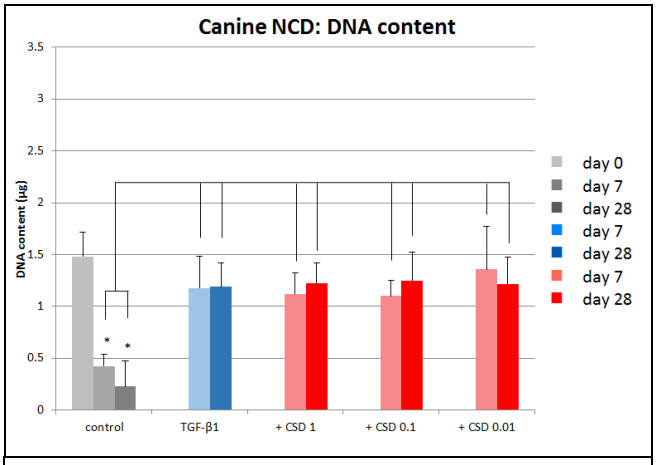


Figure 1. DNA content of the canine pellets treated with TGF-β1 (blue), TGF-β1 in combination with caveolin-1 scaffolding domain peptide (CSD) (red) or untreated (grey) for 0, 7 and 28 days. * = p<0.001

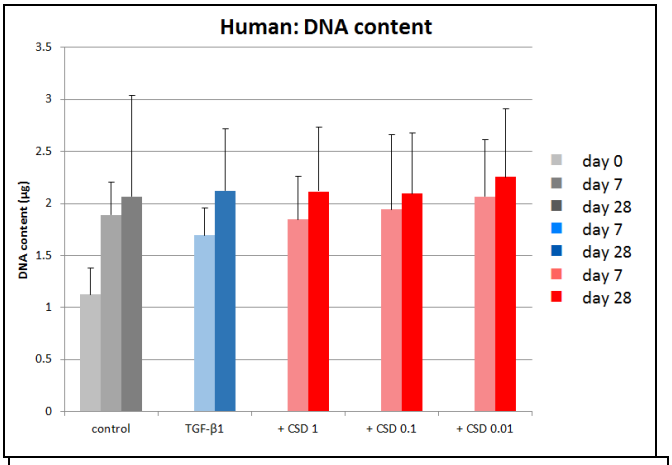


Figure 2. DNA content of the human pellets treated with TGF-β1 (blue), TGF-β1 in combination with caveolin-1 scaffolding domain peptide (CSD) (red) or untreated (grey) for 0, 7 and 28 days.

4.4.2. Extracellular matrix production

In addition to cell proliferation, extracellular matrix (GAG) production was used as an outcome parameter to measure the potential additive regenerative effects of CSD (0.01 µM, 0.1 µM and 1 µM) supplementation to TGF-β1 treatment. This parameter was quantified by monitoring the GAG content on day 7 and 28 of the culture period and by measuring the weekly GAG release of the human and canine NPC pellets in the culture medium. To support the results of the DMMB assay, extracellular matrix (GAG) content of the pellets was visualized by carrying out Safranin

O/Fast Green staining. To elaborate on differences in size between the NPC pellets, microscopic images were obtained on day 7 and day 28.

4.4.2.1. DMMB assay

In both canine and human TGF-β1 treated pellets, the GAG content increased over time ($p<0.05$) (**figures 3 and 4**). In both species, TGF-β1 treatment increased the GAG content of the pellets compared with the control pellets ($p<0.01$). However, the stimulating effect of TGF-β1 was not additionally affected by CSD. Applying a DNA correction to the GAG content of the pellets resulted in a pattern similar to the uncorrected data (with the same statistical significant differences across the culture groups), hence data is not shown.

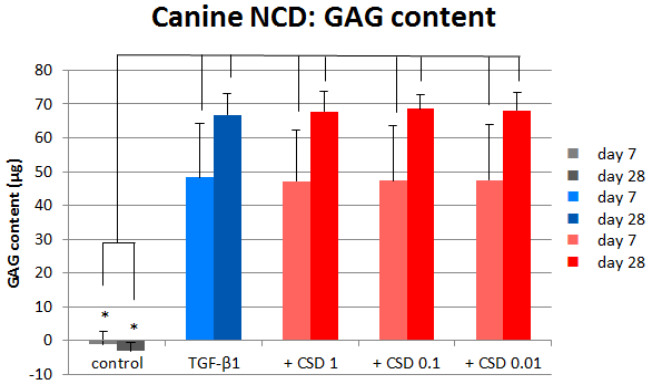


Figure 3. GAG content of the canine pellets treated with TGF-β1 (blue), TGF-β1 in combination with caveolin-1 scaffolding domain peptide (CSD) (red) or untreated (grey) for 7 and 28 days. * = $p<0.01$

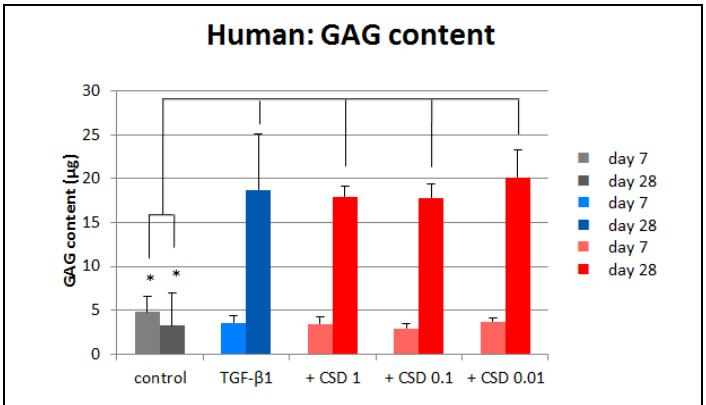


Figure 4. GAG content of the human pellets treated with TGF-β1 (blue), TGF-β1 in combination with caveolin-1 scaffolding domain peptide (CSD) (red) or untreated (grey) for 7 and 28 days. * = $p<0.01$

Next to the GAG content of the human and canine NPC pellets, the GAG release into the culture medium was measured every week of the total culture period (4 weeks). As from week 1 ($p<0.001$), the canine TGF-β1 treated pellets produced significantly more GAGs compared with the control pellets and their GAG production remained constant over time (**figure 5)**. The GAG release of the human TGF-β1 treated pellets increased over time, and was significantly higher compared to control as from week 3 onwards ($p<0.01$) (**figure 6**).

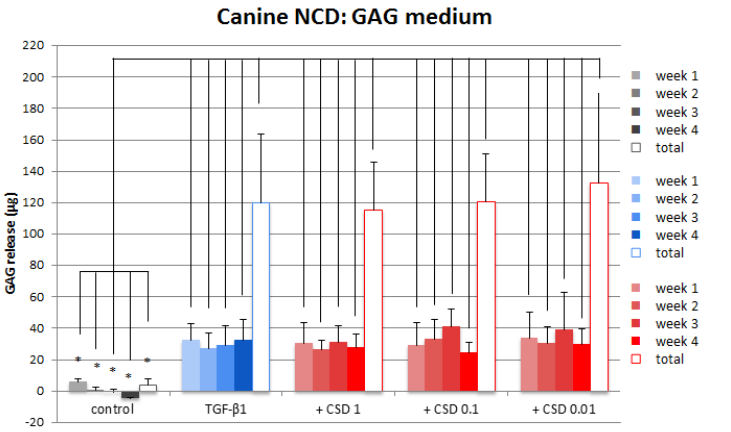


Figure 5. The weekly (1-4) GAG release of the canine pellets treated with TGF-β1 (blue), TGF-β1 in combination with caveolin-1 scaffolding domain peptide (CSD) (red) or untreated (grey). * = p<0.001

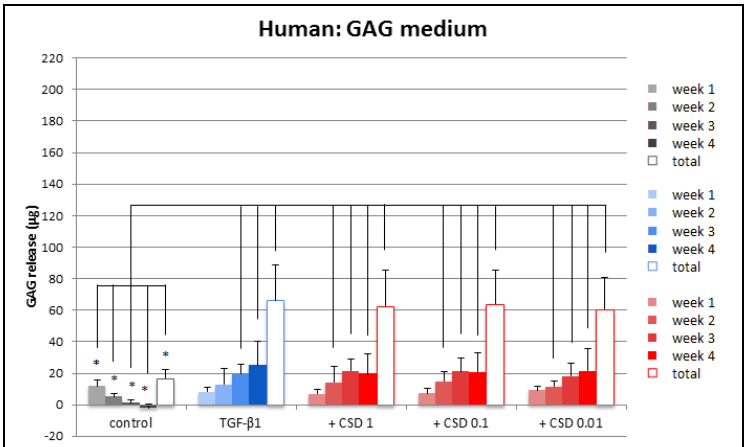


Figure 6. The weekly (1-4) GAG release of the human pellets treated with TGF-β1 (blue), TGF-β1 in combination with caveolin-1 scaffolding domain peptide (CSD) (red) or untreated (grey). * = p<0.01

4.4.2.2. Histology

In order to support the results of the DMMB assay, NPC pellets were subjected to a Safranin O/Fast Green staining on day 7 and 28 to visualize GAG production. Images of the stained pellets of canine donor 190 and human donor S13-142 (**figures 7 and 8**), are representative for all canine and human donors respectively. All control pellets (canine and human) stained negative at both day 7 and day 28 of the culture period. Canine TGF-β1 treated pellets already stained positive after 7 days of treatment. Conversely, human TGF-β1 treated pellets stained negative at day 7 but turned positive at day 28. Moreover, canine control pellets were considerably smaller compared with the treated pellets, as shown by the microscopic images of the unsectioned pellets that were obtained at days 7 and 28 of the culture period (**figure 9**). This is in contrast with the human control pellets, which retained a similar size compared with the TGF-β1 treated pellets throughout the culture period (**figure 10**).

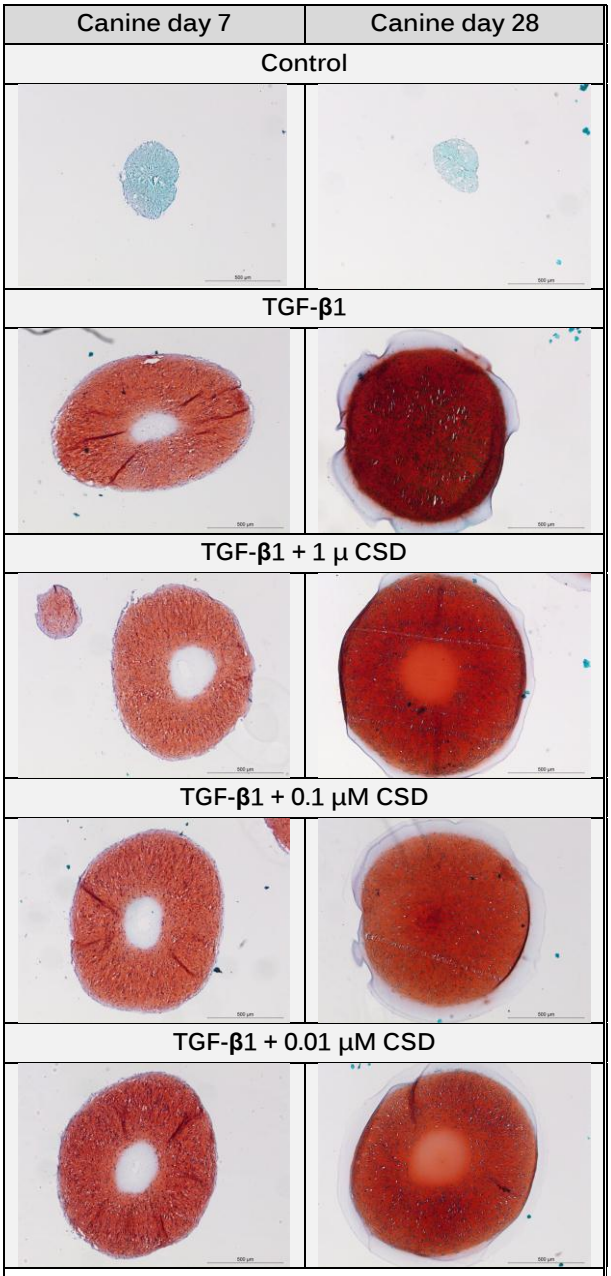


Figure 7. Images of the pellets of canine donor 190 that were stained with Safranin O/Fast Green in order to detect the presence of GAGs in pellets (red staining). The left images display the pellets that were cultured for 7 days, whereas the right images display the pellets that were cultured for 28 days.

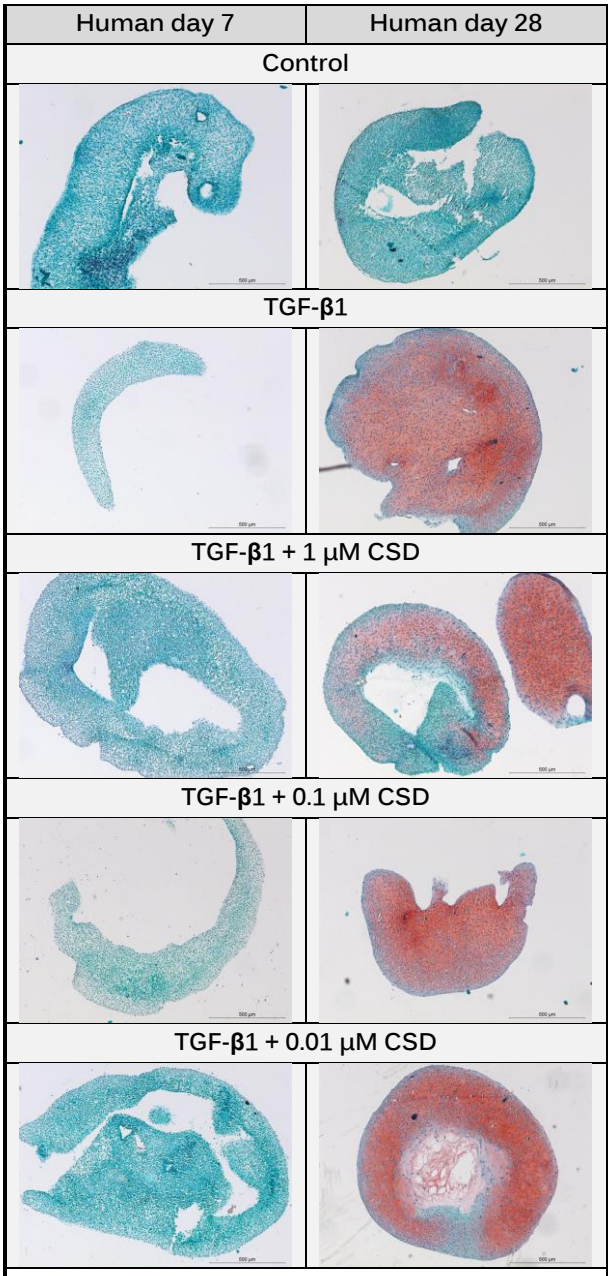


Figure 8. Images of the pellets of human donor S13-142 that were stained with Safranin O/Fast Green in order to detect the presence of GAGs in pellets (red staining). The left images display the pellets that were cultured for 7 days, whereas the right images display the pellets that were cultured for 28 days.

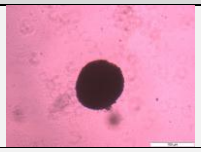
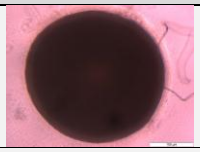
Canine: control	Canine: TGF-β1
	

Figure 9. Microscopic images of (intact) pellets from canine donor 190 at day 28. Since the solely treated TGF-β1 pellets and the TGF-β1 + caveolin-1 scaffolding domain peptide (CSD) combinatory treated pellets were equal in size, combinatory treated pellets are not shown.

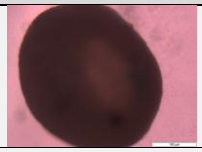
Human: control	Human: TGF-β1
	

Figure 10. Microscopic images of (intact) pellets from human donor S13-142 at day 28. Since the solely treated TGF-β1 pellets and the TGF-β1 + caveolin-1 scaffolding domain peptide (CSD) combinatory treated pellets were equal in size, combinatory treated pellets are not shown.

4.4.3. RT-qPCR

To investigate the signalling pathways underlying the effects on DNA and GAG content of the human and canine NPC pellets, the relative gene expression levels of several matrix proteins (*Aggrecan, Collagen II, Collagen I*), matrix remodelling proteins (*ADAMTS5, MMP13, TIMP1*), cell proliferation marker (*CyclinD1*), markers concerning the Wnt/β-catenin signalling (*Axin2*) and TGF-β signalling (*ALK5, ALK1, PAI1, ID1*) were determined.

The RT-qPCR data lacked statistical power for two reasons: only 3 human donors and 4 canine donors were included in the experiment and only single measurements were performed. Consequently, no significant differences were found in the human data. The canine RT-qPCR data initially showed significant differences between all TGF-β1 treated pellets and control pellets with respect to the expression of *Aggrecan*, *Collagen II, ADAMTS5, TIMP-1, CyclinD1, Collagen I, Axin2, PAI1, BAX*, and *ALK5/ALK1* and *PAI1/ID1* ratios. However, the effects of sole TGF-β1 treatment on the expression of these markers lost their statistical significance after correcting for multiple comparisons. This was caused by the fact that the P-value correction was most strict on the lowest p-values (i.e. the P-values of the effects of sole TGF-β1 treatment).

4.4.3.1. Extracellular matrix production

Proteoglycan *Aggrecan* and matrix protein *Collagen II* are largely present in healthy nucleus pulposus tissue [1,2]. On the other hand, *Collagen I* is unpliable and therefore not suitable for the shock absorbing function of the nucleus pulposus.

Aggrecan and *Collagen II* expression were both strongly upregulated in all canine and human TGF-β1 treated pellets compared with controls (*Aggrecan*: 90-fold and 6-fold and *Collagen II*: 5000-fold and 50-fold in canine and human pellets respectively) (**figures 11 and 12**). In the canine pellets that were exclusively treated with TGF-β1, *Aggrecan* and *Collagen II* expression were not significantly upregulated compared with the control pellets. The canine pellets that received additional treatment with 0.1 μM and 0.01 μM CSD presented significantly upregulated *Aggrecan* (CSD 0.1 μM p=0.039, CSD 0.01 μM p=0.029) and *Collagen II* (CSD 0.1 μM p=0.039, CSD 0,01 μM p=0.029) expression compared with the control pellets. Neither of the CSD concentrations had an additive effect on the TGF-β1 induced upregulation of *Aggrecan* (0.1 μM p=0.886, 0.01 μM p=1.000) and *Collagen II* (0.1 μM p=0.886, 0.01 μM p=0.686).

Collagen I expression was upregulated in all canine and human TGF-β1 treated pellets compared with controls (**figures 11 and 12**). Similar to the upregulation of *Aggregan* and *Collagen II* expression, this upregulation was only significant in the canine TGF-β1 treated pellets that received additional treatment with 0.1 μM CSD ($p<0.05$) and 0.01 μM CSD ($p<0.05$). None of the CSD concentrations had an additional effect on TGF-β1 induced upregulation of *Collagen I* expression (0.1 μM $p=0.886$, 0.01 μM $p=0.486$).

Thus, TGF-β1 increased the expression of both favourable matrixproteins (*Aggrecan* and *Collagen II)* and unfavourable matrixproteins (*Collagen I*) compared with controls. CSD (0.01 μM, 0.1 μM and 1 μM) had no additional effects on the expression of these matrix proteins upon TGF-β1 treatment.

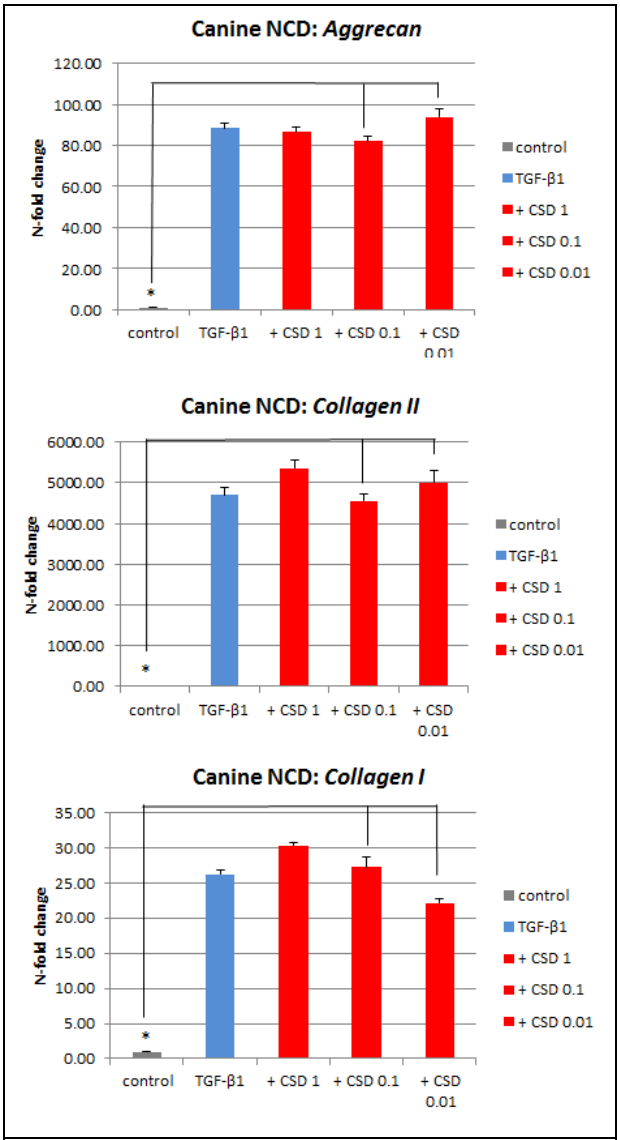


Figure 11. Relative gene expression of matrix proteins *Aggregan, Collagen II* and *Collagen I* in the canine pellets treated with TGF-β1 (blue), TGF-β1 in combination with caveolin-1 scaffolding domain peptide (CSD) (red) or untreated (grey) for 7 days. * = $p<0.05$

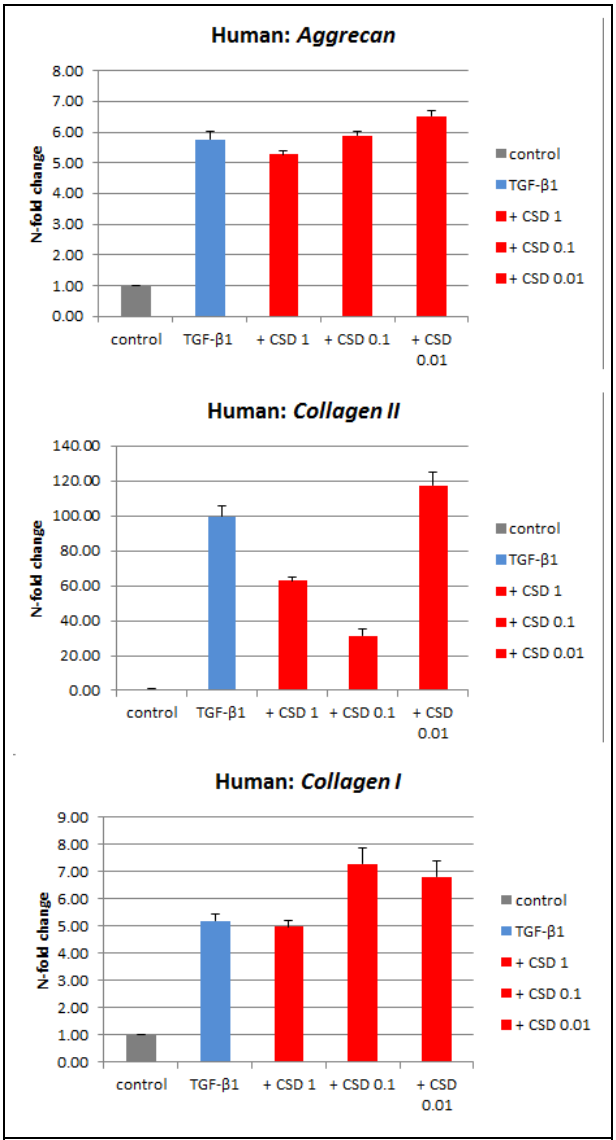


Figure 12. Relative gene expression of matrix proteins *Aggregan*, *Collagen II* and *Collagen I* in the human pellets treated with TGF-β1 (blue), TGF-β1 in combination with caveolin-1 scaffolding domain peptide (CSD) (red) or untreated (grey) for 7 days.

4.4.3.2. Matrix degradation proteins

Metalloproteinases, ADAMTS5 and MMP13 degrade extracellular matrix proteins. ADAMTS5 targets proteoglycans such as *Aggrecan*, whereas *MMP13* breaks down both proteoglycans and collagen fibers [52]. *TIMP-1 inhibits the action of such metalloproteinases* [53].

TGF-β1 treatment caused a downregulation of *MMP13* and *ADAMTS5* expression by 11-fold and 40-fold in the canine pellets compared with the controls, but this effect lacked statistical significance (**figure 13**). Only TGF-β1 treated pellets that received additional treatment with CSD revealed significantly downregulated *ADAMTS5* expression (0.1 µM, $p<0.05$ and 0.01 µM, $p<0.05$) compared with the controls. These CSD concentrations seemed to elicit an additional downregulation of the expression of these matrix proteinases, but these effects lacked statistical significance (*ADAMTS5*: 0.1 µM CSD ($p=0.886$) and 0.01 µM CSD ($p=1.000$), MMP13: 0.1 µM CSD ($p=0.886$) and 0.01 µM CSD ($p=0.686$)). TGF-β1 treatment caused a restricted downregulation of *ADAMTS5* expression in the human pellets (<1.5-fold) compared with the canine pellets, without any additional effects of CSD (**figure 14**). The expression of *MMP13* in the human pellets was too low to be detected in the RT-qPCR samples. Therefore, data concerning *MMP13* gene expression in the human pellets is not shown.

Matrix proteinase inhibitor *TIMP-1* expression was slightly downregulated in all canine TGF-β1 treated pellets (**figure 13**). Although all human TGF-β1 treated samples presented a stronger downregulation of *TIMP-1*, the TGF-β1 induced downregulation of *TIMP-1* did not reach statistical significance (**figure 14**). CSD did not have an additive effect upon the TGF-β1 induced downregulation of *TIMP-1*.

Briefly, TGF-β1 treatment decreased the expression of matrix degradation markers *ADAMTS5*, *MMP13* and *TIMP-1* in both human and canine NPC pellets. CSD did not have any additional effects on the expression of these markers upon TGF-β1 treatment.

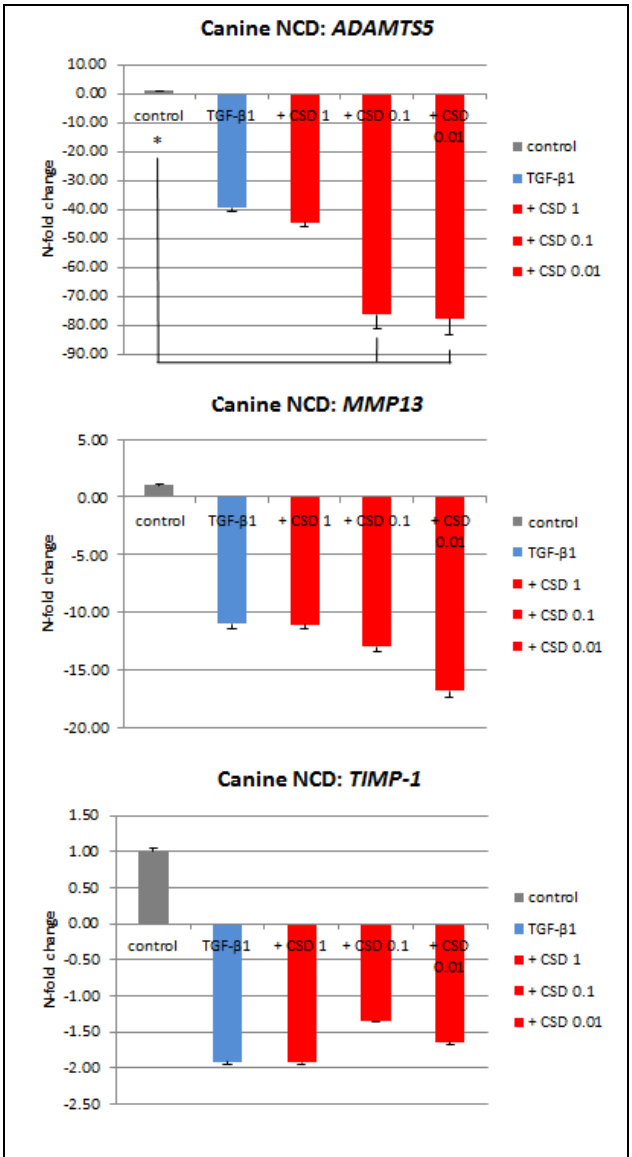


Figure 13. Relative gene expression of matrix remodelling proteins *ADAMTS5*, *MMP13* and *TIMP-1* in the canine pellets treated with TGF-β1 (blue), TGF-β1 in combination with caveolin-1 scaffolding domain (CSD) (red) or untreated (grey) for 7 days. * = p<0.05

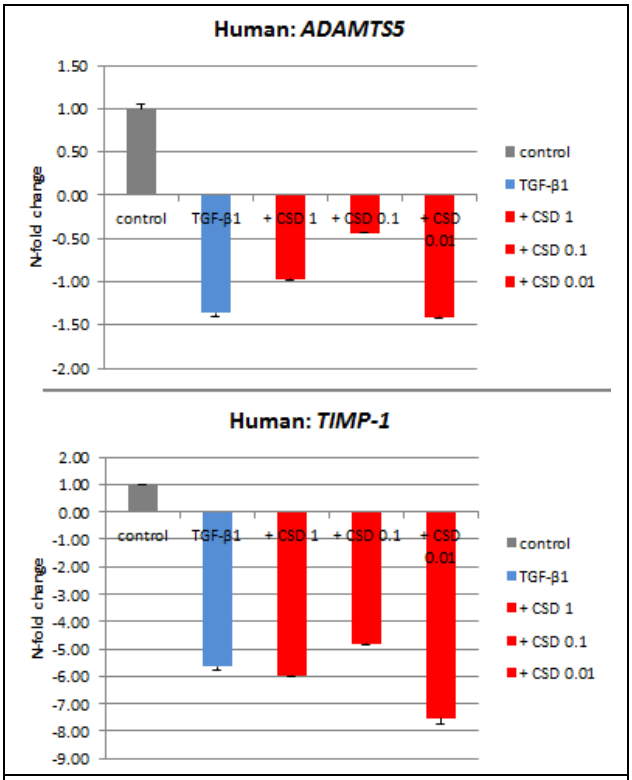


Figure 14. Relative gene expression of matrix remodelling proteins *ADAMTS5* and *TIMP-1* in the human pellets treated with TGF-β1 (blue), TGF-β1 in combination with caveolin-1 scaffolding domain peptide (CSD) (red) or untreated (grey) for 7 days.

4.4.3.3. Cell proliferation

In accordance with the increase in DNA synthesis displayed in **figure 1**, cell proliferation marker *CyclinD1* was upregulated in all TGF-β1 treated canine pellets compared with the control pellets (**figure 15**). This effect reached statistical significance in the pellets that received additional treatment of 0.1 µM CSD ($p<0.05$) and 0.01 CSD ($p<0.05$). Although no differences were observed in DNA content between the control and TGF-β1 treated pellets of the human donors, *CyclinD1* was higher expressed in the human TGF-β1 treated pellets compared with the control pellets (**figure 16**). CSD did not have an additive effect on *CyclinD1* expression in the human or canine TGF-β1 treated pellets.

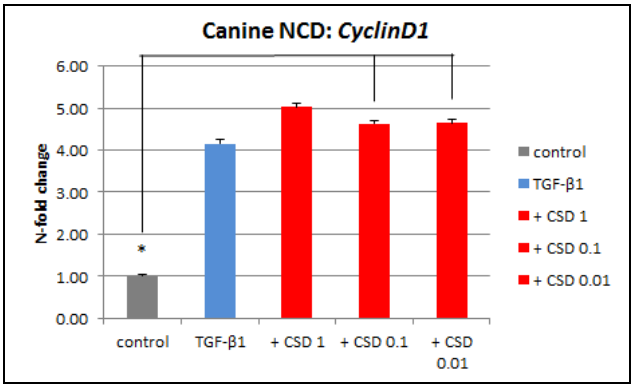


Figure 15. Relative gene expression of cell proliferation marker CyclinD1 in the canine and human pellets treated with TGF-β1 (blue), TGF-β1 in combination with caveolin-1 scaffolding domain peptide (CSD) (red) or untreated (grey) for 7 days. * = p< 0.05

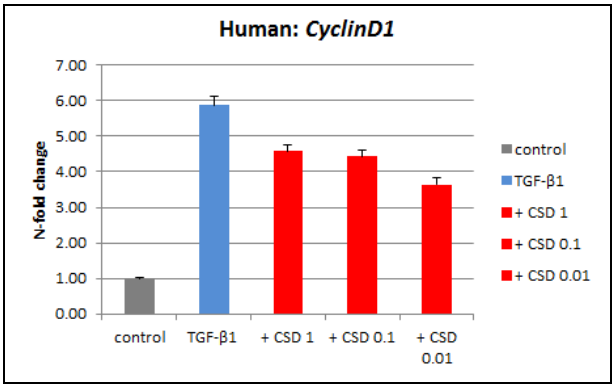


Figure 16. Relative gene expression of cell proliferation marker CyclinD1 in the canine and human pellets treated with TGF-β1 (blue), TGF-β1 in combination with caveolin-1 scaffolding domain peptide (CSD) (red) or untreated (grey) for 7 days.

4.4.3.4. Wnt/β-catenin signalling

The relative gene expression of *Axin2* (i.e. marker for active Wnt/β-catenin signalling) was measured to gain more insight into the effects of CSD on Wnt/β-catenin signalling with respect to IVD degeneration. TGF-β1 treatment decreased the expression of *Axin2* in the canine pellets, although this was not statistically significant (**figure 17**). Moreover, CSD (0.1 μM and 0.01 μM) seemed to counteract TGF-β1's effect on *Axin2* expression. In human pellets, *Axin2* expression remained steady across all culture groups (**figure 18**).

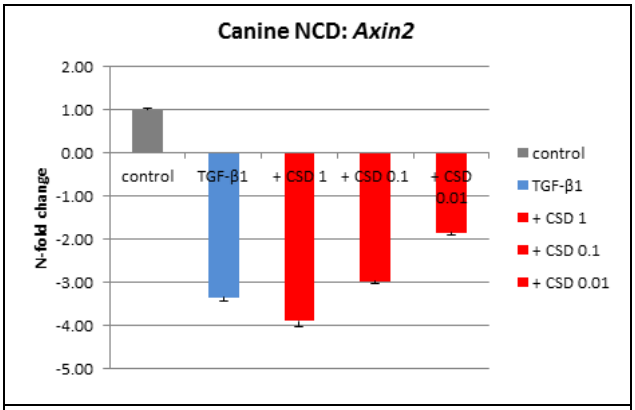


Figure 17. Relative gene expression of read-out marker for active Wnt/β-catenin signalling *Axin2* in the canine pellets treated with TGF-β1 (blue), TGF-β1 in combination with caveolin-1 scaffolding domain (CSD) (red) or untreated (grey) for 7 days.

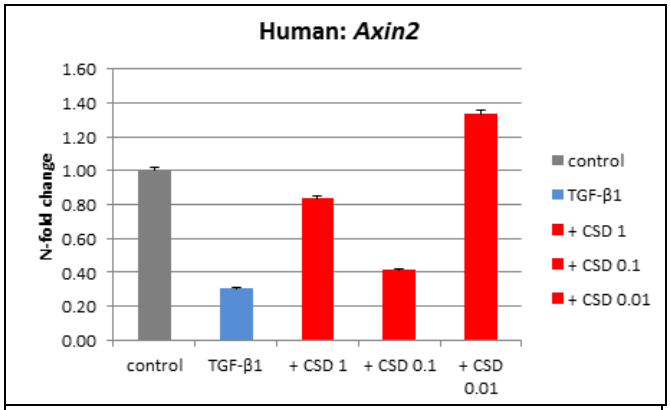


Figure 18. Relative gene expression of read-out marker for active Wnt/β-catenin signalling *Axin2* in the human pellets treated with TGF-β1 (blue), TGF-β1 in combination with caveolin-1 scaffolding domain peptide (CSD) (red) or untreated (grey) for 7 days.

4.4.3.5. TGF-β signalling

As previously mentioned, we hypothesized that caveolin-1 could induce regeneration by shifting the TGF-β signalling from the catabolic ALK1 (Smad1/5/8) pathway towards to the anabolic ALK5 (Smad2/3) pathway. To investigate this, gene expression levels of TGF-β type 1 receptors

ALK5 and *ALK1* and their respective downstream pathways read out markers *PAI1* (ALK5 (Smad2/3) pathway) and *ID1* (ALK1 (Smad1/5/8) pathway) were measured (**figures 19 and 20**).

As no human primers for *PAI1* and *ID1* were available, the expression of *PAI1* and *ID1* was solely measured in the canine pellets. TGF-β1 only slightly upregulated *ALK5* (1.3-fold) in the canine pellets compared with controls, with statistical significance when TGF-β1 was supplemented with 0.1 μM CSD ($p<0.05$) (**figure 19**). Yet this stimulating effect of TGF-β1 was considerably higher in the human pellets as it increased *ALK5* expression by 15-fold (**figure 20**). Despite the fact that in the canine pellets TGF-β1 did not have considerable effects on the expression of *ALK5*, it did have a stimulating effect on the expression of *ALK*5's downstream marker *PAI1* (with statistical significance when TGF-β1 was supplemented with 0.1 μM CSD ($p<0.05$) and 0.01 μM CSD ($p<0.05$)) (**figure 19**). No additional effect of CSD on the upregulation of *ALK5* and or *PAI1* expression was observed in the canine and human TGF-β1 treated pellets.

The expression of *ALK1* and *ID1* of the canine and human pellets were relatively unaffected by TGF-β1 treatment (irrespective of the combination with CSD), since n-fold changes ranged from 1 to -2.5. TGF-β1 treatment only slightly decreased the expression of *ALK1* (by 2.5-fold) in the canine pellets (**figure 19**), but this effect lacked statistical significance.

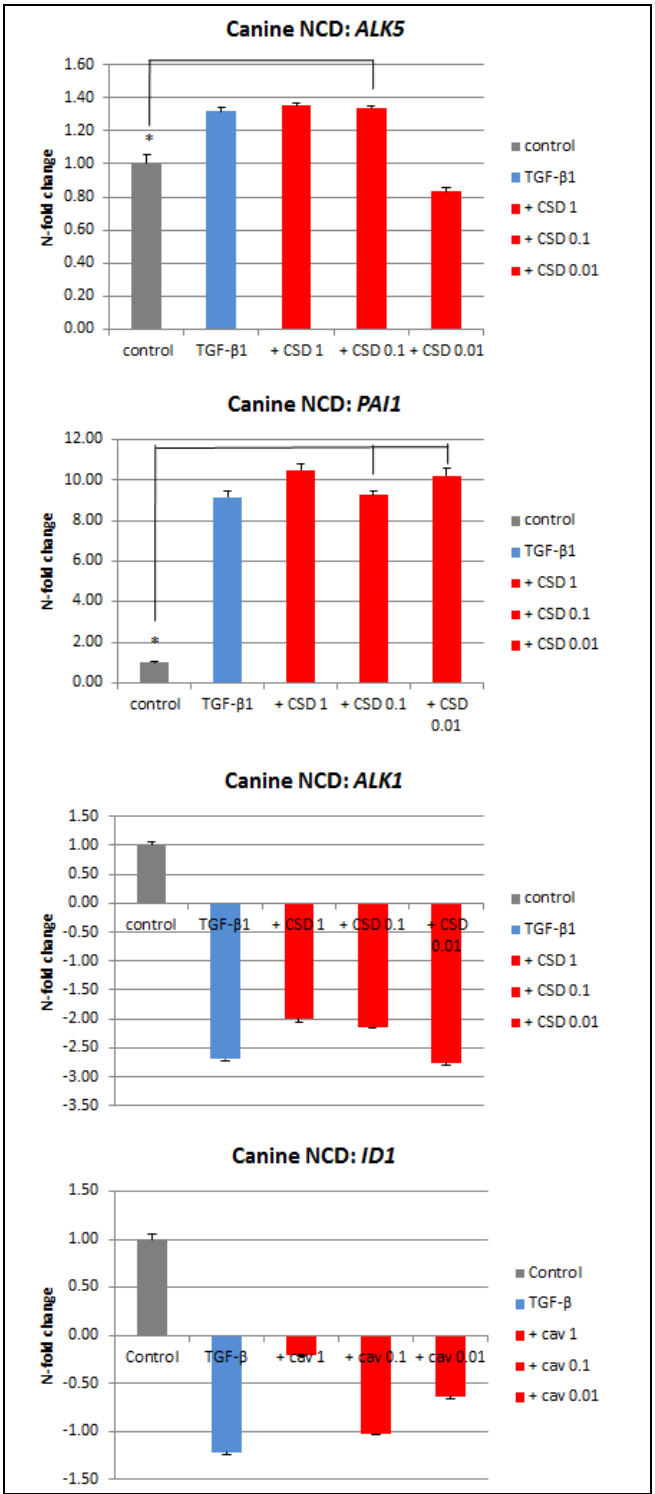


Figure 19. Relative gene expression TGF-β signalling markers *ALK5*, *PAI1*, *ALK1* and *ID1* in the canine treated with TGF-β1 (blue), TGF-β1 in combination with caveolin-1 scaffolding domain peptide (CSD) (red) or untreated (grey) for 7 days. * = p <0.05

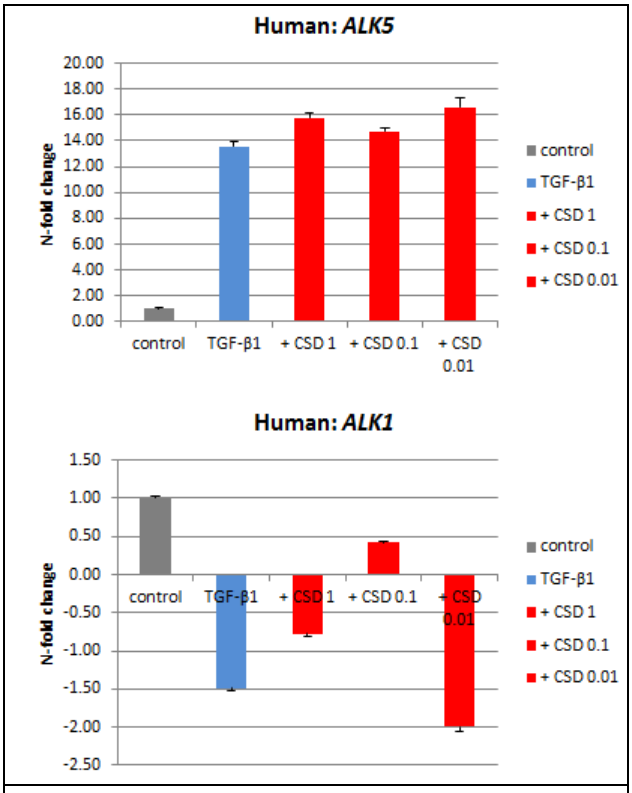


Figure 20 Relative gene expression TGF-β signalling markers ALK5, and ALK1 in the human pellets treated with TGF-β1 (blue), TGF-β1 in combination with caveolin-1 scaffolding domain peptide (CSD) (red) or untreated (grey) for 7 days.

The canine *ALK5/ALK1* ratios were comparable among the different culture groups (**figure 21**), indicating that the TGF-β1 or CSD treatment did not alter the expression levels of *ALK5* relative to *ALK1*. Moreover, TGF-β1 slightly decreased canine *PAI1/ID1* ratios compared with the

controls (with statistical significance when TGF-β1 was supplemented with 0.1 μM CSD ($p<0.05$) and 0.01 μM CSD ($p<0.05$)), indicating that TGF-β1 treatment caused an increase in *PAI1* expression relative to *ID1* expression.

Although not statistically significant, TGF-β1 decreased the *ALK5/ALK1* ratio in the human pellets, indicating a higher *ALK5* expression relative to *ALK1* expression (**figure 22**).

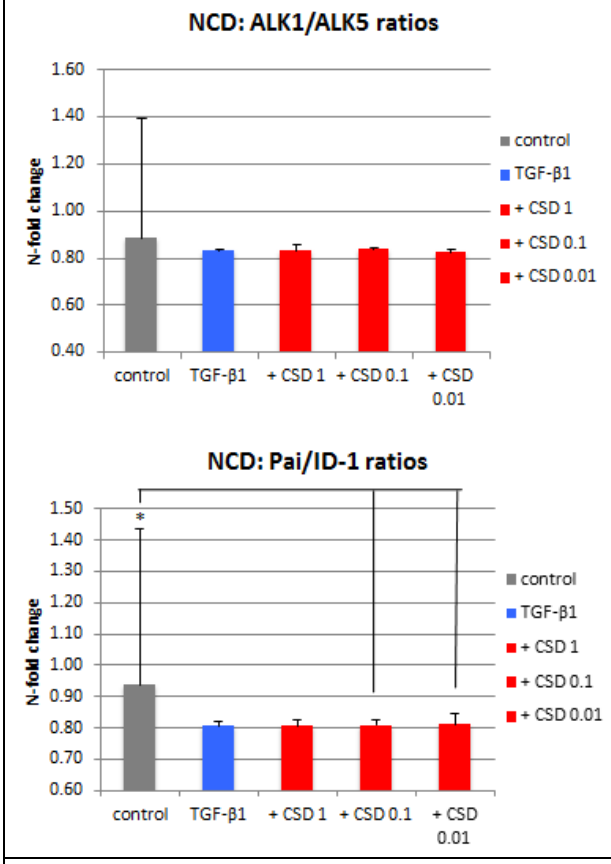


Figure 21. *ALK5/ALK1* and *PAI1/ID1* ratios in the canine pellets treated with TGF-β1 (blue), TGF-β1 in combination with caveolin-1 scaffolding domain peptide (CSD) (red) or untreated (grey) for 7 days. * = $p<0.05$

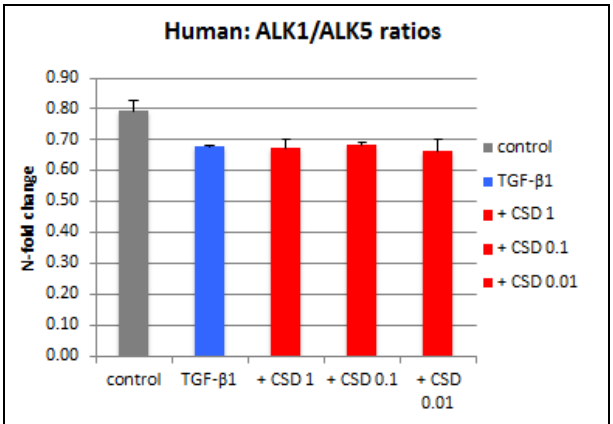


Figure 22. ALK5/ALK1 ratios in the human pellets treated with TGF-β1 (blue), TGF-β1 in combination with caveolin-1 scaffolding domain peptide (CSD) (red) or untreated (grey) for 7 days.

4.4.3.6. Apoptosis

Both apoptosis and anti-apoptosis marker *BAX* and *BCL2* were downregulated in canine and human TGF-β1 treated pellets compared with the control pellets (**figures 23 and 24**). The downregulation of *BAX* in the canine pellets was statistically significant when TGF-β1 was supplemented with 0.1 μM CSD ($p<0.05$) and 0.01 μM CSD ($p<0.05$). Addition of CSD (all tested concentrations) enhanced the TGF-β1 induced downregulation of *BCL2* expression in the human pellets (**figure 24**), but this effect lacked statistical significance. TGF-β1 treatment did not have an effect on the *BAX/BCL2* ratios and no additional effects of CSD were observed.

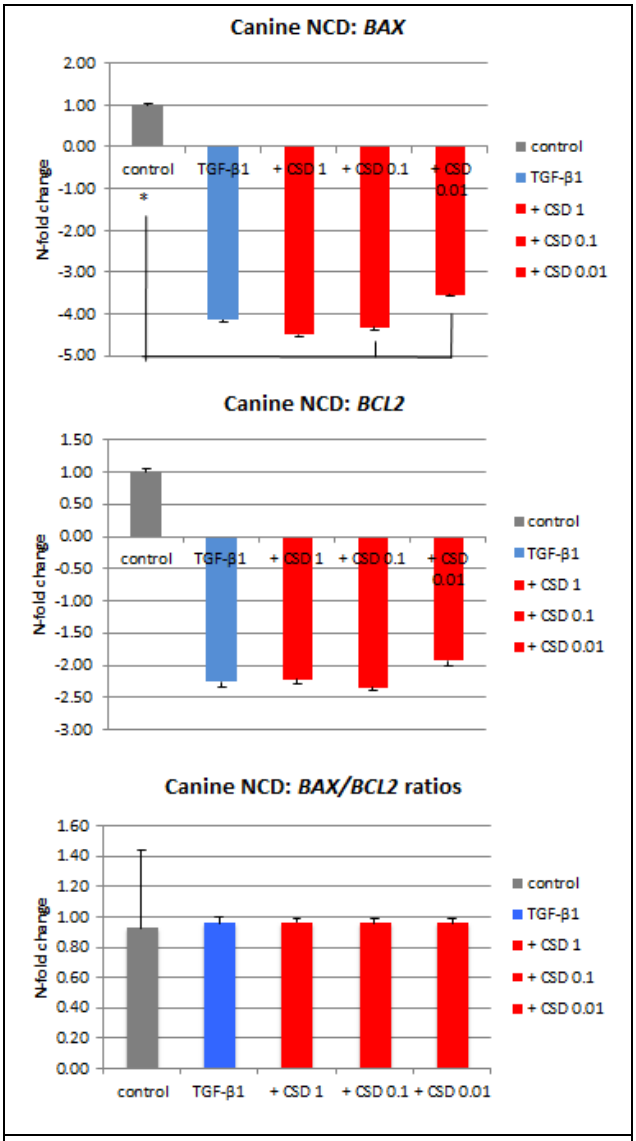


Figure 23. Relative gene expression of *BAX* and *BCL2* and *BAX/BCL2* ratios in the canine pellets treated with TGF-β1 (blue), TGF-β1 in combination with caveolin-1 scaffolding domain peptide (CSD) (red) or untreated (grey) for 7 days. * = p<0.05

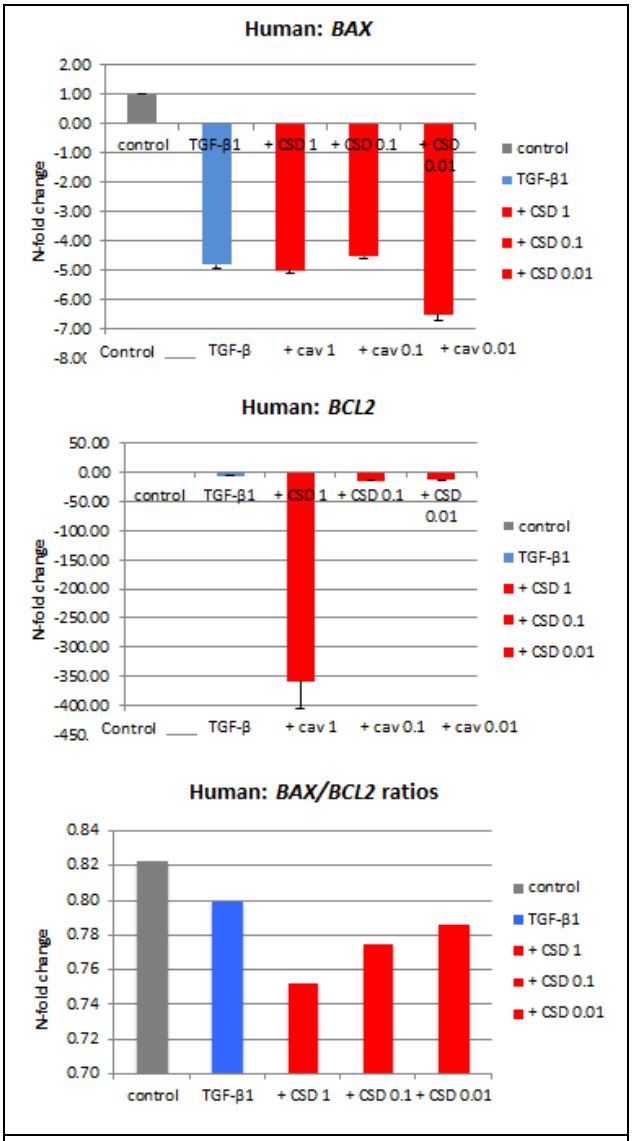


Figure 24. Relative gene expression of *BAX* and *BCL2* and *BAX/BCL2* ratios in the human pellets treated with TGF-β1 (blue), TGF-β1 in combination with caveolin-1 scaffolding domain peptide (CSD) (red) or untreated (grey) for 7 days.

4.5. Discussion

4.5.1. Regenerative effects of TGF-β1 and CSD

Consistent with previous research [27,28], TGF-β1 increased matrix production in NPCs of NCD dogs and humans as it increased the GAG content of the pellets and culture medium compared with controls (**figures 3-6**). This regenerative effect of TGF-β1 is in agreement with the qPCR results: *Aggrecan* and *Collagen II* were upregulated in all TGF-β1 treated canine and human pellets (**figures 11 and 12**). Furthermore, TGF-β1 stimulated the proliferation of the canine NPCs as it increased DNA synthesis (**figure 1**) and expression of cell proliferation marker *CyclinD1* (**figure**

15). Although TGF-β1 also increased the expression of cell proliferation marker *CyclinD1* in the human pellets (**figure 16**), this proliferative effect of TGF-β1 did not reflect into an increased DNA content of the human TGF-β1 treated pellets (**figure 2**). CSD did not have an additive effect on the human and canine TGF-β1 treated pellets on matrix production and cell proliferation. However, similar to the previous experiment (chapter 3), supplementation of 0.1 and 0.01 µM CSD seemed to enhance the TGF-β1 induced downregulation of *ADAMTS5* and *MMP13* in the canine pellets. Yet, this additional inhibition of these matrix proteinases did not affect the GAG content, GAG release or size of the CSD treated canine pellets.

RT-qPCR data showed that apart from favourable matrix proteins (*Aggrecan* and *Collagen II)*, *Collagen I* was also upregulated at day 7 and day 28. The latter is unfavourable as *Collagen I* is not pliable and therefore not beneficial to the shock-absorbing function of the nucleus pulposus [1]. This upregulation of *Collagen I* was also observed in the previous experiment (chapter 3, figure 6), yet this upregulation became a downregulation after 28 days of culturing. Since gene expression in the current study was only measured at day 7, we cannot account for a potential downregulation after day 7.

4.5.2. TGF-β signalling pathway

TGF-β1 slightly upregulated *ALK5* in the canine pellets and increased the expression of *PAI1* in these pellets (**figure 19**). The large standard deviations of the canine control *ALK5/ALK1* and *PAI1/ID1* ratios make it difficult to draw conclusions from these data. Nevertheless, TGF-β1 treatment decreased *PAI1/ID1* ratios (with statistical significance when supplemented with 0.1 µM CSD (p=0.039)). This indicates that TGF-β1 caused a shift towards the ALK5 (Smad2/3) signalling pathway rather than the ALK1 (Smad1/5/8) signalling pathway.

The expression levels of *PAI1* and *ID1* were not measured in the human pellets. This limits the possibility to determine the degree of ALK5 (Smad2/3) and ALK1 (Smad1/5/8) signalling. However, TGF-β1 considerably increased the expression of *ALK5* in the human pellets, while leaving the expression of *ALK1* unaffected (**figure 20**). This finding suggests that an increased ALK5 (Smad2/3) signalling also takes place in human NPCs. We hypothesized that CSD would cause a shift in TGF-β signalling towards the ALK5 (Smad2/3 pathway). However, these results indicate that TGF-β1 already mainly signals through the ALK5 (Smad2/3) pathway, without the influence of CSD. CSD did not cause an additional shift towards the Smad2/3 pathway, since it did not additionally affect the *ALK5/ALK1* ratios or *PAI1/ID1* ratios.

4.5.3. Wnt signalling pathways

TGF-β1 caused a downregulation of *Axin2* in the canine pellets (**figure 17**). Although not statistically significant, CSD tended to counteract TGF-β1's induced downregulation of *Axin2* in a dose-dependent manner. Addition of 1 µM CSD did not have an effect upon TGF-β1 treatment, whereas addition of 0.1 and 0.01 µM CSD increased *Axin2* expression by 0.5-fold and 1.5-fold respectively. These results indicate that TGF-β1 treated pellets inhibit the Wnt/β-catenin signalling and that CSD counteracts this inhibiting effect. The inhibiting effect of TGF-β1 agrees

with our previous findings (chapter 3) and previous research that demonstrated a TGF-β1 induced suppression of *Axin2* expression via a Smad2/3 dependent mechanism [57]. CSD's counteraction on the TGF-β1 induced inhibition of the Wnt/β-catenin signalling can only be explained by its independent stimulating effect on the Wnt/β-catenin signalling[16], since it did not affect Smad2/3 signalling levels.

Overall, no additive regenerative effect of CSD (0.01, 0.1 and 1 μM) was observed on TGF-β1 treatment (10 ng/mL) of early degenerated canine (NCD) and moderately degenerated human NPC pellets. Taken together the results of chapter 2, 3, and 4, CSD did not elicit regenerative effects in a wide range of concentrations (0.01, 0.1, 1, 2, 5, and 10 μM) on different species (human, CD and NCD dogs) and cell types (ACs and NPCs) using our specific experimental setup. One explanation might still be that at high concentrations, caveolin-1 and CDS only exert negative/degenerative effects upon NPCs in terms of cell proliferation and matrix production, and that a concentration of 0.01-1 μM CSD is too low to exert any effect at all (hereby assuming that CSD indeed mimics caveolin-1 function). However, it should be mentioned that it is also still possible that CSD functioned as a caveolin-1 antagonist instead of agonist, by interfering with caveolin-1 dependent signalling. This may indicate that caveolin-1 is needed for cell proliferation and matrix production of NPCs.

4.6. Conclusion

TGF-β1 exerted strong regenerative effects on NPCs from NCD dogs and humans. CSD, however, did not exert the hypothesized additional regenerative effects upon TGF-β1 treatment of canine and human NPCs. Despite the fact that the middle-aged donors that were used in this study ought to have increased ALK5/ALK1 signalling compared with the young CD dogs that were tested in chapter 3, CSD was still unable to induce a desired shift towards the ALK5 (Smad2/3) signalling pathway. Without the addition of CSD, TGF-β1 already signalled through the anabolic ALK5 (Smad2/3) pathway. Hence, in the present experimental set-up, the caveolin-1 scaffolding domain did not appear to be a suitable therapeutic target for IVD degeneration.

5. General discussion

The main aim of this project was to gain more insight into the regenerative effects of caveolin-1 on nucleus pulposus cells (NPCs) isolated from early degenerated intervertebral discs (IVDs) in order to assess the potential use of caveolin-1 as a therapeutic target for IVD degeneration. For this purpose, not the intact caveolin-1 protein, but a caveolin-1 scaffolding domain peptide (CSD) fused to the cell-permeable antennapedia internalization sequence was used to determine the effect of caveolin-1 on NPCs. Since this CSD peptide has been reported to upregulate the function of caveolin-1 *in vitro* and *in vivo* [13,37,36,38], we hypothesized that CSD can be used to mimic caveolin-1 function. However, it should be mentioned it is possible that the CSD at higher concentrations instead of mimicking caveolin-1 function, it may antagonize it by interfering with the intrinsic caveolin-1 dependent signalling. Initial culture experiments were performed using early degenerated NPCs isolated from Beagle dogs. The Beagle is a chondrodystrophic (CD) breed and known to show IVD degeneration at already 1 year of age and clinical signs of disc disease early in life [5]. In order to exclude the influence of genetic background on the effect of CSD treatment, additional culture experiments were performed employing NPCs isolated from non-chondrodystrophic (NCD) dogs and humans. These latter species show IVD degeneration later in life with clinical signs of disc disease at an old age. Previous research concerning the regenerative effects of asporin on IVDs of mice from different genetic backgrounds showed that genetic background can seriously affect the physiological function of a treatment (unpublished data AOspine). By testing multiple (sub)species, we limited the role of genetic background in the effect of CSD treatment.

TGF-β signalling plays an important role in the process of IVD degeneration and caveolin-1 is known to influence TGF-β signalling [9,23,55]. Therefore, the regenerative effects of CSD alone and in combination with TGF-β1 were assessed. No regenerative effects of CSD on canine NPCs were observed when it was supplemented without TGF-β1. On the contrary, supplementation of CSD in concentrations of 5 μM and higher, inhibited cell proliferation and matrix production (chapter 2, toxicity study). This can be explained by a potential dose-related effect of CSD. It could be that an overdose of CSD induces cell senescence, as demonstrated by Heathfield et al. (2008)[12]. No statistical significant additive regenerative effects of CSD (0.01, 0.1, 1, 2, 5, and 10 μM) upon TGF-β1 treatment (2 and 10 ng/mL) were observed in canine CD, NCD or human NPCs. This indicates that genetic background does not influence the biological effect of CSD. In 3 individual cases (NPC156, NPC662, donor group A), a slight additive effect of CSD upon TGF-β1 treatment was observed. However, these effects lacked statistical significance and the donor-dependent effect of CSD could not be explained from a functional point of view. It should be taken into account that these three cases might be false positive due to the multiple donor testing. A plausible explanation for finding no (additive) regenerative effect of CSD might be that - assuming that CSD indeed mimics caveolin-1 function - at high concentrations (>1μM), caveolin-1 and CDS only exert degenerative effects (decreased cell proliferation and matrix production), and that concentrations around 0.01-1 μM CSD are too low to exert any effect. On the contrary, it is also possible that CSD worked as a caveolin-1 antagonist instead of agonist,

by interfering with caveolin-1 dependent signalling. Regardless the cause, however, in the present experimental set-up, the scaffolding domain of caveolin-1 did not appear to be a promising target to develop regenerative treatments for IVD degeneration.

5.1. Potential follow-up studies

The effects of CSD in concentrations of 5 μM and 10 μM indicate that the scaffolding domain of caveolin-1 has deteriorating effects rather than regenerative effects upon IVD degeneration. Instead of supplementing CSD to NPCs, silencing caveolin-1 expression could therefore result in regenerative effects (assuming that CSD indeed mimics, and not antagonizes caveolin-1 function). An explanation for the fact that CSD failed to show any effects below 2 μM could be that these concentrations were too low to enable CSD to elicit its "true" negative effects on NPCs. To explore this possibility, a follow-up study will investigate if the knockdown of caveolin-1 gene expression leads to more cell proliferation and matrix production compared with 10 μM CSD, 1 μM CSD, and negative control (only chondrogenic culture medium) treatment. The results of this experiment should also provide more insight in the effect of CSD as a caveolin-1 mimicker and/or antagonist.

Another explanation for the fact that CSD failed to show a regenerative effect could be that its effects are transient. In the present studies NPCs were treated with CSD for at least 7 days. CSD could have already elicited an effect a few minutes/hours after administration that vanished after chronic treatment. Therefore, it may be interesting to examine CSD's effects on NPCs after short treatment (minutes, hours).

Previous research has demonstrated that caveolin-1 has anti-inflammatory effects [59,60]. For example, caveolin-1 has been demonstrated to inhibit the production of nitric oxide, cytokines and chemokines in macrophages [59]. IVD degeneration is characterized by increased levels of proinflammatory cytokines that cause progression of degeneration, disc herniation and radicular pain [61]. This suggests that CSD could still have beneficial effects (assuming that CSD indeed mimics, and not antagonizes caveolin-1 function). Not in terms of stimulating the cell proliferation and matrix production, but by means of its anti-inflammatory effects. Hence it can be worthwhile to further investigate caveolin-1's protective effects by supplementing CSD to NPCs that are situated in a catabolic environment in the presence of pro-inflammatory cytokines. One could determine the subsequent effects on apoptosis or production of inflammatory mediators and compare these with the effects on NPCs that remained in a physiological environment.

If these potential follow-up studies do not yield any positive results, our hypotheses regarding CSD and/or caveolin-1's function should be reconsidered. Thus far, caveolin-1 has been shown to be a marker for IVD cell senescence. The fact that caveolin-1 is more present in healthy notochordal cell rich nucleus pulposi[1] could be a sign of IVD stress that predicts a subsequent replacement of notochordal cells by NPCs. And lastly, as already mentioned before, at this point we still cannot exclude that the CSD peptide could have functioned as a caveolin-1 antagonist rather than a caveolin-1 mimicker. CSD could have bound to the cytosolic ligands of caveolin-1,

thereby blocking their interaction with caveolae. In this way, CSD could possibly have interfered with the action of caveolin-1 on signalling molecules that require endogenous caveolins within the caveolae for their biological function.

5.2. CD breeds versus NCD breeds versus Humans

To the best of our knowledge, we are the first to investigate and compare the effects of TGF-β1 treatment in CD dog breeds, NCD dog breeds and humans. Interestingly, these 3 donor types responded differently in our experimental set up. In contrast to the canine pellets from CD and NCD donors, the human pellets were capable of proliferating without TGF-β1 treatment as the DNA content of the human control pellets increased over time (chapter 4, figure 2). In addition, both human and canine donors responded differently to the TGF-β1 treatment. First of all, TGF-β1 treatment elicited a proliferative effect in canine pellets of both CD and NCD dogs, but lacked this effect in human pellets. More specifically, in contrast to the CD and NCD pellets, the DNA content of the human control pellets was not significantly different from the DNA content of the human TGF-β1 treated pellets throughout the culture period (**figure 1**).

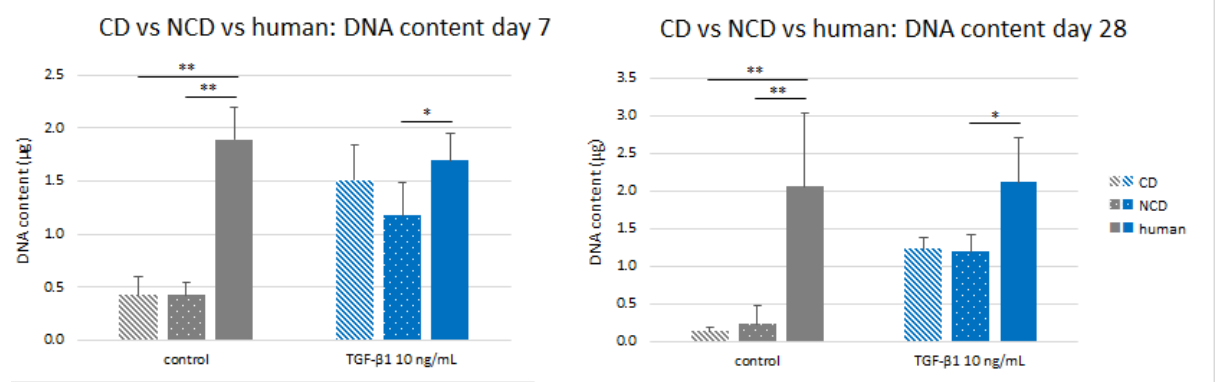


Figure 1. DNA content of the CD canine (striped), NCD canine (dotted) and human (solid) NPC cell pellets cultured for 7 days (left) and 28 days (right) in chondrogenic medium. Pellets were treated with TGF-β1 (blue) or were left untreated (grey). * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. CD = chondodystrophic, NCD = non-chondodystrophic.

This difference in effect could be explained by differences in sensitivity to TGF-β1 treatment between canine and human NPCs. It could also be explained by the difference in size of the canine and human pellets. Although human and canine pellets were cultured with an equal amount of cells (starting density of 200.000 NPCs/well), the human pellets were larger in size compared with the canine pellets as from day 1 of the culture period (**figure 2)**. Consequently, TGF-β1 could have failed to sufficiently reach the inner part of the human cell pellets. As a result, TGF-β1 could have only affected the cells residing in the terminus of the pellets. This explanation seems to be plausible, since TGF-β1 did show a proliferative effect on smaller human pellets (35.000 cells) (unpublished data by our research group).

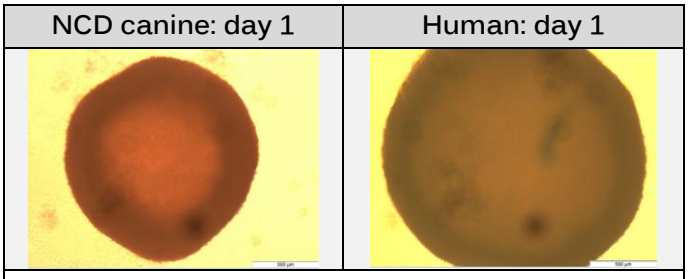


Figure 2. Starting from day 1 of the culture period, the NCD canine pellets (left) was smaller in size compared with the human (right) pellets.

Interestingly, the CD, NCD and human donors responded with different intensity to the TGF-β1 treatment concerning matrix production. Apart from the fact that the increase in matrix production had a delayed onset in the human pellets, GAG content of the human TGF-β1 treated pellets was significantly lower compared with the canine TGF-β1 treated pellets ($p<0.01$) after 7 and 28 days of treatment (**figure 3**). These findings correspond with the expression profiles of matrix proteins *Aggrecan* and *Collagen II*: the TGF β1 treated human pellets show a lower upregulation of *Aggrecan* (6-fold) and *Collagen II* (100-fold) expression compared with the canine NCD pellets (90-fold and 5000-fold) (chapter 4, figure 12). Furthermore, TGF β1 induced a stronger downregulation of matrix proteinases *ADAMTS5* (40-fold) and *MMP13* (11-fold) in the canine NCD pellets compared with the human pellets (*ADAMTS5*: 1.4-fold). The higher GAG content of the canine pellets in comparison with the human pellets could therefore be the result of a higher net effect of more matrix production in combination with less matrix degradation in the canine pellets. This difference in level of responsiveness to TGF-β1 could be explained by differences in stage of IVD degeneration. The IVDs of the human donors were Thompson grade III, indicating that the cells are further degenerated and that the cells may subsequently be less responsive to TGF-β1 compared with the canine donors (mostly grade II).

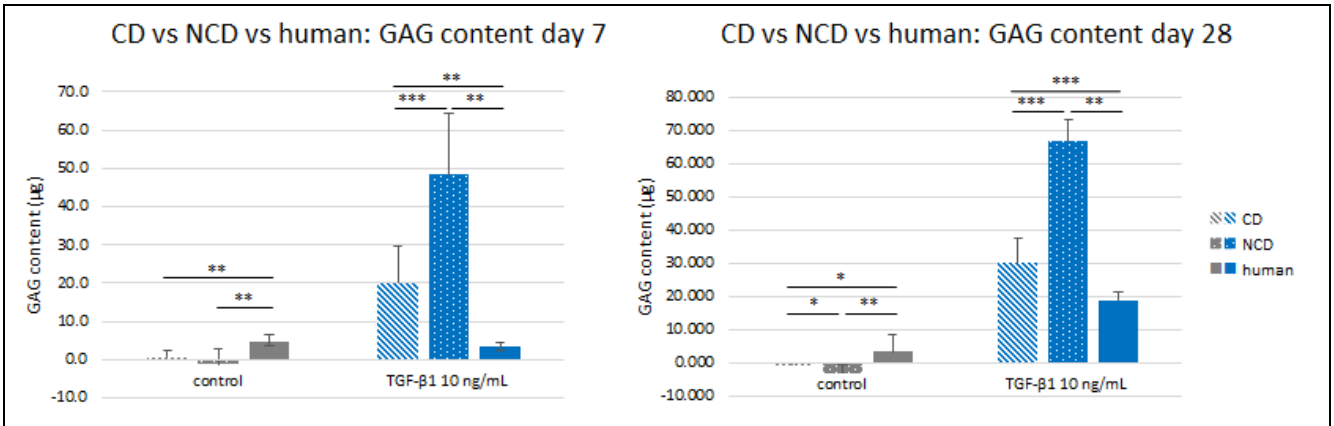


Figure 3. GAG content of the CD canine (striped), NCD canine (dotted) and human (solid) NPC cell pellets cultured for 7 days (left) and 28 days (right) in chondrogenic medium. Pellets were treated with TGF-β1 (blue) or left untreated (grey). * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$. CD = chondodystrophic, NCD = non-chondodystrophic.

In addition, differences were observed between pellets from NCD dogs and pellets from CD dogs. Cells from NCD dogs responded with more intensity to the TGF-β1 treatment concerning

GAG production compared with the CD dog breeds ($p<0.001$). More specifically, TGF-β1 caused a 30-fold increase in GAG content of the CD pellets compared with a 65-fold increase of the GAG content in the NCD pellets after 28 days of treatment (**figure 3**). Interestingly, increased GAG content levels of the CD pellets more closely resembled the GAG content levels of the human pellets than the NCD pellets (**figure 3**). This finding suggests that the responsiveness of CD dogs to TGF-β1 treatment is more similar to that of humans, indicating that CD dogs may provide a better model for human IVD degeneration than NCD dogs. This is in contrast to the current belief that NCD dogs provide a better animal model [6]. This could be explained by the fact that this current consensus is based on similarities in etiology and histological and microscopic findings between IVDs of humans and NCD dogs, rather than growth characteristics of the human and canine NCD NPCs.

5.3. Challenges and Study limitations

We experienced several challenges and study limitations during this project. First of all, in the dose optimization pilot (chapter 2) we experienced difficulty in obtaining sufficient quantities of RNA from the cell pellets in order to initiate cDNA reactions. In the follow up studies, this problem was tackled by pooling duplo pellets thereby doubling the RNA yields. However, this solution caused additional problems as the lack of a duplo measurement reduced statistical power of the RT-qPCR results (as seen in the study discussed in chapter 4). Therefore, additional duplo pellets should be cultured in future experiments to be able to perform RT-qPCR with sufficient statistical power.

Another limitation of this study was the lack of proof concerning the assumption that CSD was capable of reaching and entering the NPCs, since it did not influence TGF-β signalling. However, the demonstrated dose-dependent effects of CSD in the toxicity study indirectly support this assumption. Moreover, the CSD peptide used was previously demonstrated to be effectively taken up into blood vessels and endothelial cells in *in vitro* and *in vivo* experiments [13]. In order to continue unravelling caveolin-1's effects, it is necessary to fully confirm the capability of CSD in reaching and entering the NPCs in the current study set-up. In order to do this, the CSD peptides could be labelled prior to administration with a fluorescent dye, followed by the detection of fluorescence in the NPCs using confocal microscopy. Besides, the nitric oxide synthase (NOS) activity should be determined in CSD-treated NPC lysates to establish whether CSD inhibited NO production by NOS. Furthermore, the *in vitro* experiments with high concentrations of CSD (5 and 10μM) should be repeated with low concentrations of DMSO (in which CSD is dissolved), since high DMSO concentrations could have interfered with the observed negative effect of CSD on the ACs/NPCs (decreased cell proliferation and matrix production).

Courses

During my Honours Programme year I seized the opportunity to follow several courses and additional education:

- "Molecular methods"
    - Course instructors: Dr. Louis Penning and Dr. Peter Leegwater
    - 2 days, part time (September 17 and September 19)
- "Introductory statistics and RStudio/R"
    - Course instructors: Hans Vernooij and Jan van den Broek
    - 2 days, full time (December 11 and December 12)
- "Modern Methods in Data Analysis"
    - Course instructors: Drs. Cas Kruitwagen and Jan van den Broek
    - 3 weeks, full time (January 6 – January 24)
    - 4 ECTS
- "Presenting in English for PhD researchers"
    - Course instructor: Drs. Margo de Wolf
    - 2 days, full time (April 15 and April 22)
    - 1 ECTS
- "Writing for academic publication in the veterinary and life sciences"
    - Course instructor: Dr. Linda McPhee
    - 10 weeks, part time (3 hours per week), April 17 – June 26
    - 3 ECTs

Furthermore I visited the Veterinary Science Day (November 14) and I presented my research at the European Veterinary Voorjaarsdagen 2014 (April 17 – April 19).

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Literature

1. Smolders LA, Meij BP, Onis D, et al. 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 Res. Ther.* 2013;15(1):R23. doi:10.1186/ar4157.

2. Bergknut N, Smolders LA, Grinwis GCM, et al. Intervertebral disc degeneration in the dog. Part 1: Anatomy and physiology of the intervertebral disc and characteristics of intervertebral disc degeneration. *Vet. J.* 2013;195(3):282-91. doi:10.1016/j.tvjl.2012.10.024.

3. Bach FC, Willems N, Penning LC, Ito K, Meij BP, Tryfonidou MA. Potential regenerative treatment strategies for intervertebral disc degeneration in dogs. *BMC Vet. Res.* 2014;10:3. doi:10.1186/1746-6148-10-3.

4. S R. Disc morphology in health and disease. 2001. Available at: http://www.biochemsoctrans.org/bst/030/0864/bst0300864.htm. Accessed August 6, 2014.

5. Smolders LA, Bergknut N, Grinwis GCM, et al. Intervertebral disc degeneration in the dog. Part 2: chondrodystrophic and non-chondrodystrophic breeds. *Vet. J.* 2013;195(3):292-9. doi:10.1016/j.tvjl.2012.10.011.

6. Bergknut N, Rutges JPHJ, Kranenburg H-JC, et al. The dog as an animal model for intervertebral disc degeneration? *Spine (Phila. Pa. 1976).* 2012;37(5):351-8. doi:10.1097/BRS.0b013e31821e5665.

7. Liu P, Rudick M, Anderson RGW. Multiple functions of caveolin-1. *J. Biol. Chem.* 2002;277(44):41295-8. doi:10.1074/jbc.R200020200.

8. Parton RG, Simons K. The multiple faces of caveolae. *Nat. Rev. Mol. Cell Biol.* 2007;8(3):185-94. doi:10.1038/nrm2122.

9. Gvaramia D, Blaauboer ME, Hanemaaijer R, Everts V. Role of caveolin-1 in fibrotic diseases. *Matrix Biol.* 2013;32(6):307-15. doi:10.1016/j.matbio.2013.03.005.

10. Zou H, Stoppani E, Volonte D, Galbiati F. Caveolin-1, cellular senescence and age-related diseases. *Mech. Ageing Dev.* 132(11-12):533-42. doi:10.1016/j.mad.2011.11.001.

11. Baker N, Tuan RS. The less-often-traveled surface of stem cells: caveolin-1 and caveolae in stem cells, tissue repair and regeneration. *Stem Cell Res. Ther.* 2013;4(4):90. doi:10.1186/scrt276.

12. Heathfield SK, Le Maitre CL, Hoyland JA. Caveolin-1 expression and stress-induced premature senescence in human intervertebral disc degeneration. *Arthritis Res. Ther.* 2008;10(4):R87. doi:10.1186/ar2468.

13. Bucci M, Gratton JP, Rudic RD, et al. In vivo delivery of the caveolin-1 scaffolding domain inhibits nitric oxide synthesis and reduces inflammation. *Nat. Med.* 2000;6(12):1362-7. doi:10.1038/82176.

14. Galbiati F, Volonte D, Brown AM, et al. Caveolin-1 expression inhibits Wnt/beta-catenin/Lef-1 signaling by recruiting beta-catenin to caveolae membrane domains. *J. Biol. Chem.* 2000;275(30):23368-77. doi:10.1074/jbc.M002020200.

15. Yamamoto H, Komekado H, Kikuchi A. Caveolin is necessary for Wnt-3a-dependent internalization of LRP6 and accumulation of beta-catenin. *Dev. Cell* 2006;11(2):213-23. doi:10.1016/j.devcel.2006.07.003.

16. Chun J, Hyun S, Kwon T, Lee EJ, Hong S-K, Kang SS. The subcellular localization control of integrin linked kinase 1 through its protein-protein interaction with caveolin-1. *Cell. Signal.* 2005;17(6):751-60. doi:10.1016/j.cellsig.2004.10.016.

17. Huang H, He X. Wnt/beta-catenin signaling: new (and old) players and new insights. *Curr. Opin. Cell Biol.* 2008;20(2):119-25. doi:10.1016/j.ceb.2008.01.009.

18. Smolders LA, Meij BP, Riemers FM, et al. Canonical Wnt signaling in the notochordal cell is upregulated in early intervertebral disk degeneration. *J. Orthop. Res.* 2012;30(6):950-7. doi:10.1002/jor.22000.

19. Ukita K, Hirahara S, Oshima N, et al. Wnt signaling maintains the notochord fate for progenitor cells and supports the posterior extension of the notochord. *Mech. Dev.* 2009;126(10):791-803. doi:10.1016/j.mod.2009.08.003.

20. Hiyama A, Sakai D, Tanaka M, et al. The relationship between the Wnt/β-catenin and TGF-β/BMP signals in the intervertebral disc cell. *J. Cell. Physiol.* 2011;226(5):1139-48. doi:10.1002/jcp.22438.

21. Hiyama A, Sakai D, Risbud M V, et al. Enhancement of intervertebral disc cell senescence by WNT/β-catenin signaling-induced matrix metalloproteinase expression. *Arthritis Rheum.* 2010;62(10):3036-47. doi:10.1002/art.27599.

22. Heldin CH, Miyazono K, ten Dijke P. TGF-beta signalling from cell membrane to nucleus through SMAD proteins. *Nature* 1997;390(6659):465-71. doi:10.1038/37284.

23. Kwon Y-J, Lee J-W, Moon E-J, Chung YG, Kim O-S, Kim H-J. Anabolic effects of Peniel 2000, a peptide that regulates TGF-β1 signaling on intervertebral disc degeneration. *Spine (Phila. Pa. 1976).* 2013;38(2):E49-58. doi:10.1097/BRS.0b013e31827aa896.

24. Lee K-I, Moon S-H, Kim H, et al. Tissue engineering of the intervertebral disc with cultured nucleus pulposus cells using atelocollagen scaffold and growth factors. *Spine (Phila. Pa. 1976).* 2012;37(6):452-8. doi:10.1097/BRS.0b013e31823c8603.

25. Gruber HE, Fisher EC, Desai B, Stasky AA, Hoelscher G, Hanley EN. Human intervertebral disc cells from the annulus: three-dimensional culture in agarose or alginate and responsiveness to TGF-beta1. *Exp. Cell Res.* 1997;235(1):13-21. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9281347. Accessed August 6, 2014.

26. Hayes AJ, Ralphs JR. The response of foetal annulus fibrosus cells to growth factors: modulation of matrix synthesis by TGF-β1 and IGF-1. *Histochem. Cell Biol.* 2011;136(2):163-75. doi:10.1007/s00418-011-0835-x.

27. Thompson JP, Oegema TR, Bradford DS. Stimulation of mature canine intervertebral disc by growth factors. *Spine (Phila. Pa. 1976).* 1991;16(3):253-60. Available at: http://www.ncbi.nlm.nih.gov/pubmed/2028297. Accessed August 6, 2014.

28. Tilwani RK, Bader DL, Chowdhury TT. Biomechanical Conditioning Enhanced Matrix Synthesis in Nucleus Pulposus Cells Cultured in Agarose Constructs with TGFβ. *J. Funct. Biomater.* 2012;3(1):23-36. doi:10.3390/jfb3010023.

29. Finnson KW, Parker WL, ten Dijke P, Thorikay M, Philip A. ALK1 opposes ALK5/Smad3 signaling and expression of extracellular matrix components in human chondrocytes. *J. Bone Miner. Res.* 2008;23(6):896-906. doi:10.1359/jbmr.080209.

30. Blaney Davidson EN, Remst DFG, Vitters EL, et al. Increase in ALK1/ALK5 ratio as a cause for elevated MMP-13 expression in osteoarthritis in humans and mice. *J. Immunol.* 2009;182(12):7937-45. doi:10.4049/jimmunol.0803991.

31. Lee EK, Lee YS, Han I-O, Park SH. Expression of Caveolin-1 reduces cellular responses to TGF-beta1 through down-regulating the expression of TGF-beta type II receptor gene in NIH3T3 fibroblast cells. *Biochem. Biophys. Res. Commun.* 2007;359(2):385-90. doi:10.1016/j.bbrc.2007.05.121.

32. Santibanez JF, Blanco FJ, Garrido-Martin EM, Sanz-Rodriguez F, del Pozo MA, Bernabeu C. Caveolin-1 interacts and cooperates with the transforming growth factor-beta type I receptor ALK1 in endothelial caveolae. *Cardiovasc. Res.* 2008;77(4):791-9. doi:10.1093/cvr/cvm097.

33. Razani B, Zhang XL, Bitzer M, von Gersdorff G, Böttinger EP, Lisanti MP. Caveolin-1 regulates transforming growth factor (TGF)-beta/SMAD signaling through an interaction with the TGF-beta type I receptor. *J. Biol. Chem.* 2001;276(9):6727-38. doi:10.1074/jbc.M008340200.

34. Mathew R. Cell-specific dual role of caveolin-1 in pulmonary hypertension. *Pulm. Med.* 2011;2011:573432. doi:10.1155/2011/573432.

35. Attisano L, Wrana JL. Signal transduction by the TGF-beta superfamily. *Science* 2002;296(5573):1646-7. doi:10.1126/science.1071809.

36. Bernatchez PN, Bauer PM, Yu J, Prendergast JS, He P, Sessa WC. Dissecting the molecular control of endothelial NO synthase by caveolin-1 using cell-permeable peptides. *Proc. Natl. Acad. Sci. U. S. A.* 2005;102(3):761-6. doi:10.1073/pnas.0407224102.

37. Tourkina E, Richard M, Gööz P, et al. Antifibrotic properties of caveolin-1 scaffolding domain in vitro and in vivo. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 2008;294(5):L843-61. doi:10.1152/ajplung.00295.2007.

38. Zhang G-Y, Yu Q, Cheng T, et al. Role of caveolin-1 in the pathogenesis of tissue fibrosis by keloid-derived fibroblasts in vitro. *Br. J. Dermatol.* 2011;164(3):623-7. doi:10.1111/j.1365-2133.2010.10111.x.

39. Miyasato SK, Loeffler J, Shohet R, Zhang J, Lindsey M, Le Saux CJ. Caveolin-1 modulates TGF-β1 signaling in cardiac remodeling. *Matrix Biol.* 2011;30(5-6):318-29. doi:10.1016/j.matbio.2011.05.003.

40. Schmitz M, Zerr I, Althaus HH. Effect of cavtratin, a caveolin-1 scaffolding domain peptide, on oligodendroglial signaling cascades. *Cell. Mol. Neurobiol.* 2011;31(7):991-7. doi:10.1007/s10571-011-9694-1.

41. Williams TM, Lisanti MP. The Caveolin genes: from cell biology to medicine. *Ann. Med.* 2004;36(8):584-95. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15768830. Accessed August 6, 2014.

42. Williams TM, Lisanti MP. The caveolin proteins. *Genome Biol.* 2004;5(3):214. doi:10.1186/gb-2004-5-3-214.

43. Kathuria H, Cao YX, Ramirez MI, Williams MC. Transcription of the caveolin-1 gene is differentially regulated in lung type I epithelial and endothelial cell lines. A role for ETS proteins in epithelial cell expression. *J. Biol. Chem.* 2004;279(29):30028-36. doi:10.1074/jbc.M402236200.

44. Williams TM, Medina F, Badano I, et al. Caveolin-1 gene disruption promotes mammary tumorigenesis and dramatically enhances lung metastasis in vivo. Role of Cav-1 in cell invasiveness and matrix metalloproteinase (MMP-2/9) secretion. *J. Biol. Chem.* 2004;279(49):51630-46. doi:10.1074/jbc.M409214200.

45. Song L, Ge S, Pachter JS. Caveolin-1 regulates expression of junction-associated proteins in brain microvascular endothelial cells. *Blood* 2007;109(4):1515-23. doi:10.1182/blood-2006-07-034009.

46. Boscher C, Nabi IR. Caveolin-1: role in cell signaling. *Adv. Exp. Med. Biol.* 2012;729:29-50. doi:10.1007/978-1-4614-1222-9_3.

47. Hunter CJ, Matyas JR, Duncan NA. Cytomorphology of notochordal and chondrocytic cells from the nucleus pulposus: a species comparison. *J. Anat.* 2004;205(5):357-62. doi:10.1111/j.0021-8782.2004.00352.x.

48. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* 2001;29(9):e45. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=55695&tool=pmcentrez&rendertype=abstract. Accessed July 31, 2014.

49. Qi W, Ding D, Salvi RJ. Cytotoxic effects of dimethyl sulphoxide (DMSO) on cochlear organotypic cultures. *Hear. Res.* 2008;236(1-2):52-60. doi:10.1016/j.heares.2007.12.002.

50. Navarro A, Anand-Apte B, Parat M-O. A role for caveolae in cell migration. *FASEB J.* 2004;18(15):1801-11. doi:10.1096/fj.04-2516rev.

51. Lee CR, Sakai D, Nakai T, et al. A phenotypic comparison of intervertebral disc and articular cartilage cells in the rat. *Eur. Spine J.* 2007;16(12):2174-85. doi:10.1007/s00586-007-0475-y.

52. Tetsunaga T, Nishida K, Furumatsu T, et al. Regulation of mechanical stress-induced MMP-13 and ADAMTS-5 expression by RUNX-2 transcriptional factor in SW1353 chondrocyte-like cells. *Osteoarthritis Cartilage* 2011;19(2):222-32. doi:10.1016/j.joca.2010.11.004.

53. Nagase H, Visse R, Murphy G. Structure and function of matrix metalloproteinases and TIMPs. *Cardiovasc. Res.* 2006;69(3):562-73. doi:10.1016/j.cardiores.2005.12.002.

54. Lires-Deán M, Caramés B, Cillero-Pastor B, Galdo F, López-Armada MJ, Blanco FJ. Anti-apoptotic effect of transforming growth factor-beta1 on human articular chondrocytes: role of protein phosphatase 2A. *Osteoarthritis Cartilage* 2008;16(11):1370-8. doi:10.1016/j.joca.2008.04.001.

55. Blaney Davidson EN, Remst DFG, Vitters EL, et al. Increase in ALK1/ALK5 ratio as a cause for elevated MMP-13 expression in osteoarthritis in humans and mice. *J. Immunol.* 2009;182(12):7937-45. doi:10.4049/jimmunol.0803991.

56. Goumans M-J, Valdimarsdottir G, Itoh S, Rosendahl A, Sideras P, ten Dijke P. Balancing the activation state of the endothelium via two distinct TGF-beta type I receptors. *EMBO J.* 2002;21(7):1743-53. doi:10.1093/emboj/21.7.1743.

57. Dao DY, Yang X, Chen D, Zuscik M, O'Keefe RJ. Axin1 and Axin2 are regulated by TGF- and mediate cross-talk between TGF- and Wnt signaling pathways. *Ann. N. Y. Acad. Sci.* 2007;1116:82-99. doi:10.1196/annals.1402.082.

58. Norrdin RW, Histand MB, Sheahan HJ, Carpenter TR. Effects of corticosteroids on mechanical strength of intervertebral joints and vertebrae in dogs. *Clin. Orthop. Relat. Res.* 1990;(259):268-76. Available at: http://www.ncbi.nlm.nih.gov/pubmed/2208865. Accessed August 25, 2014.

59. Medina FA, de Almeida CJ, Dew E, et al. Caveolin-1-deficient mice show defects in innate immunity and inflammatory immune response during Salmonella enterica serovar Typhimurium infection. *Infect. Immun.* 2006;74(12):6665-74. doi:10.1128/IAI.00949-06.

60. Wang XM, Kim HP, Song R, Choi AMK. Caveolin-1 confers antiinflammatory effects in murine macrophages via the MKK3/p38 MAPK pathway. *Am. J. Respir. Cell Mol. Biol.* 2006;34(4):434-42. doi:10.1165/rcmb.2005-0376OC.

61. Risbud M V, Shapiro IM. Role of cytokines in intervertebral disc degeneration: pain and disc content. *Nat. Rev. Rheumatol.* 2014;10(1):44-56. doi:10.1038/nrrheum.2013.160.