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GENERAL DISCUSSION

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AIMS AND SUMMARY OF THE THESIS

The role of the intervertebral disc is predominantly mechanical, as it provides limited flexibility to the spine and acts as a shock absorber by distributing loads over the spine. For effective disc functioning, the composition of the extracellular matrix is of great importance. Organized collagen type I fibrils in the stiff annulus fibrosus enclose a network of proteoglycans and collagen type II in the gelatinous nucleus pulposus. The a-vascular nature of the intervertebral disc limits its self-regenerating properties and so far no clinical relevant therapy exists to stop or reverse degeneration of the intervertebral disc. The general aim of this thesis was to improve different techniques required for a thorough and quantitative investigation of the effects of new regenerative medicine strategies in the treatment of intervertebral disc degeneration, with the ultimate goal to better understand and supplement the (lack of) self-regenerating properties of the intervertebral disc. For this purpose, five studies were conducted with the following specific sub-aims:

- To optimize the RNA isolation process of four cartilaginous structures for subsequent real-time RT-PCR analyses (Chapter 2)
- To evaluate the potential of multivariate Fourier Transform Infra-Red spectroscopic imaging for investigation of intervertebral disc degeneration (Chapter 3)
- To assess the use of luciferase mediated bioluminescence imaging for monitoring the temporal and spatial behavior of adipose derived stem cells in the intervertebral disc (Chapter 4).
- To assess the effects of an increased osmolality of the IVD pressure on the biomechanical and cellular behavior (Chapter 5)
- To regenerate *in vivo* mildly degenerated IVDs with use of a hydrogel and bone morphogenetic protein growth factors (Chapter 6)

Proteoglycans form the main component of the extracellular matrix of cartilaginous tissues. These proteoglycans not only tend to co-purify with the RNA, they also inhibit the real-time RT-PCR analysis. For homogenization of the tissue, we suggest the use of the MagnaLyser. Further, we recommend the commercially available RNeasy Fibrous kit as the best option for RNA isolation of the nucleus pulposus, annulus fibrosus and articular cartilage. For RNA isolation of meniscal tissue, we advise the RNeasy Lipid kit. Although RNA isolated with the golden standard Phenol-Chloroform (Trizol) resulted in a higher yield, the quality was not comparable with RNeasy kits and showed large variations in subsequent real-time RT-PCR analysis.

Real-time RT-PCR analysis only gives details on the response of the cells to its environment and not the actual protein production. It also requires disruption of the tissue and thereby

inhibits the evaluation of the local distribution of extracellular matrix proteins. We therefore investigated whether Fourier Transform Infra-Red (FTIR) microscopic imaging is a promising tool to study the biochemical composition of the intervertebral disc within a single section. This technique provides information on the biochemical composition of a tissue based on the vibration frequency of covalent bonds. We could reveal five matrix components with FTIR spectroscopy, and were able to match the distribution maps of both the FTIR and histologically obtained data. Healthy and mildly degenerated IVDs showed a distinct distribution of two different kinds of proteoglycans. Important is the finding that multivariate FTIR imaging can also be used for semi quantitative information of collagen and proteoglycan content. We found significant moderate to high correlations between histological grading, glycosaminoglycan content and MRI T2* measurements with data obtained from FTIR spectroscopic imaging.

Adipose stem cells gained a great interest over the last few years for their application in regenerative medicine. The cells are easy to harvest from the patient's adipose tissue, in large clinical relevant quantities, they proliferate rapidly and possess multi lineage differentiation potential. However, upon injection into the intervertebral disc, or any other tissue, the cells cannot be monitored anymore. We showed that luciferase mediated bioluminescence imaging is a promising tool to monitor the spatial and temporal distribution the adipose stem cells in the intervertebral disc. The cells were not negatively influenced by the lentiviral transduction with the luciferase vector. Both the photons emitted by the Firefly and *Gaussia* luciferase oxidation reaction could be detected through several millimeters of vertebral bone for several consecutive days, although signal intensity was much higher for the *Gaussia* luciferase.

We substantiated our insight in the role of the osmotic pressure of the extracellular matrix of the disc using the loaded disc culture system. We found that increasing the osmolality of the disc by reducing the water content did not result in a degenerative gene expression profile as we previously observed in our group by mechanical overloading. An unexpected decrease in the expression of the Tonicity Enhancer Binding Protein (TonEBP) was observed which is in contrast to the current literature. An important difference might be the experimental set up; we used an *ex vivo* cultured complete IVD subjected to loading, whereas most other researchers only studied isolated cells in 2D cultures.

Finally, we attempted regeneration of the mildly degenerated intervertebral disc with the use of an extracellular matrix mimicking hydrogel composed of fibrin and hyaluronic acid to which we covalently bound the bone morphogenetic proteins 2 and 7. This slow release system for growth factors was tested in a goat model for disc degeneration as a follow up time of twelve weeks exceeds the maximum culture period of the loaded disc culture system.

Moreover, this enabled us to use a novel clinical evaluation tool, MRI T2*. The application is this slow release system for the bone morphogenetic proteins 2 and 7 was shown to be safe for the goat intervertebral disc. Unfortunately, no regeneration of the disc was observed on either MRI T2* analysis, macroscopic and histological evaluation and biochemical assays in any of the intervention groups.

DISCUSSION

Intervertebral disc degeneration is frequently associated with low back pain, the preeminent cause of medical complaints worldwide. With four out of five people suffering from severe low back pain at least once in their life, high direct medical and indirect costs are the result, putting a major social-economic burden on society[1–4]. Therapeutic options are limited and thus far no minimal invasive treatment aimed at tissue regeneration is available in the clinic. The general aim of this thesis was to improve different techniques required for a thorough and quantitative investigation of the effects of new regenerative medicine strategies in the treatment of intervertebral disc degeneration, with the ultimate goal to better understand and supplement the (lack of) self-regenerating properties of the intervertebral disc. In this thesis, we worked towards tissue engineering of the intervertebral disc. However, to be able to do this in a scientifically sound manner with appropriate quantitative and standardized parameter evaluations, we concluded that we should start off with the improvement of adequate molecular biological, biochemical, and histological techniques under controlled *ex vivo* conditions. Using this "tool box", we continued by investigating the role of hyperosmolality, and finished with an attempt to regenerate the disc *in vivo*.

The importance of the composition of the extracellular matrix of the IVD is highlighted in many studies[5–7]. Proteoglycans are not only literally the most abundant component of the IVD, they also are a key factor within disc research[6,8,9]. Also in this thesis the proteoglycans mark their way throughout all chapters. The negatively charged glycosaminoglycan side chains of the proteoglycans attract positive ions into the nucleus pulposus, thus generating an osmotic gradient. Subsequently, water molecules are pulled into the disc by this osmotic gradient resulting in a swelling pressure. This swelling pressure gives the intervertebral disc the ability to withstand the daily compressive loading. Already 40 years ago the important role of the proteoglycans and swelling pressure for effective biomechanical functioning was pointed out by Urban *et al.*[7,10,11]. However, although we know that with aging and degeneration proteoglycans and water are lost, we still have not found a therapeutic intervention restoring the extracellular matrix of the disc and concomitant its functioning[12–14]. The process of disc degeneration can be viewed in analogy of a flat tire; the water and proteoglycans can be compared to the air which escapes the tire, slow and hardly noticeable in the beginning but progressively increasing when the inner tube becomes leak, finally resulting in a damaged tire which is not reparable. For repair of the tire different options are available, simple and cheap at the beginning of the process but gradually becoming more difficult and expensive. For the treatment of disc degeneration, however, we only have an end state treatment which comprises spinal fusion and can be compared with changing your tire with a wooden wheel from the very first developed bicycles. Given the modern times with more knowledge and improved communication possibilities, we should not have to reach out to "ancient"

and inferior therapies but search for minimal invasive therapies restoring tissue properties.

Although we established an optimal protocol for the isolation of RNA from IVD tissue in chapter 2, due to the low cell density and abundancy of proteoglycans it remains very difficult to perform PCR analysis on IVD tissue. In chapter 5 the expression of some genes could not be quantified because detection levels were too low for reliable quantification. While this could be caused by the actual lack of expression at that moment, i.e. inflammatory and matrix degrading genes are not continuously expressed in healthy IVDs[15], the shortage of sufficient RNA due to the low cell density could also be a reason. We also showed differences between a physiological experimental set up in comparison to 2D cultured cells. Although more research is performed with the use of *ex vivo* culture systems, analysis of gene expression still occurs mostly on isolated cells[16]. In some cases, complete IVDs are investigated but RT-PCR related problems are circumvented by isolating the cells from the tissue via an enzymatic degradation of the extracellular matrix, after which RNA isolation and subsequent PCR analysis are performed on the single cells[17]. Although the cells are not cultured, enzymatic degradation of the matrix during several hours will influence gene expression. On the other hand, it can be debated whether PCR analysis is the proper way to analyse the effects of regenerative therapies for the IVD, because the correlation between mRNA expression and protein level has been questioned for many years[18,19]. FTIR spectroscopy can be used as a valuable analysis tool providing information regarding different proteins within a single section showing good correlations with MRI T2* weighed images, histological grading and biochemical analysis of the disc. As the main function of the IVD is biomechanical[5], this should be a critical outcome parameter in the research of IVD de- and regeneration, advocating a physiological experimental setting using complete IVDs.

The intervertebral disc used in the experiments described in this thesis are obtained from goats. Although these animals are quadrupeds, many similarities with the human IVD exist, which makes them a proper model to study the degeneration and the regeneration of the intervertebral disc. First, the size and shape of disc are corresponding and the main loading of the disc is in the axial direction as is the case for the human IVD[20,21]. Another important similarity is the lack of notochordal cells in the human and goat IVD as this is a progenitor cell with high regenerative capacity by producing proteoglycans and collagen type II[22,23]. The presence of these cells may overrate the therapeutic effects of a treatment modality in other animal models like the mouse, rat and rabbit. The bioreactor for discs used in this study, the loaded disc culture system, was previously developed and validated by our group[24,25]. This system allows precise control of loading, nutrient and oxygen supply and the applied loading and displacement of the disc are both recorded. The typical diurnal human loading pattern can be simulated in the loaded disc culture system and comprises 8 hours of low load during the sleep and 16 hours of activity where the loading is alternated in magnitude.

Moreover, overloading and static loading regimes can be applied resembling heavy physical work and sitting for a prolonged time. In contrast to animal models, it is possible to vary only one parameter, *e.g.* the osmolality of culture medium (chapter 5). Moreover, this system provides a physiological experimental set up without the need to disrupt the tissue, allowing evaluation of the effects of an intervention in a complete IVD. With *in vitro* cell cultures, enzymatic digestion of the tissue, lost connection with the extracellular matrix and consequently mechanotransductive pathways and dedifferentiation upon culturing could bias the obtained results[26–29]. While *in vitro* studies may be easier to conduct and potentially relevant for initial testing of hypotheses, subsequent testing in *ex vivo* bioreactors should be an important step prior to translation into animal models. This will reduce the use and costs of animal models and is conform the general guideline according the "3R principle" in animal testing; replacement, reduction and refinement. In chapter 5 we observed a remarkable difference in gene expression when comparing our *ex vivo* cultures with the current *in vitro* cultures. Retrospectively, the evaluation of the slow release system for bone morphogenetic proteins 2 and 7 might have been more applicable and worthwhile to conduct first in the loaded disc culture system before testing in animal models, as now done in chapter 6. Along with the adverse effects observed in a goat model investigating a nucleus replacement[30] this shows the importance of physiological testing conditions before proceeding to animal models.

Considering the regeneration of the IVD, we should start with the beginning: as a tire does not properly function without sufficient air, the IVD needs the proteoglycans to create a swelling pressure necessary to withstand the daily loading. Recovery of the swelling pressure of the disc and subsequent intradiscal pressure will increase the disc height and tensions the annulus fibers[31]. The disc's most abundant proteoglycan, aggrecan, is formed by the binding of keratin and chondroitin sulphated glycosaminoglycans to a central core protein. Up to 100 aggrecan molecules can link to a single hyaluronic acid, together forming a proteoglycan[6]. Already at the level of a single aggrecan molecule, inter individual variations exist as the number of attachment sites for chondroitin sulphate in the CS1 region ranges from 13 to 33. A low number of repeats can negatively influence the aggrecan function making individuals more susceptible for early disc degeneration[8,32,33]. Although this variation is not the optimal target for treatment, it does point out the importance of genetic predisposition. With degeneration, the aggrecan molecule is subjected to proteolytic degradation, cleaving the core protein or disassembling the link with hyaluronic acid. The resulting small fragments then may diffuse out of the disc. Although both the glycosaminoglycans (=charge) and the size of the aggregations are important for aggrecan function, the small fragments are still charged and can attribute to the osmotic pressure[8,9]. Preventing these fragments to diffuse into the annulus and finally away from the IVD by some sort of sealing of the (inner) annulus fibrosus could decelerate the degeneration process as the osmotic pressure

stays intact preventing a shift towards more shear stress exerted on the disc cells[31]. On the other hand, preventing proteolytic activity may be even more successful. Link-N, naturally stabilizing aggrecan to hyaluronic acid, not only enhances aggrecan production but also down regulates the transcription of matrix metalloproteinases (MMPs) and is furthermore an economically attractive growth factor[34–36]. As Link-N has to be administrated to the NP, a needle puncture through the annulus is essential. This could, however, lead to injury and alter the biomechanics of the disc, and multiple injections may be needed for prolonged benefits[8,37]. The recent breakthrough in genome engineering with the discovery of CRISPR Cas9 might also be beneficial for disc degeneration. A CRISPR-Cas9 based transcriptional activation system has recently been created for endogenous human genes (e.g. IL-1RN, NANOG). Guide RNA molecules were created to recognize the DNA target promotor sites and resulted in the enhanced expression of the specific target genes. This system may be copied to design Link-N gene activation. Not only can Cas9 be used for gene activation but also gene suppression is possible by hindering transcription of RNA polymerase and MMPs. Also inflammatory genes may be a possible target for gene suppression[38,39]. At this moment, the CRISPR Cas9 system is not yet investigated for disc degeneration but with the current exponentially increasing experiments and promising results this is just a matter of time.

Getting back to the analogy of disc degeneration and the flat tire, a simple yet highly effective solution to inflate an air tube is using CO_2 cartridges. For the treatment of disc degeneration, a similar proteoglycan mimic able to be compressed but rapidly expanding upon release would be beneficial. Artificial synthetic proteoglycans might offer an excellent solution as these are not subjectable to proteolytic activity of the MMPs in the catabolic environment of the degenerating IVD[14]. The recurrent issue remains administration of the product, where a tire has an effective and strong connection between the internal and external environment, annulus closure after a needle puncture is still not possible[14,40]. Patches to cover the outer annulus, sealants to occlude fissures, biomaterials to fill up the puncture hole and sutures to close the hole in the annulus all still not possess the demanding biomechanical properties[40,41]. Biomaterials stiffer in comparison to the annulus fibrosus will be extruded while too soft materials cannot restore biomechanical properties. In addition, adhesion and integration within the annulus tissue are needed to counteract the shear forces occurring in the annulus fibrosus. Currently, needle punctures used for *in situ* testing of annulus closures have a large diameter (2,5-5 mm)[40,42] in order to match the dimensions caused by nucleus pulposus herniation defects. For minimal invasive treatment of mild disc degeneration, much thinner needles (26 - 29 G = 0.34 - 0.46 mm) can be used for the delivery of nucleus substitutes[43,44]. Combinations of a sealant filling up the needle track and a suture on the outside might offer a solution of annulus closure for the aforementioned therapies. Another possibility is using the transpedicular approach to reach the nucleus, thereby leaving the annulus intact[45]. Although promising, application in a goat model did result in protrusion of

nucleus pulposus substitute via the drill hole[30].

With the many therapies currently investigated aiming at the regeneration of mildly degenerated IVDs, two important remarks have to be made. First; how are we going to address mild degeneration? In other words: how can we effectively identify mild disc degeneration, how do we approach them and how do we evaluate treatment outcomes? Not in all cases mild degeneration results in symptoms and vice versa low back pain is not always caused by disc degeneration[46,47]. MRI T2 weighted images scored according to the Pfirmann grade is the current golden standard to establish disc degeneration, but they lack information on the biochemical composition and only show weak to moderate correlation with biomechanical functioning of disc[48–50]. Techniques such as T1ρ mapping and T2* mapping might offer better options as more information on the extracellular matrix can be obtained[48,49,51,52]. Another factor to take into account is the moment at which MRI scans are made. Due to the daily loading, 25% of the water in the disc is expelled and reabsorbed every day[53]. Subsequent MRI scans should be obtained according to a standardized time protocol to be able to observe the possible small changes in the disc as a consequence of mild degeneration. When we succeed in identifying mild disc degeneration, a more ethical question arises; should all patients with signs of mild degeneration but without pain symptoms be treated? Medical cost will rise tremendously, a cost-benefit calculation should be made, investing whether the money saved by reduction of spinal fusion treatments and work absenteeism will outweigh each other. Additionally, improvement in quality of life should also be taken into account. Although this might seem a problem for the future, it will cause a division among society and economic ethical decisions have to be made.

Stem cells form a central cell source within the field of regenerative medicine as these cells are multipotent and able to differentiate in various tissues[54–56]. Cell senescence is increasing with disc degeneration, resulting in a reduced matrix synthesis and repair of the disc[4,6]. In the treatment for disc degeneration stem cells are employed for both their stimulating trophic effects and the potential to differentiate into nucleus pulposus cells with the goal to stimulate proteoglycan synthesis in the disc[45,57,58]. Different *in vivo* studies showed the capacity of the implanted stem cells to produce extracellular matrix proteins, especially glycosaminoglycans[56,59–61]. Clinical trials show a reduction in pain and disability scores and in a few cases an increase in disc hydration was observed on MRI[62,63]. However, all studies lack appropriate controls and do not correct for potential placebo effect. Furthermore, information obtained from T2-weighted MRI images can be doubted as hydration status of the disc changes over the day and lacks correlation with the biomechanical response, and thus functioning, of the IVD[50,53]. Although in clinical trials no adverse effects are observed, a goat model of disc degeneration injected with a freshly isolated and directly applied stem cell preparation, *i.e.* the stromal vascular fraction, resulted in a severe inflammatory response combined with lymphocyte infiltration, neovascularisation and endplate destruction[64]. Discs

in the same study injected with cultured adipose derived stem cells did not resulted in adverse effects. Another important factor taken into consideration for (stem) cell treatment of the IVD is the environment of the degenerated disc, were low pH, oxygen and nutrient concentrations combined with a decreased intradiscal pressure and high shear forces do not provide a "comfortable" welcome for the cells[65–67]. Concomitantly, the number of cells injected varies in each study and no optimal number has been identified so far. Studies investigating the use of stem cells for cartilage regeneration found the structure and abundance of aggrecan and the architecture of the extracellular matrix of immature quality9. In contrast, *in vitro* research shows an improved aggrecan molecule when synthesized by stem cells compared the aggrecan molecules present in the adult cartilage[68,69]. Although a growing number of clinical trial investigates the effects of (stem) cell therapy for the treatment of disc degeneration, clear evidence of tissue regeneration is lacking and the use of stem cells can be questioned[61,63].

In contrast to all investigated therapeutic interventions, unloading of the joints is shown to increase proteoglycan content in both the IVD and articular cartilage[70–72]. Additionally, a recent study showed that static overloading predisposes the intervertebral disc for posterior herniation. In contrast to dynamic overloading, static overloading mainly affected the cell viability and extracellular matrix integrity of the posterior region of the annulus, the location where most lumbar hernias originate, whereas the nucleus was saved[73]. Currently, most people have a sedentary lifestyle resulting in prolonged static (over) loading of the disc which increases cell death and the risk of disc degeneration[25,74,75]. Loading and unloading of the IVD causes water to flow in and out of the disc, along with the water nutrients will enter and waste products will leave the intervertebral disc[31]. This suggest that alternating periods of static overloading (sitting in a prostrate position behind the computer) with dynamical loading (walking around) may be beneficial for the intervertebral disc by stimulating fluid flow. Furthermore, standing desks and desk bikes may reduce the low back pain experienced by many sedentary people.

Regeneration of the intervertebral disc is an important field in biomedical research as low back pain forms a major socioeconomic burden in the western society. And although the process of disc degeneration can be explained in analogy to a flat tire, treatment of the IVD is much more complex. Currently the research field investigating treatment of disc degeneration is very broad; different cell types, NP replacing hydrogels, AF closure and whole IVD replacement are all scrutinized. Are we currently searching for the most innovative and fanciest treatment options and thereby disregard the simple yet effective options? Perhaps we should take a step back, considering the main function of the disc, i.e. transferring loads over the spine, for which an appropriate intradiscal pressure is needed. This pressure is generated by the negatively charged glycosaminoglycan side chains of the

proteoglycans. Why not focus on the development of an injectable hydrogel which is not degradable by proteolytic activity, which immediately restores the intradiscal pressure, mimics the swelling capacity of aggrecan, and is injectable via a small (26-29G) needle in combination with a unloading regime of the disc? This way the main function of the disc is restored and a hydrostatic pressure is created to stimulate proteoglycan production by the own NP cells. From there, improvements can be made including the addition of (stem) cells and biological growth factors.

In this thesis different steps were taken to improve techniques required for a thorough and quantitative investigation of the effects of new regenerative medicine strategies in the treatment of intervertebral disc degeneration. The ultimate goal was to better understand and supplement the (lack of) self-regenerating properties of the intervertebral disc, thereby working towards tissue engineering of the disc. Starting with the improvement of analyzing techniques for real-time RT-PCR investigating cellular behavior, infrared spectroscopy for the biochemical composition of disc and monitoring cells in the disc for evaluation of cellular therapy, we continued to investigate the effect of a hyperosmolar disc on biomechanical and cellular behavior in an *ex vivo* organ culture model. This thesis is completed by testing a slow release system of bone morphogenetic protein 2 and 7 for disc regeneration where no adverse effects but also no regenerative effects were found.

Combining all the studies and obtained knowledge over the past years I may conclude that the intervertebral disc is a highly complex organ and both the de- and regeneration processes comprise a myriad of interacting factors which are currently not fully understood. With every experiment, more questions arise than are answered. The development of *ex vivo* organ culture systems may provide important new clues by enabling systematic studies in identifying the role of individual factors, while keeping all other conditions unaltered. This will likely be pivotal in developing adequate treatment strategies, but it is a time-consuming and elaborate process. According to my expectations, it will still take many years before regenerative medicine strategies will become a feasible and validated, evidence-based treatment modality for the disc degeneration. Therefore, this thesis is just the beginning, with much more to follow. . . .

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