Chemical and physical factors to regenerate the nucleus pulposus

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Chemical and physical factors to regenerate the nucleus pulposus

Chemische en fysische factoren voor het regenereren van de nucleus pulposus
(met een samenvatting in het Nederlands)

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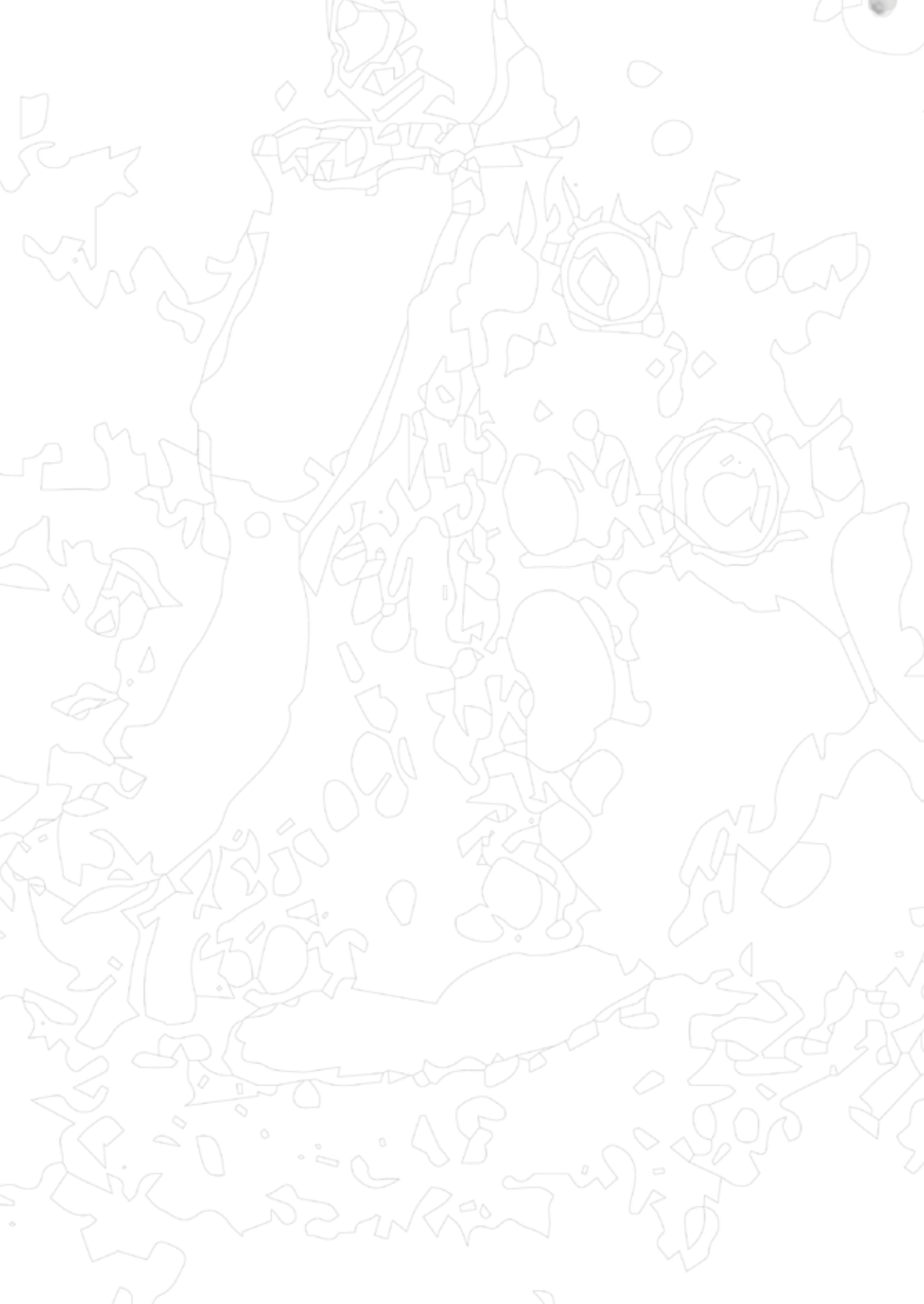

General introduction and research aims

Chapter 1

The intervertebral disc

Low back pain is one of the leading causes of disability worldwide, and as it affects many people, costs of medical care and work absenteeism involve billions per annum[1-2]. In particular chronic low back pain is associated with intervertebral disc (IVD) degeneration[3-4]. The IVD is a cartilaginous tissue and the largest avascular structure in the human body[5]. The IVDs enable the spine to withstand the large loads to which it is subjected constantly and allow for movement[6]. The IVD consists of a gelatinous core, the nucleus pulposus (NP), which is surrounded by a fibrous ring, the annulus fibrosus (AF). Posterior and inferior, the IVD is bordered by two cartilage endplates that connect it to the adjacent vertebrae[7]. In the healthy IVD, nerves and vessels are only present in the outer layer of the AF[7]. The NP usually displays the first signs of degeneration[8-10] and as such will be the focus of this thesis, but degeneration of the IVD involves all of its structures[9] (Figure 1). One of the major changes in the degenerative NP is the loss of proteoglycans[8], which has direct consequences limiting the amount of water that can be imbibed and thereby the pressures the IVD can withstand[10]. The predominant collagen in the NP, type II collagen, is gradually replaced by type I collagen[8]. Furthermore, the lamellae that make up the AF become irregular and disorganized[7], and change its mechanical properties, making it less able to contain the NP[10]. As a result, the total disc height may also decrease[7]. Sclerosis of the subchondral plate and occlusion of vascular buds under the endplate leads to decreased nutrient and oxygen transport into the IVD, which causes and enhances degeneration[11-12]. Increasing mechanical instability resulting from the loss of tension in the IVD may also lead to further degeneration by fissure formation in the NP and AF[10]. Inside these fissures, ingrowing blood vessels may be found, frequently accompanied by sensory nerves[7,13].

The exact causes of disc degeneration are unknown, but it is clear that several factors play important roles, as each of these may induce or accelerate degeneration. Mechanical overloading or immobilization[14], reduced nutrient, oxygen, and waste product transport due to endplate occlusion[12], and inflammatory factors are involved in the onset and exacerbation of degeneration. Inflammation also plays

a role in the ingrowth of blood vessels and nerves[15]. Genetic predisposition might increase the degenerative response to these factors[16-20]. Degeneration increases with age, but age in itself is not a cause of degeneration[14].

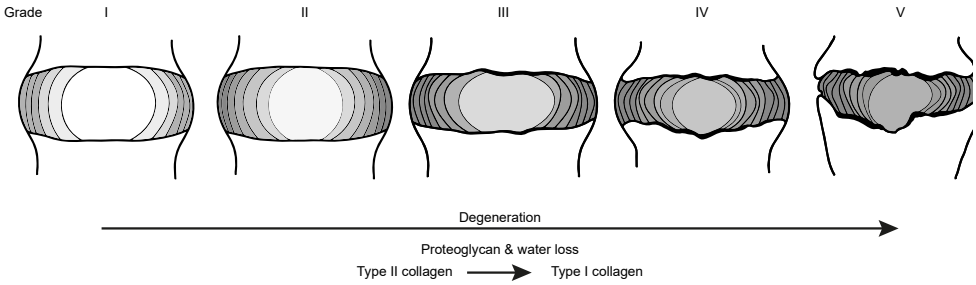

Figure 1 – Degeneration stages of the intervertebral disc from healthy to severely degenerated (Thompson grades I to V)[21].

Current treatments

Physical therapy and exercise are usually the first treatments for patients with low back pain. Although research in animal models and organ cultures indicates that controlled exercise can indeed improve IVD tissue quality by increased extracellular matrix (ECM) content[22-23], a large number of patients do not respond to these conservative treatments and still experience debilitating pain. For those patients, surgical treatment is often the only alternative, including removal of the diseased disc and fusion of the two vertebra[24] or replacement by an artificial disc[25]. Both procedures are very invasive and are almost equally effective, slightly favoring disc replacement[26]. Especially fusion of vertebrae has been suggested to increase the risk of adjacent disc degeneration[27], but long term data on disc replacements are only available in a limited number of clinical trials[26]. Moreover, minimally invasive surgery is becoming increasingly used, which may change the long-term outcomes of fusion procedures[28-29]. Nonetheless, all of these treatments provide a temporary solution and fail to achieve biologic repair, as they do not solve the underlying cause: IVD degeneration. Instead, regeneration of the IVD could provide a long-term solution.

Regenerative treatment strategies

Regenerative medicine enhances the human body's own capacity to repair and regenerate diseased or missing tissues[30]. This capacity is aided or induced by the addition of biomaterials, cells, growth factors, or a combination thereof. NP cells are being considered for regeneration[31], but problems with cell numbers and dedifferentiation during expansion arise[32-33] and use of autologous NP cells is limited to herniated IVDs. However, in most chronic low back pain patients, herniation does not play a role. Therefore, mesenchymal stromal (stem) cells (MSCs) are also being investigated as a cell source for IVD regeneration and more evidence emerges indicating they are safe to use[34-39], although data on their effectiveness in sufficiently powered randomized trials are scarce. Biomaterials and hydrogels are increasingly important for regenerative medicine in general[40] and are very interesting for IVD regeneration[41]. Biomaterial implants can be used to provide (temporary) mechanical stability of the IVD and increase the disc height reduced as a consequence of degeneration[42-43], as carriers for cells or therapeutic agents, and might also have the capacity to induce regeneration on their own[44-45]. The transplanted and/or resident NP cells present in the degenerative disc environment might need additional cues to induce sufficient regeneration. Growth factors relay messages and instruct cells to divide, differentiate, produce matrix, or produce more growth factors. As such, they are powerful tools to guide IVD regeneration[46-47]. In this respect, the bone morphogenetic proteins (BMPs), which are members of the transforming growth factor β (TGF-β) superfamily, are well-known inducers of chondrogenesis and relevant for cartilaginous tissues such as the IVD[48-49].

Regeneration of the intervertebral disc

The intervertebral disc environment

The intervertebral disc is a tissue with low cell numbers[5]. In the healthy IVD, the NP

cells reside in an environment that is rich in water and ECM (mostly proteoglycans and type II collagen). These properties ensure water is imbibed and with the NP being confined by the AF, the osmotic pressure in the core of the IVD is high compared to other tissues. The average tissue osmolality in mammals is 300 mOsm/kg[50], while in the NP it ranges from 450 in the morning to 550 in the evening and decreasing back to 450 during the night[5]. Depending on the loading conditions, NP tissue has a compressive modulus that varies between 0.1 and 5 MPa and displays properties of both a liquid and a solid[51-52]. Moreover, the tissue always experiences load, even at rest, as the muscles exert forces on the spine[53]. During IVD degeneration, the concentration of proteoglycans in the NP decreases and with that also the osmolality and the hydrostatic pressure, altering disc biomechanics[10]. While normally nutrients and oxygen are present at low concentrations, this decreases even further with degeneration, whereas the concentrations of waste products such as lactate increase and cause a decrease in the pH values[54]. Altogether, the degenerated NP is a harsh environment for resident cells, and possibly even more for cells to be transplanted for regeneration.

Regeneration by modulating cell environment

In vitro research is needed in order to obtain knowledge on potential regenerative treatments, before they can be tested in pre-clinical *in vivo* experiments or in a clinical setting. However, the cellular environment provided in the laboratory is essentially different from a living body, and from the moment a cell is taken out of its tissue it will respond to those differences[33]. NP regeneration is often studied using cells isolated from their tissue and expanded on tissue culture plastics in the absence of biomechanical loading. There is no ECM present, such as proteoglycans and collagens, which normally provides NP cells with environmental signals through integrin-binding and focal adhesions[55]. As a consequence, NP cells dedifferentiate, thus many redifferentiation models are far from the *in vivo* situation. During redifferentiation, NP cells are also often cultured in pellets[56] and hence are in very close contact to each other, which is not the case in the matrix-rich hypocellular IVD in vivo. This also alters cell behavior and may influence the results of any study into regeneration that is carried out in such a system[57-58]. Cells

of the chondrogenic lineage, like NP cells, are commonly cultured in medium that contains essential nutrients that cells need to divide or produce matrix rich in type II collagen. Noteworthy, healthy NP matrix contains about 25 times more proteoglycans than collagens by weight[59] facilitating high tissue osmolality. Cells for NP regeneration might need these specific cues to help maintain their phenotype and their ability to regenerate the correct tissue. Some of this can be achieved by adding growth factors like basic fibroblast growth factor (bFGF) during expansion, which maintains the redifferentiation capacity, and members of the TGF-β superfamily during redifferentiation[60-61]. Furthermore, there are other properties of the culture medium that can also be tuned, such as osmolarity. All factors involved, such as cells, biomaterials and growth factors likely need to act in synergy in order to achieve successful regeneration (Figure 2).

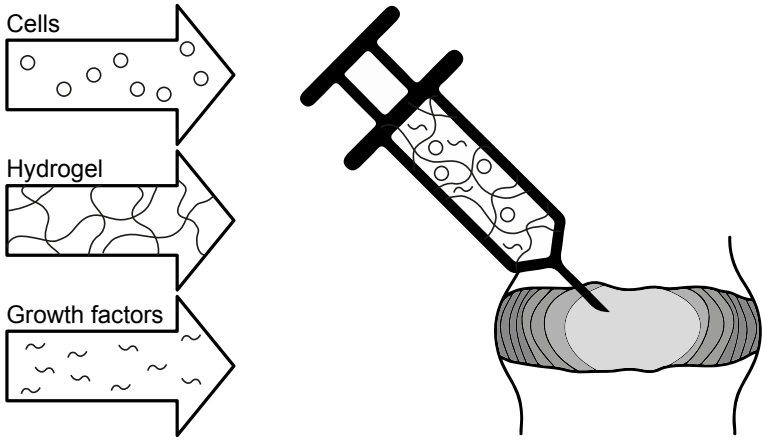

Figure 2 – Strategies for regeneration of the intervertebral disc, including cells, hydrogels and growth factors.

Cells

Implantation of cells into the NP can induce regeneration of the tissue. Several cell types are being considered for implantation, such as NP cells, chondrocytes, and MSCs[62]. NP cells have shown to slow down degeneration and increase regeneration[31]. Harvesting NP cells from an intact disc, however, will cause (further) degeneration, as the procedures to obtain them are invasive and it is known that

even punctures and contrast fluid injection for the purpose of discography to confirm an intact AF can cause further damage and degeneration to the IVD[63]. Using cells from herniated disc tissue would circumvent this issue, but these cells might not have optimal regeneration capacity and would not be applicable for patients with chronic low back pain without preceding hernia[32]. Allogeneic NP cells may be a promising alternative[64]. A cell type that shares many characteristics with NP cells, such as the type of matrix they produce, is the chondrocyte. However, some differences also exist, such as the mechanical properties and the content of the ECM, especially the aggrecan to collagen ratio, which is 25:1 in the NP and 3:1 in cartilage[65]. So far chondrocytes for NP regeneration have only been tested in animal models[66] and a recent clinical trial in human with juvenile cartilage cells has been discontinued[67]. MSCs have shown induction of regeneration by differentiation into or stimulation of NP cells[36-37,68-69], and so far several clinical trials have even demonstrated the safety of allogeneic MSCs[34-35]. MSCs thus seem a feasible option for treatment of IVD degeneration.

Biomaterials

Biomaterials can provide cells with cues that enhance regeneration. Hydrogels can provide experimental platforms to assess regenerative processes of cells and are useful for clinical applications, as they provide a condition that closely resembles the natural environment of NP cells[70]. The first clinical trials have been conducted with inert materials that are only intended to provide mechanical stability and increase disc height[42-43]. Research also focuses on instructive biomaterials as cell-carriers that can be further functionalized with the addition of growth factors[41]. *In vitro* culture in hydrogels can also help redifferentiate expanded cells to their more chondrocyte-like state[33]. Hydrogel properties that affect cell behavior such as the stiffness, fixed charge density, and porosity, can be tuned[41]. Understanding how the NP cell interacts with its environment would enable efficient and smart use of hydrogels for regeneration. Currently, many different polymers are used for NP culture or regeneration, but it is unknown what the optimal hydrogel for regeneration by NP cells would be[41]. A standard hydrogel would be a useful tool for comparison of newly developed materials, enabling comparison of research in

different laboratories.

Soluble factors

Growth factors can be used to direct matrix production by target cells. In this case, the resident NP cells, either or not in combination with transplanted cells can be stimulated to produce proteoglycans and thus repair the degenerated NP tissue[48]. Choosing the right growth factor is a challenge, as many growth factors can have different functions, which is cell- and tissue-dependent. One group of growth factors that are particularly interesting for NP regeneration, are the BMPs[49]. They have many different functions throughout development and maintenance of different tissues, including bone and cartilaginous tissues[71]. As a consequence, they can exert different effects on NP cells[72], including unwanted effects such as hypertrophic differentiation and even bone formation[73]. The choice of growth factor to be implemented in a treatment strategy for disc regeneration should thus be made after careful consideration and research.

Aim of this thesis

The general aim of this thesis is to gain insight in local and external, chemical, and physical cues in NP regeneration as research tool and therapeutic application and to explore several regeneration strategies.

Specific aims

1. Improve the regenerative conditions of NP cells.
    a. Identify the role of osmolality in regeneration by NP cells.
    b. Identify the role of osmolality during isolation and expansion of NP cells.
2. Define a reference hydrogel as comparator for hydrogel-based NP regeneration research.
3. Understand the mechanism behind the response to different hydrogels by NP cells in 3-dimensional culture.
4. Identify the most promising BMP for NP regeneration.

Thesis outline

In order to determine the role of osmolarity during regeneration, in *chapter 2* NP cells were used in regeneration cultures on transwell filters with redifferentiation medium of normal and 3 higher osmolalities. Outcome parameters were tonicity-dependent gene expression and matrix production.

In *chapter 3*, we explored regeneration cultures with NP cells in an environment that better mimics the IVD. For this purpose NP cells were cultured in six different, commonly used hydrogels based on natural polymers. Histological and biochemical evaluation of matrix production was performed to determine NP phenotype and regenerative capacity. In addition, the absence of serum in the medium was investigated. Furthermore, in order to confirm the role of tonicity, NP cells were isolated and expanded in normal medium, but also in high osmolality medium (normal values for NP) and cultured in regeneration cultures in the 2 best

performing hydrogels in different osmolalities (normal and hyperosmotic).

The mechanism behind the differential response of NP cells to different hydrogels and different stiffness was investigated in *chapter 4*. To determine whether this response is dependent on focal adhesion formation, a focal adhesion kinase inhibitor was employed to reduce the amount of focal adhesions. The effect on matrix production and gene expression of the NP cells in hydrogels with and without integrin-binding motifs was investigated to assess the influence of focal adhesions on the regenerative capacity of NP cells.

Different BMPs were added to transwell filter regeneration cultures of NP cells in *chapter 5* and compared to TGF-β as a control. Differences between responses to the BMPs were measured in terms of matrix production. Furthermore, as a first step towards translation and having in mind that MSCs are already in clinical use, matrix production was also measured in a pellet co-culture system of NP cells and MSCs with the addition of BMPs.

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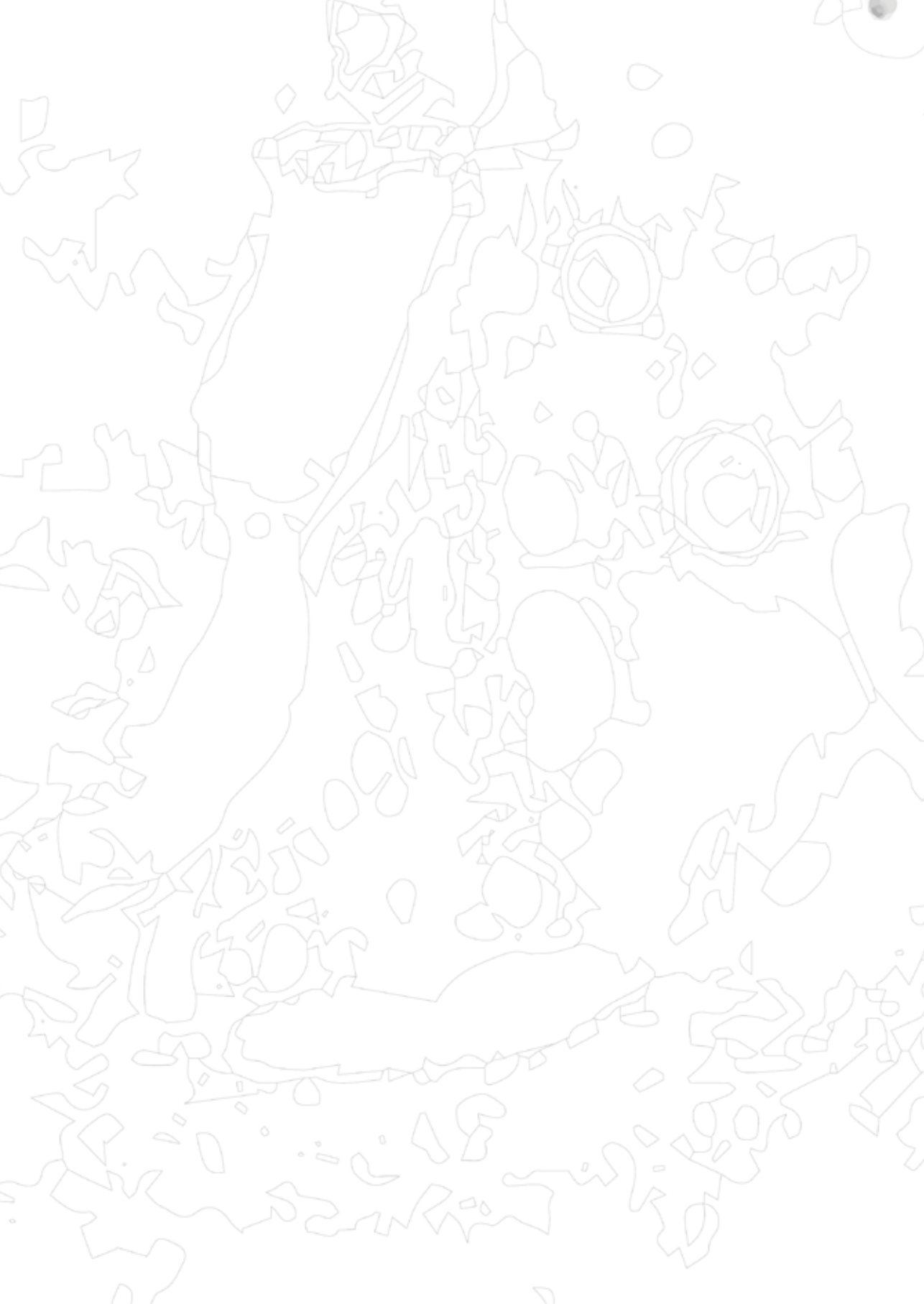

No Effects of Hyperosmolar Culture Medium on Tissue Regeneration by Human Degenerated Nucleus Pulposus Cells Despite Upregulation Extracellular Matrix Genes

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Chapter 2

Abstract

Study Design. An in vitro study using human degenerated nucleus pulposus cells.

Objective. To determine the effect of osmolality and different osmolytes on the regeneration by human nucleus pulposus cells through gene expression and extracellular matrix production.

Summary of Background Data. Intervertebral disc (IVD) degeneration is a major problem in developed countries. Regeneration of the IVD can prevent pain and costs due to diminished work absence and health care, and improve quality of life. The osmotic value of a disc decreases during degeneration due to loss of proteoglycans and might increase degeneration. It is known that gene expression of matrix genes of nucleus pulposus (NP) cells increases when cultured in hyperosmotic medium. Thus, increasing the osmolality of the disc might be beneficial for disc regeneration.

Methods. In the current study, isolated degenerated human NP cells were used in regeneration culture with medium of different osmolalities, adjusted with different osmolytes. NaCl, urea and sucrose. The cells were cultured for 28 days and expression of matrix genes and production of glycosaminoglycans and collagen II were measured.

Results. Gene expression for both collagen II and aggrecan increased with increasing osmolality using NaCl or sucrose, but not urea. Protein production however, was not affected by increasing osmolality and was decreased when using urea and sucrose. Expression of genes for Col1A1, MMP13, and MMP14 decreased with increasing osmolality, whereas expression of LOXL2 and LOXL3 increased. Transient expression of TonEBP was found 6 hours after the start of culture, but not at later time points.

Conclusion. Although expression of matrix genes is upregulated, hyperosmolality does not enhance matrix production by nucleus pulposus cells. Raising osmolality can potentially increase matrix production, but in itself is not sufficient to accomplish regeneration in the current in vitro culture system.

Key words: aggrecan, collagen II, DNA., GAG, hyperosmotic, intervertebral disc, nucleus pulposus, osmolality, osmolyte, sodium chloride, sucrose, urea.

Introduction

Intervertebral disc (IVD) degeneration is one of the leading causes of chronic low back pain and is a major cause of decreased productivity. The direct and indirect costs in the Netherlands add up to €7.6 billion annually1. Degenerative changes can be observed very early in life in the center of the intervertebral disc2, the nucleus pulposus (NP). In the healthy NP, negatively charged glycosaminoglycans (GAGs) attracting positively charged cations provide the disc with the ability to retain water and withstand pressure. The osmolality in a healthy disc ranges from 450 to 550 mOsm/kg3. It increases due to mechanical loading during the day and decreases again during the night. During degeneration, GAGs are gradually lost and as a result, water-binding capacity is reduced and tissue osmolality decreases4.

GAG production and expression of matrix genes in human and bovine NP cells increase with osmolality[5–7]. Therefore, restoration of osmolality in the degenerated IVD may be a prerequisite for successful regeneration. However, the effect on long term protein and hence tissue production has not been investigated in human degenerate NP cells before and may provide a final answer to the necessity of increasing osmolality.

In addition to the commonly used sodium chloride (NaCl)[5–6] also urea[8] and sucrose[6,9] have been used as osmolytes in NP cell and tissue culture. Urea is a small, nontoxic osmolyte that is formed by the liver for removing waste ammonia from the body and since it is nonionic, it can freely enter the cell. Sucrose is a disaccharide of glucose and fructose and can only be actively absorbed by intestinal cells[10]. Although the effects of adding these agents are commonly ascribed to changes in osmolality, it appears that several osmolytes also affect cell behavior through other mechanisms. Gene expression of cells in bovine NP tissue explants were shown to more closely resemble native tissue when cultured at high osmolality with sucrose compared to NaCl[6]. Raising osmolality using a NaCl/potassium chloride (KCl) mixture or sorbitol was shown to reduce nucleus pulposus cell proliferation, as opposed to urea[8]. Insight into the role of osmolality versus the osmolyte used on the regenerative capacity of human NP cells may further improve regeneration of the IVD by using the right osmolyte at the right concentrations.

Therefore, in this study, several osmolytes were used to raise the osmolality of the culture medium to different concentrations to study the effects on extracellular matrix (ECM) production by NP cells from human degenerated discs at both the gene expression and protein level.

Materials and Methods

Cell Isolation and Expansion

Human intervertebral discs were removed during postmortem examination (Local Medical Ethical Committee (METC) number 12–364, see Table 1 for donor details). NP and AF tissue were macroscopically dissected using scalpel and forceps by visual inspection. Nucleus pulposus cells from 5 different donors (aged 36 to 77, mean age 63, Thompson grade III) were isolated as described before[11]. Cells were expanded using Dulbecco's Modified Eagle Medium (DMEM, Gibco, Life Technologies, Carlsbad, CA) + 10% heat inactivated fetal bovine serum (HyClone, Life Technologies) + 2% antibiotics mixture (20 kU/ml penicillin, 20 kU/ml streptomycin, 50 mg/L amphotericin-B and 50 mg/L gentamycin (Lonza, Basel, Switzerland)) and 10 ng/ml recombinant human basic fibroblast growth factor (bFGF) (R&D Systems, Minneapolis, MA). During the second passage anti-microbial agents were reduced to 1% penicillin/streptomycin (Gibco). Cell viability was always at least 98%. To verify whether pooling cells of donors of different age would favor young over old cells, we evaluated time to confluence for 51 donors and found no effect of age on cell proliferation (Supplemental Digital Content Figure 1).

Table 1 – Sex, age and cause of death of donors used in this study. Cells from donor 1&2 were pooled before because of their limited quantity.

Donor	Sex	Age	Cause of death
1 + 2 (mixed)	M	65	Respiratory insufficiency (after non-hodgkin lymphoma)
	F	36	Coronary atherosclerosis
3	F	60	Cardiac arrest
4	M	66	Cardiac arrest
5	M	77	Bleeding from aortoduodenal fistula

Regeneration Culture

Cells of all donors were used in regeneration culture. Cells were seeded as described before[12] at 1 * 10^6 cells/cm^2. Osmolality of standard culture medium (DMEM low glucose, pyruvate, L-glutamine (Gibco) + 2% insulin-transferrin-selenium-X (Gibco) + 2% ascorbic acid-2-phosphate (Sigma) + 2% human serum albumin (Sanquin, Amsterdam, The Netherlands) + 1% penicillin/streptomycin (Gibco) + 10 ng/ml TGF-β2 (R&D Systems)) was adjusted from isosmotic 340 mOsm/kg to hyperosmotic 400, 450, and 500 mOsm/kg with either NaCl (27, 47, and 63 mmol added), urea (60, 110, and 166 mmol added) or sucrose (60, 112, and 165 mmol added) (all from Sigma). Medium was changed 3 times per week and stored at -80°C until further analysis.

Lactate Dehydrogenase (LDH) Assay

LDH activity as commonly used indicator of cytotoxicity was measured to check for toxic effects of the osmolytes, using a Cytotoxicity Detection Kit (Roche) according to the manufacturer's protocol. Duplicates were measured on a Versamax microplate reader (Molecular Devices LLC, Sunnyvale, CA).

Gene Expression

Total RNA was isolated using RNEasy MiniKit (Qiagen, Venlo, The Netherlands). Quality and quantity were checked using a Nanodrop (Thermo Scientific). Reverse transcription was performed using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Life Technologies). Gene expressions for collagen II (COL2A1), aggrecan (ACAN) and tonicity-responsive enhancer binding protein (TonEBP) were quantified using quantitative polymer chain reaction (qPCR) in a ViiATM Real-Time PCR System (Applied Biosystems). The primers can be found in Table 2. Expression levels of genes coding for enzymes involved in collagen biosynthesis for cells treated with NaCl were analyzed with custom made microfluidic card-based low density array (Applied Biosystems) in a ViiATM Real-Time PCR System. For each sample 50ml of diluted cDNA was mixed with 50ml of TaqMan PCR master mix (Applied Biosystems). Reactions were performed according to recommended protocol: 50°C for 2 min, 95°C for 10 min and for 40 cycles at 95°C for 12 s and 60°C for 1 min. All data were analyzed using ViiATM Real-Time PCR System Software v1.1 (Applied Biosystems). Gene expression levels in all experiments were normalized to GAPDH.

Table 2 – Primers used for qPCR. All primers were designed using the Roche assay design centre. The 10 µl reaction containing SYBRGreen Supermix (Bio-Rad, Hercules, CA), 6 µM forward primer, 6 µM reverse primer and 5 ng of cDNA. Reactions were performed at 95°C for 15 sec, 60°C for 30 sec, 72°C for 30 sec, for 40 cycles.

Primer	Forward	Reverse
ACAN	5'-GGGTTTTCGTGACTCTGAGG-3'	5'-GAAATAGCAGGGGATGGTGA-3'
COL2A1	5'-TGGTGCTAATGGCGAGAAG-3'	5'-CCCAGTCTCTCCACGTTCAC-3'
TonEBP	5'-TCAGACAAGCGGTGGTGA-3'	5'-AGGGAGCTGAAGAAGCATCA-3'
GAPDH	5'-AGCCACATCGCTCAGACAC-3'	5'-GCCCAATACGACCAAATCC-3'

GAG and DNA Analysis

Cultured samples were digested overnight in 250 mg/ml papain and 1.57 mg/ml L-cysteine (Sigma). Sulfated GAG content was measured with a 1,9-dimethyl-methylene blue (DMMB, Sigma) assay with chondroitin-6-sulfate as the standard using a Versamax microplate reader. Values at 525nm were divided by the measurement at 595 nm.

DNA quantity was measured by Picogreen assay (Life Technologies) on a fluorescence spectrophotometer (Fluoroskan Ascent FL, Thermo Scientific, Waltham, MA) with excitation at 485nm and emission at 538 nm. GAG was corrected for DNA. GAGs produced during culture were measured in medium collected and pooled per week.

Procollagen II ELISA

The carboxyterminal propeptide of type II collagen (PIICP) content in culture medium as a parameter for collagen II production during culture[13] was measured using an enzyme-linked immunosorbent assay (ELISA, Cloud-Clone Corp, Houston, TX) according to the manufacturer's protocol using a Versamax microplate reader.

Histology

After regeneration culture for 28 days, formed neotissue samples were fixed overnight in 3.7% formalin (J.T. Baker, Avantor Performance Materials, Center Valley, PA) at room temperature. After dehydration the tissues were embedded in paraffin (Stemcowax, Adamas Instruments, Amerongen, The Netherlands) and cut into 5mm sections with a microtome (Thermo Scientific). After deparaffinization, safranin-O (Merck) staining for GAGs and fast green (Merck) for collagen and Weigert's Hematoxylin (Klinipath, Duiven, The Netherlands) for cell nuclei were performed, or immunohistochemistry for collagen II with mouse monoclonal primary antibody collagen type II (DSHB II-II6B3 (DSHB, Iowa City, IA). Mouse isotype IgG was used as negative control. Images were taken with a light microscope, DP 70 camera and cell^F software (Olympus, Tokyo, Japan).

Statistical Analysis

Statistical analysis was performed using SPSS software (IBM, Armonk, NY) and included univariate analysis of variance with randomized block design and post hoc Tukey t-test. Outliers were detected using the 3*sigma rule; a value is considered an outlier when it is outside the mean plus or minus 3 times the standard deviation range.

Results

Cytotoxicity

LDH levels at day 2 for cells cultured with iso- and hyperosmotic medium with sucrose were lower than for cells cultured with iso-osmotic medium or medium with osmolality

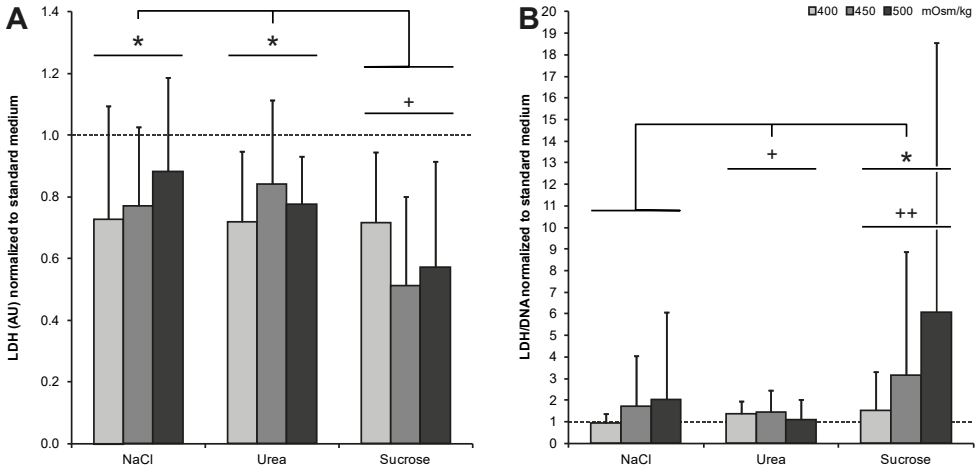


Figure 1 – LDH of 4 donors was measured during culture of NP cells with 3 different osmolytes and compared to standard medium. **A**: LDH production for cells cultured with sucrose on day 2 was lower than for hypo-osmotic medium (+ p = 0.02) and medium adjusted with NaCl or urea (* p < 0.001). **B**: LDH/DNA levels on day 28 for cells cultured with sucrose was higher than for cells with hypo-osmotic medium (++ p = 0.045) or medium with NaCl (* p < 0.001). Moreover, LDH/DNA of urea was higher than NaCl (+ p = 0.025).

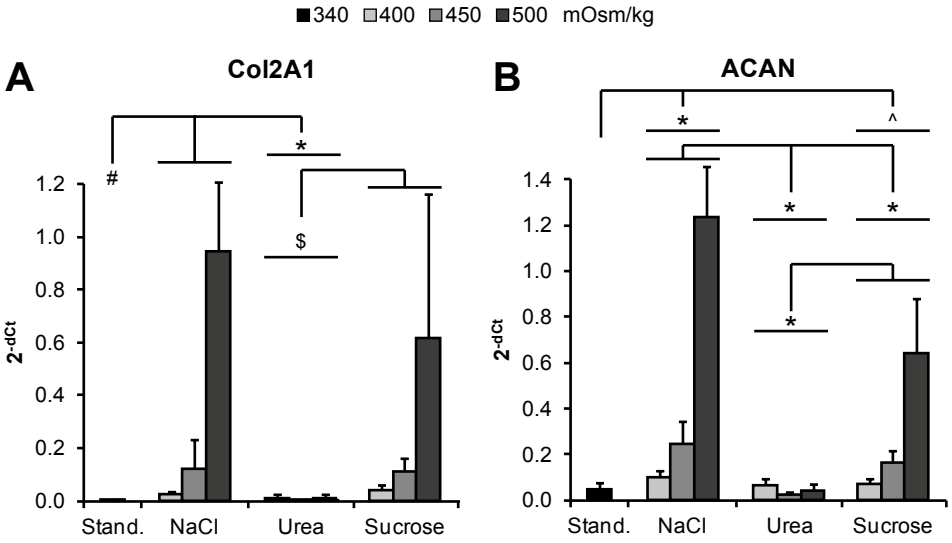


Figure 2 – Gene expression of cells cultured for 28 days with standard medium or medium with adjusted osmolality. **A**: COL2A1 was upregulated in cells cultured with NaCl and sucrose, regardless of concentration (* p ≤ 0.001, # p = 0.035, $ p = 0.019, compared to cells cultured with standard medium or medium with urea). **B**: Expression of ACAN was increased in cells cultured with NaCl and sucrose (* p < 0.001, ^ p = 0.025).

increased by NaCl or urea ($p < 0.002$, Figure 1A). LDH normalized to DNA on day 28 was higher for sucrose than in iso-osmotic medium and medium with increased concentrations of NaCl, and activity in medium conditioned by cells cultured with urea were higher than NaCl ($p < 0.045$), regardless of concentration (Figure 1B).

Extracellular Matrix Gene Expression

Gene expression analysis showed the most prominent effect for NP cells cultured in hyperosmotic media adjusted with NaCl. COL2A1 was increased approximately 200-fold or 25-fold for cells cultured in 500 mOsm/kg medium with NaCl or sucrose, respectively,

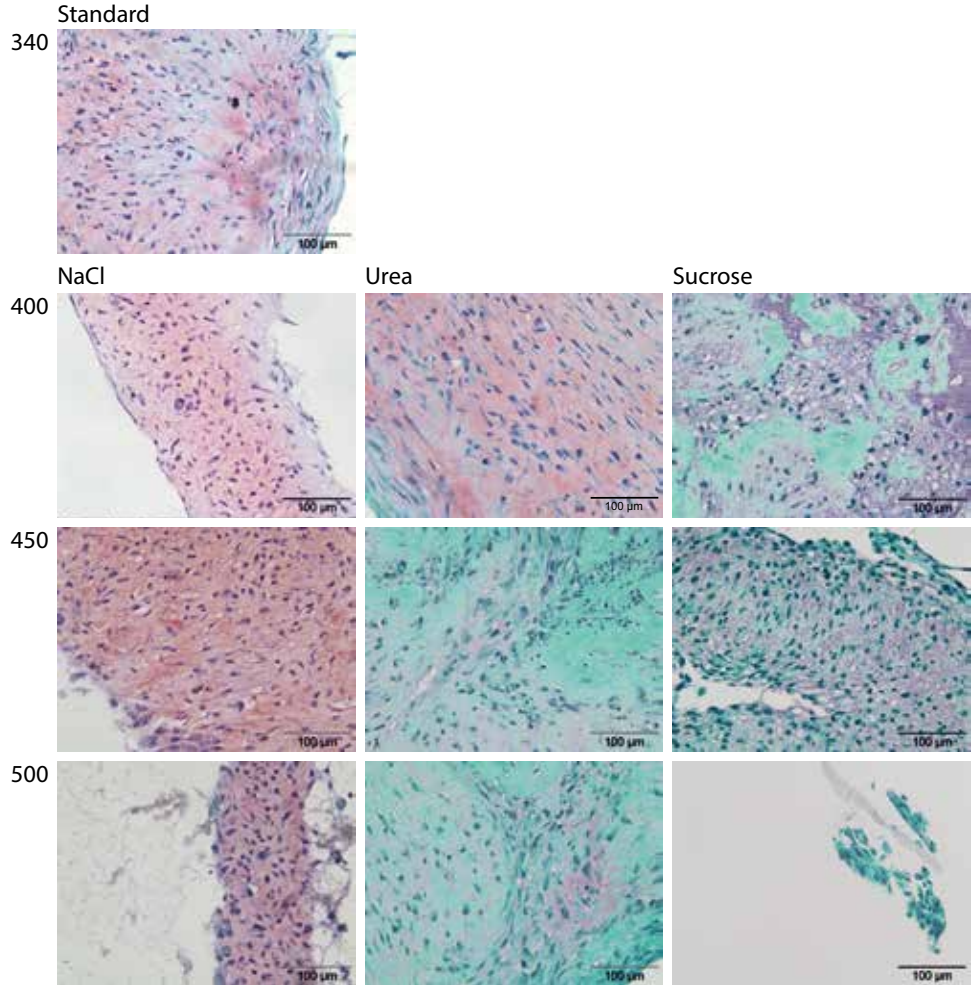


Figure 3 – Safranin-O shows more intense staining for NaCl samples. In urea 450 and 500 mOsm/kg and all sucrose concentrations, Fast Green staining for collagens is more pronounced than Safranin-O.

in comparison to standard medium or medium with urea, regardless of concentration (Figure 2A). Expression levels of ACAN were increased 150-fold in 500 mOsm/kg medium with NaCl and 12-fold in cells cultured in 500 mOsm/kg medium with sucrose (Figure 2B). Hyperosmotic medium with urea did not show any effect on COL2A1 or ACAN expression. No effects were found at 400 or 450 mOsm/kg.

Histology and Collagen II Staining

Safranin-O staining (Figure 3) was more intense in the tissue cultured in medium of NaCl-adjusted osmolality compared to the sucrose and urea-containing media. No clear effect

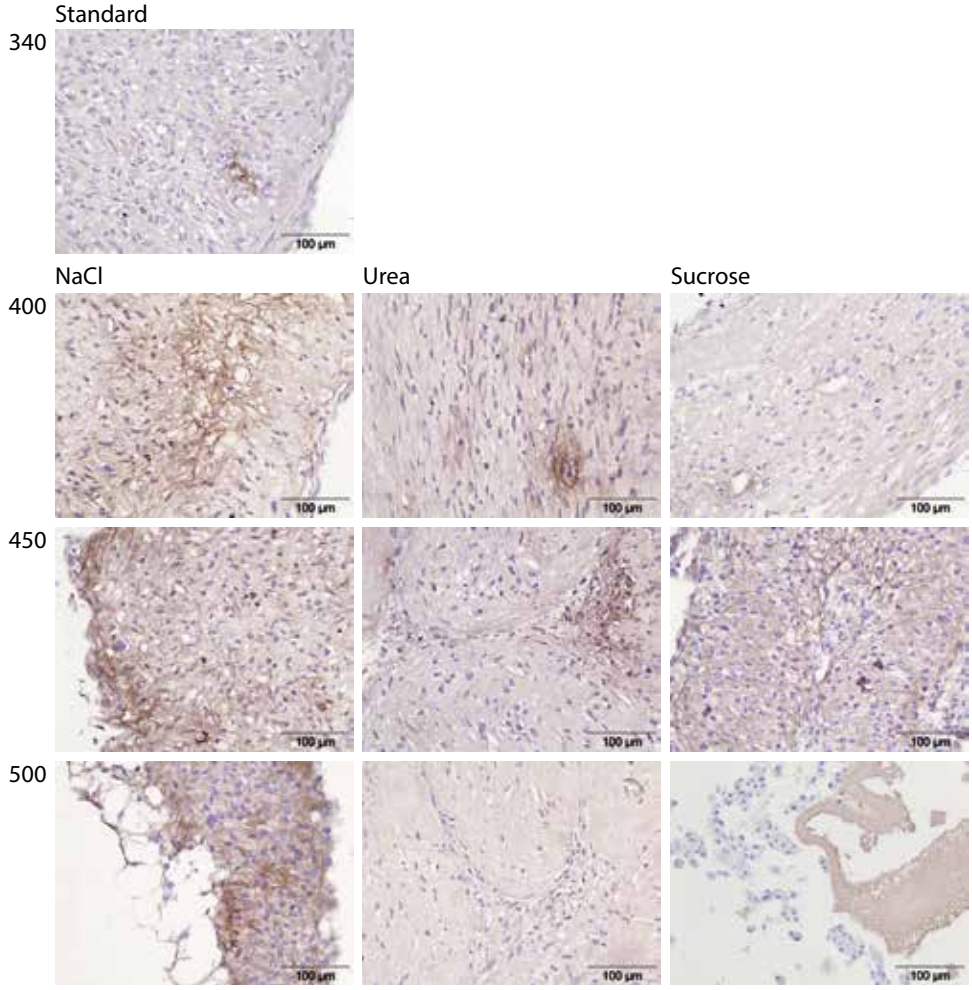


Figure 4 – Immunohistochemistry for collagen II showing areas of collagen II formation. Staining appears most intense in samples cultured with NaCl-adjusted osmolality and does not appear different between concentrations.

was seen of increasing osmolality. Collagen II immunohistochemistry (Figure 4) for NaCl 400 mOsm/kg demonstrated the presence of collagen II in all tissues, which appeared to be more intense in NaCl adjusted medium. No effect of increasing osmolality was seen.

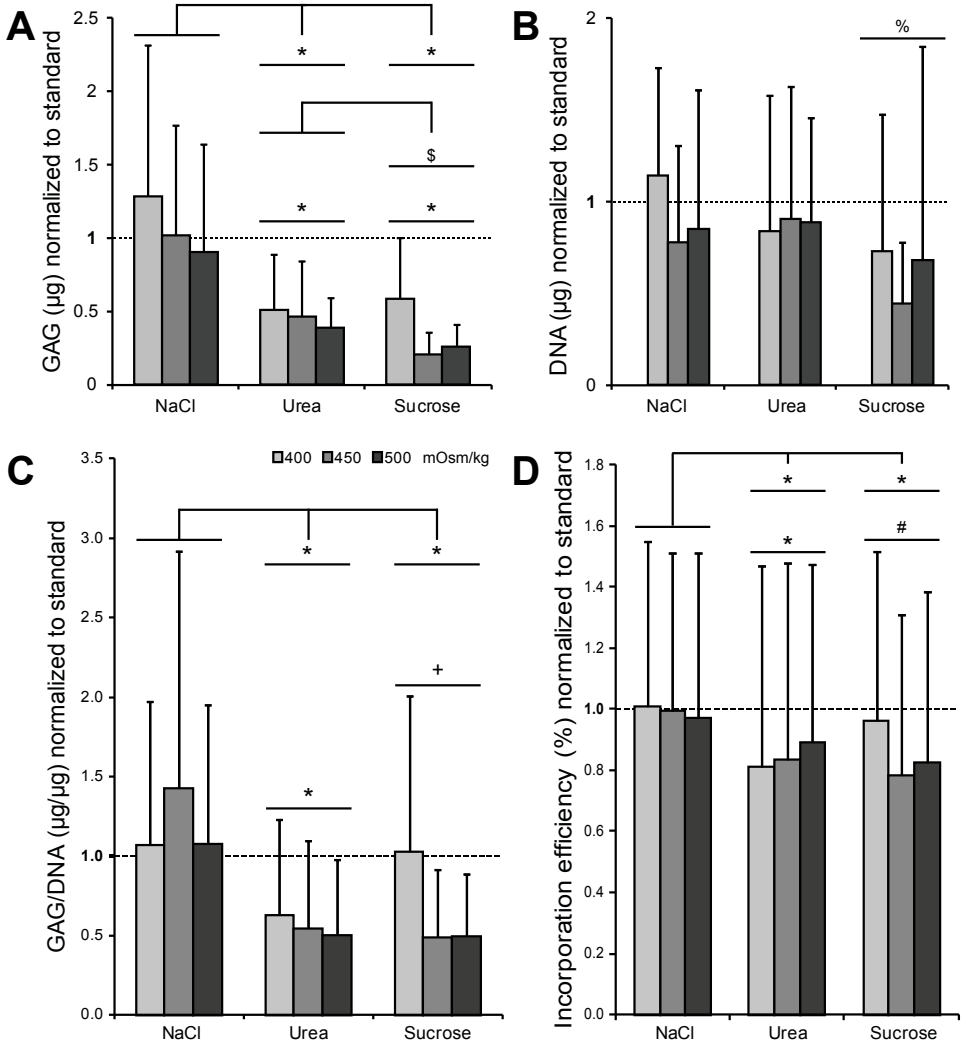


Figure 5 – GAG and DNA in regenerated tissue at day 28 were all normalized to standard medium. **A**: GAG in regenerated tissue at day 28 for cells cultured with urea or sucrose was lower than hypo-osmotic medium or medium with NaCl (* $p \leq 0.001$). Sucrose was also lower than urea ($ $p = 0.35$). **B**: DNA values in regenerated tissue for sucrose were lower than for standard medium (% $p \leq 0.04$). **C**: Cells cultured with urea or sucrose produced lower amounts of GAG per DNA than cells cultured with standard medium. (* $p \leq 0.001$, + $p = 0.024$) or cells treated with NaCl (* $p \leq 0.001$). **D**: Incorporation of GAGs versus release into the medium was less efficient for cell cultured with urea or sucrose compared to standard medium (* $p \leq 0.001$, # $p = 0.002$) or NaCl (* $p \leq 0.001$).

GAG Content

GAG content of tissue formed on day 28 was significantly higher for cells cultured in medium with NaCl compared to cells cultured with urea and sucrose ($p < 0.05$, Figure 5A), independent of osmolality. Moreover, the GAG content of tissue produced by the cells in the presence of urea was higher than by cells cultured with sucrose ($p = 0.035$). DNA content was lower for sucrose than for standard medium ($p \leq 0.04$, Figure 5B). No effect of increasing osmolality was found on GAG normalized to DNA, for any osmolyte (Figure 5C). Incorporation efficiency of GAGs in tissue in percentage was calculated by determining the ratio of GAG content to total GAG production (released into the medium + tissue GAG content). Incorporation efficiencies were higher for standard medium and NaCl adjusted media compared to urea and sucrose ($p < 0.05$, Figure 5D). The use of 450 or 500 mOsm culture medium, regardless of osmolyte, significantly decreased incorporation efficiency compared to standard medium ($p = 0.008$ and $p = 0.04$ respectively).

Collagen II Production

Collagen II was produced throughout the entire culture (Figures 6A and C). After week 3, apparently production slowed down and was lowest in the 4th week of culture. No differences were found when comparing different osmolarities adjusted with NaCl, nor when the different osmolytes at 400 mOsm/kg were compared with standard iso-osmotic medium (Figures 6B and D).

Collagen Low Density Array

Expression levels of matrix metalloproteinase (MMP) 13 and 14 genes decreased with increasing osmolality. Gene expression levels coding for enzymes involved in collagen crosslinking, lysine hydroxylases and lysine oxidases (PLODs, LOX, LOXLs) were not negatively affected by increasing osmolality, LOXL2 and 3 were even significantly increased (Figure 7).

Time course of matrix gene expression and protein production at different osmolality using NaCl

ACAN expression increased after 1 week of culture and stayed high until the end of the culture period (Figure 8A). The expression of TonEBP was upregulated after 6 hours of culture in response to high osmolality, thereafter returning to baseline (Figure 8B). At the protein level, most of the GAGs were produced in the first 2 weeks of culture, similar to the timing of collagen production found. The total amounts of GAG and DNA increased during culture, but GAG/DNA did not change between 14 and 28 days (data not shown).

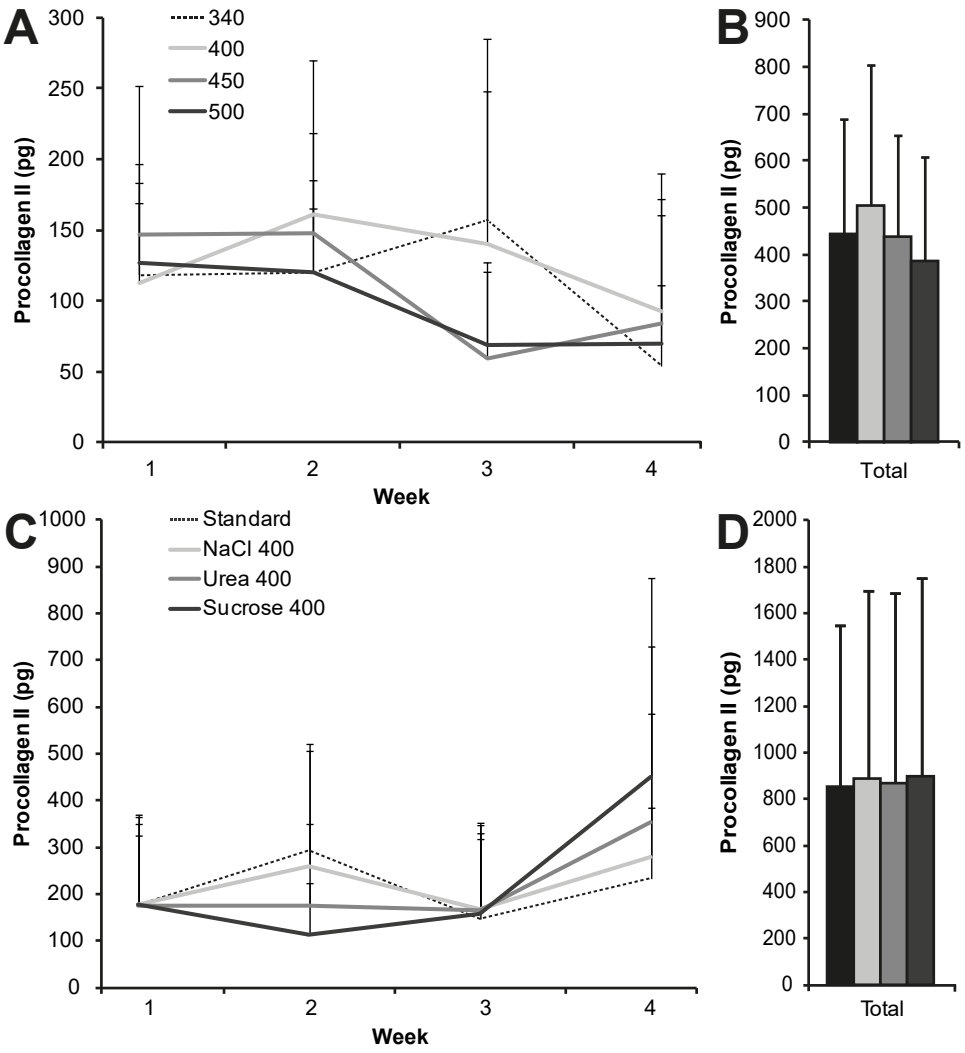
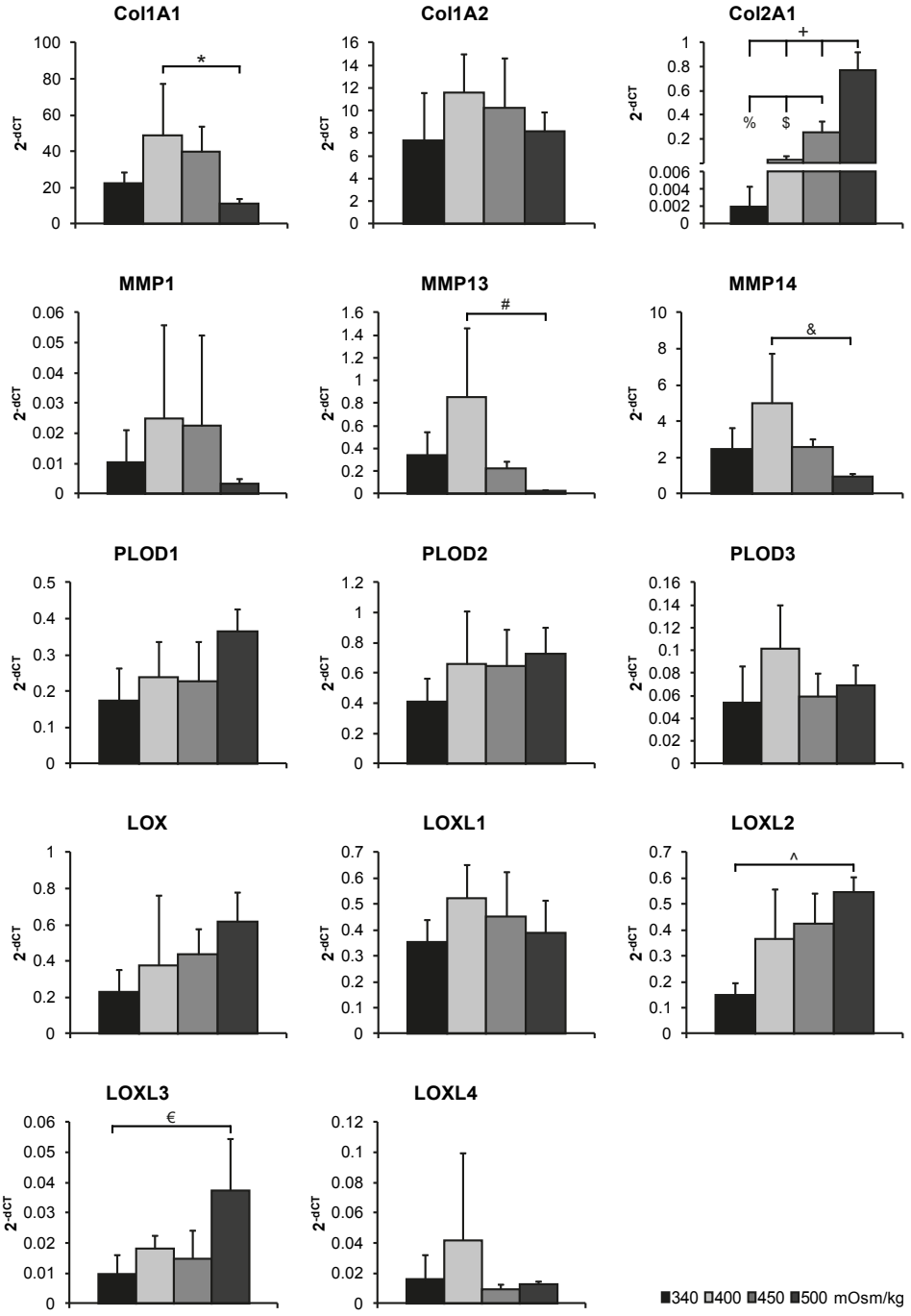


Figure 6 – Procollagen II (PIICP) was measured in medium during culture. **A**: Procollagen II released per week for cells cultured with standard medium or medium with NaCl 400, 450, or 500 mOsm/kg. **B**: Total amount of procollagen II during 28 days of culture. **C**: Procollagen II released per week for cells cultured with standard medium or medium with NaCl, urea or sucrose, all of 400 mOsm/kg. **D**: Total amount of procollagen II during 28 days of culture.

Figure 7 – Genes for collagen and collagen related genes were measured with a low density array. Col1A1 expression was lower for 500 mOsm/kg compared to 400 (* $p = 0.41$). Col 2A1 expression increased for increasing osmolalities: expression at 450 mOsm/kg was higher than at 340 (% $p = 0.025$) or 400 ($ $p = 0.031$) and expression at 500 mOsm/kg was higher than all others (+ $p < 0.001$). MMP13 and MMP14 gene expression levels were higher for NaCl 400 mOsm/kg compared to 500 mOsm/kg (# $p = 0.022$ and & $p = 0.016$, respectively). Expression of LOXL2 and LOXL3 were higher for 500 mOsm/kg compared to iso-osmotic medium (^ $p = 0.006$ and € $p = 0.026$, respectively).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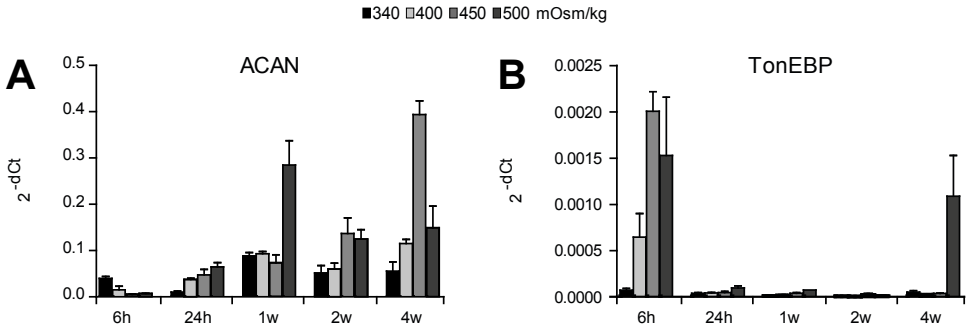

Figure 8 – **A**: Gene expression of aggrecan increased during culture of cells with NaCl. **B**: TonEBP showed a transient upregulation in the first 6 hr of culture.

Discussion

The current study is the first to investigate in detail the role of osmolality and different osmolytes in tissue regeneration by degenerated human NP cells. Upregulation of extracellular matrix genes was found with increasing osmolality. At the protein level however, no increase of matrix deposition was found. Using sucrose or urea, even a decrease in matrix production with increased osmolality was observed. The discrepancy between matrix gene expression and actual protein deposition as a function of NaCl-mediated increase of osmolality in the newly formed tissue seemed not to be related to enhanced degradation of the matrix, as a decrease in MMP gene expression was found and gene expression of enzymes related to crosslinking of collagen was not affected.

The increase in ACAN gene expression with increasing osmolality in the current study is in line with several publications[6–7]. A decrease in proteoglycan deposition by bovine NP cells cultured in medium with sucrose is in line with our data, suggesting some similarity in response between healthy bovine and degenerated human NP cells[14]. However, others have found contrasting results at the protein level[15] or even decreased GAG production with increasing osmolality[5,16].

Differential effects may be due to the use of healthy cells, to a species difference or even culture system. It may be argued that the NP cells used in this study are not sensitive to osmolality changes, either because they are derived from degenerated discs or because they have been passaged. However, this seems unlikely since cells still respond to osmolality changes by upregulating TonEBP. Moreover, aquaporin 2, a tonicity dependent water channel regulated by TonEBP[17] is strongly expressed in degenerate IVD tissue. The discrepancy between RNA and protein production found for NaCl-mediated hyperosmolality has been reported in many fields of research[18–21] and may arise from altered protein half-life or intracellular degradation. MMP expression (MMP1, 13 or

14) and hence matrix degradation did not seem to be involved, nor a loss of collagen network functionality due to diminished crosslinking.

Despite the transient nature of the upregulation of TonEBP, the increase in aggrecan expression in the presence of increased concentrations of NaCl was maintained throughout culture, suggesting at least part of this effect was independent of osmolality. Indeed, no clear upregulation of ECM expression with osmolality in the presence of urea was found and in general ECM production in the presence of urea and sucrose was lower. These effects therefore may have been related to NaCl specific signaling. The endothelial sodium channel (ENaC) found in human chondrocytes, mesenchymal stromal cells and osteoblasts[22] is postulated to be involved in mechanosensing in cartilage and bone may have a similar function in NP cells. However, the response of human articular chondrocytes to increasing osmolality was shown previously not to be dependent on sodium signaling[23]. Urea was previously shown to inhibit the colony forming efficiency of cell lines[24], growth plates of rats[25], and increase MMP13 expression in chondrocytes[26], so also urea may exert effects on NP cells independent of osmolality. Osmolyte specific effects of sucrose are more difficult to support, and sucrose has been used before as an inert agent to raise medium osmolality[6,27]. In the current study cell numbers were lower and toxicity higher in tissues produced with sucrose compared to standard medium, in line with previous data[8,23]. However, others have reported effects of sucrose on cells not to be different from those of NaCl[9,23,28].

Not just the osmotic pressure, but also changes in osmolality (which occur during mechanical loading) affect cells. For example, transient receptor potential cation channel subfamily V member 4 (TRPV4)-mediated Ca^{2+} signaling increased in cells exposed to a decrease in osmolality[29]. The in vitro culture model used here is characterised by two changes in osmolality, namely isolation and expansion at 340 mOsm/kg and hyperosmolality upon the start of regeneration culture. When bovine chondrocytes were expanded up to passage 3 at higher osmolality, they produced constructs that contained slightly more ECM and were also stronger than constructs from cells that were expanded at low osmolality, as well as higher aggrecan and collagen II gene expression[23,30]. Also chondrocyte expansion in hypertonic medium (380 vs 280) yielded higher aggrecan gene expression, higher collagen II gene expression and protein production, and lower collagen I gene expression and protein production than expansion at 280 mOsm/kg, but cell viability decreased at osmolalities over 380 mOsm/kg[23]. This is in line with the observation that high osmolality induces DNA damage in murine kidney cells[31] and bovine NP cells[8]. At the same time, DNA repair was also higher in NP cells at higher osmolality adjusted with NaCl and to a lesser extent with sorbitol. Therefore, raising osmolality may have opposing effects, which could be partly dependent on the different stages of cell growth and tissue regeneration. Ideally, the NP-specific nature of isolated and cultured cells is checked by using specific markers. However, the few markers found until now were either only present in a subpopulation of NP cells[32] or only in healthy cells[33], whereas degenerated cells were used in the current study. Several factors have

been found exclusively expressed in AF cells, but these data are difficult to reproduce or to confirm with protein data and might differ between species[33]. Altogether, conservative dissection of NP tissue from inner AF and endplate tissue is currently most likely the best approach toward NP cell isolation.

The observation that increasing osmolality does not seem to be sufficient in NP-mediated regeneration, suggests that other factors need to be taken into account when delivering NP cells or stimulate resident cells in degenerated IVDs. However, to what extent these findings can be translated toward the in vivo conditions is not clear. For regenerative strategies in intervertebral disc degeneration, it seems that osmolality is not a factor that would need to be addressed for regeneration by degenerated NP cells. This implicates that for therapeutic application hydrogel carriers for endogenous or exogenously added cells may not need to be adjusted for osmolality.

In conclusion, in the current culture system, the increased expression of matrix genes with osmolality appears to be osmolyte rather than osmolality-dependent. Expression levels of matrix related genes increase with increasing osmolality. However, the absence of an effect at the protein level suggests adjusting osmolality with NaCl to enhance matrix production by IVD cells in vitro is not sufficient for regeneration.

Key Points

- Increasing osmolality increases expression of Col2A1 and ACAN genes in human degenerated NP cells.
- The effects of osmolality are dependent on the osmolyte used, suggesting also osmolality independent mechanisms.
- Increasing osmolality does not increase extracellular matrix protein deposition.

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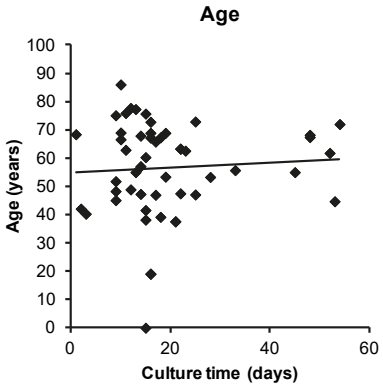
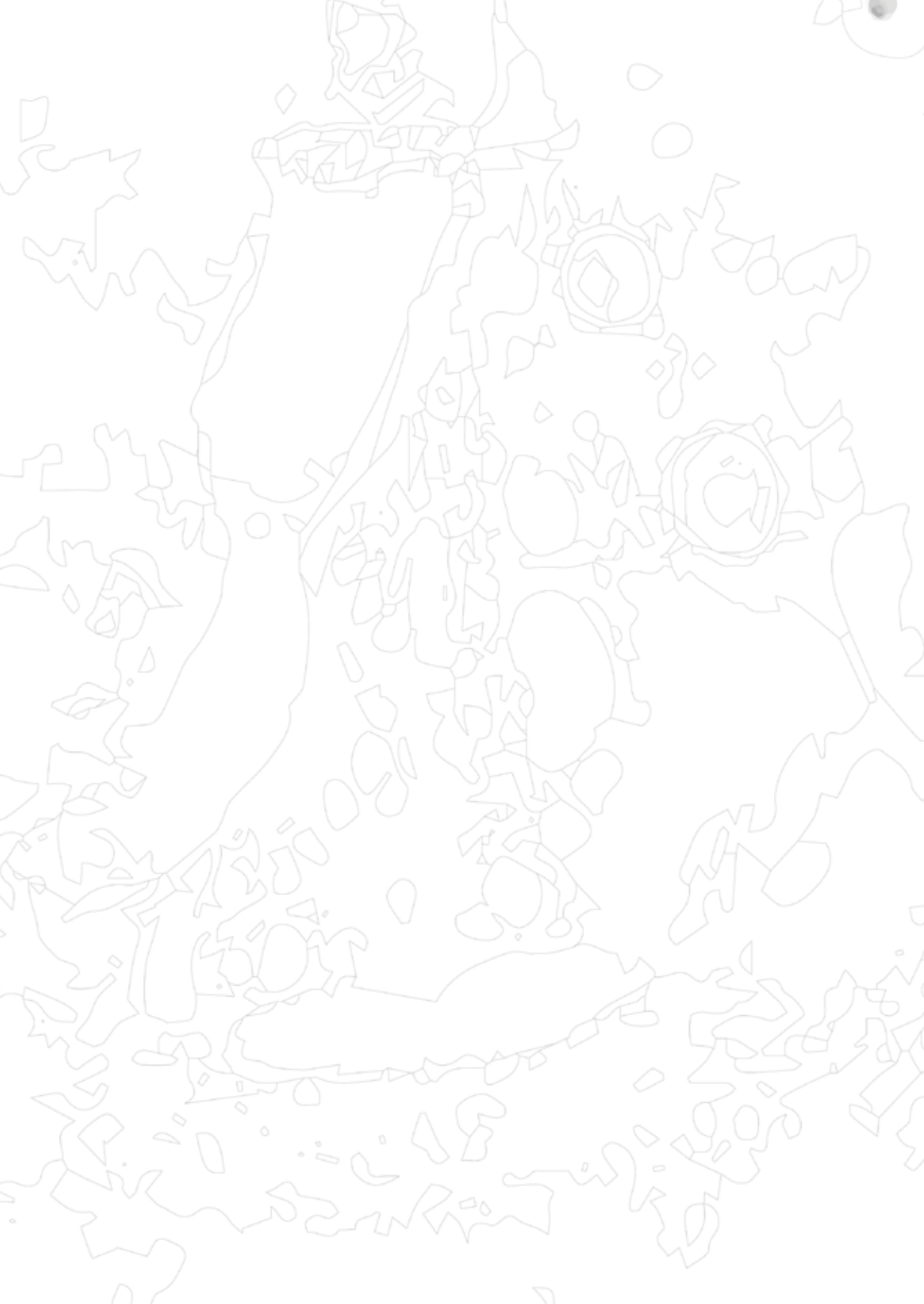
Supplementary data



Supplementary figure S1 – The Pearson correlation between age and culture time to confluence was 0.067 with p=0.638, indicating that there is no correlation.



Comparing Hydrogels for Human Nucleus Pulposus Regeneration: Role of Osmolarity During Expansion

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Chapter 3

Abstract

Hydrogels can facilitate nucleus pulposus (NP) regeneration, either for clinical application or research into mechanisms of regeneration. However, many different hydrogels and culture conditions for human degenerated NP have been employed, making literature data difficult to compare. Therefore, we compared six different hydrogels of natural polymers and investigated the role of serum in the medium and of osmolarity during expansion or redifferentiation in an attempt to provide comparators for future studies. Human NP cells of Thompson grade III discs were cultured in alginate, agarose, fibrin, type II collagen, gelatin methacryloyl (gelMA), and hyaluronic acid–poly(ethylene glycol) hydrogels. Medium containing fetal bovine serum and a serum-free (SF) medium were compared in agarose, gelMA, and type II collagen hydrogels. Isolation and expansion of NP cells in low compared to high osmolarity medium were performed before culture in agarose and type II collagen hydrogels in media of varying osmolarity. NP cells in agarose produced the highest amounts of proteoglycans, followed by cells in type II collagen hydrogels. The absence of serum reduced the total amount of proteoglycans produced by the cells, although incorporation efficiency was higher in type II collagen hydrogels in the absence than in the presence of serum. Isolation and expansion of NP cells in high osmolarity medium improved proteoglycan production during culture in hydrogels, but variation in osmolarity during redifferentiation did not have any effect. Agarose hydrogels seem to be the best option for in vitro culture of human NP cells, but for clinical application, type II collagen hydrogels may be better because, as opposed to agarose, it degrades in time. Although culture in SF medium reduces the amount of proteoglycans produced during redifferentiation culture, isolating and expanding the cells in high osmolarity medium can largely compensate for this loss.

Keywords: nucleus pulposus, hydrogel, regeneration, osmolar concentration, humans

Introduction

Chronic low back pain is the leading cause of disability worldwide and is associated with degeneration of the intervertebral disc (IVD)[1,2]. Degeneration of the IVD is assumed to be mostly initiated in the nucleus pulposus (NP)[3], whereby a fibrotic type of tissue replaces the normal matrix containing high amounts of negatively charged proteoglycans that attract cations. This normal matrix rich in anionic polymers enables the tissue to imbibe large amounts of water, and thereby the healthy NP resembles a hydrogel, defined as "a polymeric network, which can absorb large quantities of water, while remaining insoluble in aqueous solutions[4]." Therefore, hydrogels are extensively being studied for repair of the NP. Although the ontogeny of human NP cells is still not completely undisputed, in adult IVDs, they resemble chondrocytes in cell morphology, protein expression, and tendency to dedifferentiate into a fibroblast-like cell upon tissue degeneration[5]. Similar to chondrocytes, NP cells grown in hydrogels have been shown to produce extracellular matrix (ECM)[6–16] and redifferentiate better in a three-dimensional (3D) environment compared to two-dimensional (2D) systems[17,18] and "semi-3D" high density cultures[19]. However, since important differences between chondrocytes and NP cells remain, NP cells should be investigated independently and proven concepts using articular chondrocytes cannot be translated per se. In addition to their application as part of regenerative treatments, hydrogels are employed in preclinical research in vitro to mimic the native environment for NP cells and study mechanisms of ECM production as part of regeneration.

As yet, there is no consensus on the most suitable hydrogel for in vitro mechanistic studies on NP regeneration, or for regeneration in vivo[6]. Commonly, studies on hydrogel-based regeneration are performed with one particular hydrogel. However, this study setup does not enable assessment to what extent such a novel hydrogel suitable for further use, that is, represents an improvement over other previously studied hydrogels. Various hydrogels are currently being used for NP cell culture and ECM production. Natural polysaccharides, mimicking the glucosamine-rich part of the NP matrix, and collagen-derived matrices, have been obvious choices. Alginate is a polysaccharide derived from brown algae and can be ionically cross-linked by the addition of divalent ions such as Ca^{2+}, Ba^{2+}, or Mg^{2+}. This gentle cross-linking method makes it a very suitable material for cell culture. It has been shown to support matrix formation by NP cells in vitro[7–9]. The seaweed-based polysaccharide agarose is receiving increasing attention in the field of IVD regeneration[10–13]. Hyaluronic acid (HA) is a glycosaminoglycan (GAG) and component of the ECM of cartilaginous tissues such as the NP, and thus HA-based hydrogels provide a natural cell environment[14,15]. Type II collagen hydrogels and type I collagen-derived scaffolds such as gelatin have also been shown to support the chondrogenic NP phenotype, that is, secretion of collagen type II and aggrecan[16,20]. Gelatin can be functionalized with methacryloyl groups (gelatin methacryloyl [gelMA]) that allow subsequent cross-linking, resulting in a more stable and strong hydrogel, while

still supporting matrix formation[21]. Although not a native part of the IVD, fibrin also has been used for chondrocyte[22], annulus fibrosus (AF)[23], and NP cell encapsulation[24–27], due to its extremely good biocompatibility as evidenced by a lack of inflammation, foreign body response, or fibrosis, and thereby widespread clinical applicabilition[28–30].

Although hyperosmolality is an important feature of the native NP tissue, most of the above-mentioned hydrogels have no intrinsic swelling pressure, and if they do (e.g., the swelling properties of alginate are known to depend on the type of alginate and cross-linking method[31,32], and those of agarose on concentration[33]), they are usually used in nonconfined culture conditions. Controversy remains on the requirement of hyperosmolality for regeneration. Although at the mRNA level, hyperosmolality increased aggrecan and type II collagen expression in human NP cells, no effects were shown at the protein level[19]. However, as these data were based on NP cells expanded in monolayer in normosmotic conditions and redifferentiated in high-density culture without hydrogels, possibly the NP cells lost their responsiveness to hyperosmolality or required a natural local milieu. Articular chondrocytes expanded in high osmolality, showed a higher ECM producing capacity[34], and produced stronger constructs, although in this study, hyperosmolality during redifferentiation even had negative effects[35]. To our knowledge, the role of hyperosmolarity during expansion has not yet been studied for NP cell-mediated regeneration.

To provide the NP research field with a possible comparator for future research on NP regeneration in hydrogels, this study compares the above-mentioned six commonly used natural hydrogels for ECM production by NP cells. These gels were used in the presence of serum or in a more defined, serum-free (SF) differentiation medium. Furthermore, the role of osmolality during expansion and redifferentiation culture in ECM production was investigated in the two best performing hydrogels.

Materials and Methods

Culture

Cell isolation and expansion

Human IVDs were collected during autopsy (Local Medical Ethical Committee [METC] number 12–364). NP was conservatively separated from AF tissue by visual inspection. NP cells from 10 different donors (aged 47–69, mean age 63; Thompson grade III) were isolated in Dulbecco's Modified Eagle's Medium (DMEM) (31885; Gibco, Life Technologies, Carlsbad, CA) with 14 U/mL pronase (Roche, Basel, Switzerland) + 110 U/ mL DNAse (Sigma, St. Louis, MO) for 1 h followed by incubation with 13.7 U/mL collagenase

(Roche) + 110 U/mL DNAse overnight. Cells were passed through a 70 mm cell strainer, washed twice with medium, and collected by centrifugation. Cells were expanded in 2D culture using DMEM + 10% heat-inactivated fetal bovine serum (FBS; HyClone, Life Technologies) + 2% antibiotics mixture (final concentration 200 U/mL penicillin, 200 U/mL streptomycin, 50 mg/ L amphotericin-B, and 50 mg/L gentamicin [Lonza, Basel, Switzerland]) and 10 ng/mL recombinant human basic fi- broblast growth factor (R&D Systems, Minneapolis, MA). After the first week, antimicrobial agents were reduced to 100 U/mL penicillin + 100 mg/mL streptomycin (pen/strep; Gibco). Expansion typically took 3–6 weeks and to minimize dedifferentiation, all cells were used at passage 2. Cell numbers and viability were determined manually with a Bürker-Türk hemocytometer and Trypan blue (Sigma). Cell viability was always at least 98%.

Hydrogel preparation

Hydrogels of six different polymers were used in NP cell culture in concentrations of polymer as suggested by the manufacturer or previously used in literature[6] (Table 1). Alginate (alginic acid sodium salt, medium viscosity; Sigma) 3% (w/v) solutions were mixed with the NP cell suspension (passage 2) to a final concentration of 2% (w/v) alginate and were added dropwise into a sterile 102 mM $CaCl_2$ solution in which the beads were allowed to cross-link for 10 min. Alginate hydrogels required weekly recross-linking (i.e., incubation with $CaCl_2$) for stability since otherwise, they would become softer due to ion leaching. Agarose (low electroendosmosis; Sigma) hydrogels were prepared by autoclaving a 3% (w/v) solution and maintaining it at 45°C until use. The temperature was lowered to 39°C before mixing in the NP cells (final concentration agarose 2% [w/v]), after which gelation was allowed to occur at room temperature (RT). GelMA was prepared as described before by reacting gelatin from porcine skin type A (Sigma) with methacrylic anhydride for 1 h at 60°C, followed by dialysis and freezedrying.[36] Irgacure 2959 (0.05%; BASF, Ludwigshafen, Germany) was added to a 15% (w/v) gelMA solution and mixed 2:1 with the NP cell suspension to a final hydrogel concentration of 10%. GelMA hydrogels were prepared by injection into a Teflon mold[37] and UV cross-linking for 15 min at 365 nm at an intensity of 2.6 mW/cm^2. Fibrin (commercially available as Tissucol; Baxter, Deerfield, IL) hydrogel was prepared according to the manufacturer's instructions. In short, fibrin was mixed with cells and an equal part of thrombin solution was added. Cross-linking occurred at 37°C for 15 min. HA/poly(ethylene glycol) (PEG) (HA/PEG commercially available as HyStem; Esibio, Alameda, CA) solution was mixed with cross-link solution and left to cross-link at 37°C for 30 min. Type II collagen hydrogels (commercially available as the 3D collagen culture kit; EMD Millipore, Billerica, MA) were prepared by neutralizing the pH of the acidic 6 mM type II collagen solution in acetate buffer. Cells were mixed into this solution and left to cross-link at 37°C for 1 h. All hydrogels contained $2 \cdot 10^6$ cells/mL and were 50 µL each. To provide an indication of biomechanical properties of these gels, in an unrelated set of experiments with the same hydrogels, the elastic modulus was measured. For alginate, agarose, GelMA, fibrin, HA/

PEG, and collagen II these were shown to be 4±1, 29±12, 11±1.5, 4, 4±2, 4±1, and 2±1 kPa, respectively[41].

Table 1 – Polymer and Hydrogel Properties of the Different Materials.

Material	Concentration (% w/v)	Cross-linking method
Alginate	2%	Ca^{2+} ions
Agarose	2%	Thermal gelation
GelMA	10%	UV-cross-links
Fibrin	≈7-11%	Covalent cross-links by factor XIII
HA/PEG	1%	Thiol-based cross-linking of HA and PEG
Type II collagen	0.1%	pH-dependent gelation

Hydrogel culture with NP cells

NP cells from seven donors were cultured in hydrogels in a medium with FBS (DMEM + 10% FBS + 0.2 nM ascorbic acid-2-phosphate [ASAP; Sigma] + 1% pen/strep) or in an SF differentiation medium (DMEM + 2% insulin–transferrin–selenium-X [Gibco] + 0.2 nM ASAP + 2% human serum albumin [Sanquin, Amsterdam, The Netherlands] + 1% pen/strep) for 28 days. To assess the effect of osmolality during expansion, NP cells of three donors were expanded in a medium of 340 mOsm/kg or in a medium with osmolality adjusted to 400 mOsm/kg by the addition of NaCl. After expansion for two passages, cells were incorporated in agarose, gelMA, or type II collagen hydrogels and cultured in the SF differentiation medium adjusted to 400, 450, or 500 mOsm/kg with NaCl (2, 3.7, or 5.5 mg/mL) and confirmed with a freezing point micro-osmometer (Advanced Instruments, Inc., Norwood, MA). In all experiments, empty hydrogels without cells were cultured and taken along as controls in all analyses.

Histology and immunohistochemistry

Live/dead staining (Life Technologies) of two hydrogels of each type was performed after 7 days of culture. Hydrogels were washed in phosphate-buffered saline (PBS) and incubated in PBS containing 2 mM calcein AM and 4 mM ethidium homodimer-1 for 20 min at 37°C. The staining solution was replaced by PBS and the hydrogels were examined with a fluorescent BX51 microscope, DP 70 camera, and cell^F software (Olympus, Tokyo, Japan). After 28 days of culture, hydrogels were fixed overnight in 4% buffered formalin, dehydrated, and embedded in paraffin. Five micrometer sections were mounted on Superfrost Ultra Plus microscope slides (Thermo Scientific). Before staining, sections were rehydrated through a graded alcohol series to demi water. For Safranin-O/Fast Green staining, sections were incubated with Mayer's hematoxylin

(Merck, Kenilworth, NJ) for 5 min, washed in tap water for 10 min, and stained with 0.4% (w/v) Fast Green (Merck) for 4 min. After washing in 1% (v/v) acetic acid, 5 min staining by 0.125% (w/v) Safranin-O (Merck) was followed by dehydration and mounting. For aggrecan immunohistochemistry, antigen retrieval was performed by incubating in citrate buffer (10 mM sodium citrate, pH 6.0) at 96°C for 20 min and cooling to RT for an additional 20 min. For type II collagen, antigen retrieval consisted of incubation with pronase (1 mg/mL in PBS; Roche) followed by hyaluronidase (10 mg/mL in PBS; Sigma) (both at 37°C for 30 min). Immunostaining protocols were the same after the antigen retrieval. Sections were blocked with PBS + 5% bovine serum albumin (PBS–BSA) for 30 min at RT and incubated overnight at 4°C with the primary antibody diluted in PBS–BSA (aggrecan [1:150; 6-B-4 [Novus Biologicals, Littleton, CO]) or type II collagen (0.4 mg/mL, DSHB II-II6B3 [DSHB, Iowa City, IA]). The EnVision kit (a-mouse; Dako, Glostrup, Denmark) was used for visualization according to the manufacturer's protocol. Microscopy pictures were taken with a BX43 upright microscope and CellSens software (Olympus). Exposure was adjusted in Photoshop (Adobe, San Jose, CA) for descriptive analysis of the images.

Biochemistry

After 28 days of culture, 3–5 hydrogels per donor and hydrogel type were incubated overnight at 60°C in a papain digestion buffer with 250 mg/mL papain (Sigma) and 1.57 mg/mL l-cysteine (Sigma). GAG content was determined with a 1,9-dimethyl-methylene blue (DMMB; Sigma) assay with pH adjusted to 1.0 because of interference of alginate and gelMA hydrogels at pH 3.0, which also eliminates signal generated by the unsulfated GAGs of HA. Values were determined using a chondroitin sulfate standard curve. DNA content was determined with a Picogreen assay and compared to a λDNA standard (Life Technologies). In both assays, measurements were performed in duplicate.

Statistics

All statistical analyses were carried out with SPSS 21 (IBM, North Castle, NY). Outliers determined with the 5*s rule were removed (a value is considered an outlier when it is outside the mean plus or minus five times the standard deviation range). For all data, Kruskal–Wallis tests were performed, with post hoc multiple Mann-Whitney tests with a Benjamini-Hochberg correction. All graphs display data as mean – standard deviation.

Results

Comparison of different hydrogels for NP cell culture

Hydrogel handling

Cross-linking of type II collagen hydrogels resulted in shrinking of the hydrogels with fluid expelled on top of the hydrogels. The type II collagen hydrogels were also very soft and difficult to handle, as were the HA/PEG hydrogels. During culture, degradation was seen of type II collagen and HA/PEG hydrogels, and most notably for fibrin. The fibrin hydrogels had completely disappeared after 2–3 weeks of culture, ending up with a cell pellet in the presence of FBS. Alginate, agarose, and gelMA hydrogels were very stable. Collagen gels already showed degradation within the first week of culture. After 28 days, this resulted in clearly smaller constructs in the differentiation medium and even in hydrogel-free pellets upon culture in the presence of FBS (Supplementary figure S1).

Histology and immunohistochemistry

All cell-free hydrogels were negative for background immunohistochemical staining (data not shown). Cell-free alginate and HA/PEG hydrogels stained positive for Safranin-O, as both have a net negative charge. Fast Green stains collagenous fibers and thus also stained cell-free gelMA and type II collagen hydrogels green. Figure 1 displays live/dead staining performed after 7 days of culture, and histology and immunohistochemistry after 28 days. In all hydrogels, primarily live cells were present. Microscopic tissue-like clusters were formed by several cells in lacuna-like cavities inside alginate and HA/PEG hydrogels. These clusters were positive for Safranin-O, suggesting the presence of proteoglycans, although the background staining found cannot fully confirm this. However, immunohistochemistry indicated the presence of aggrecan and type II collagen. Also, around individual cells in alginate, positive aggrecan immunostaining was visible. Diffuse pericellular Safranin-O staining was present in the agarose hydrogels, indicating GAG and hence proteoglycan production, and this matrix was also immunopositive for aggrecan and type II collagen. Very little matrix seemed to be deposited in gelMA hydrogels, as no staining was seen for any type of (immuno)staining, except for Fast Green. Since the fibrin had completely disappeared, histology resembled cells grown in pellets. Safranin-O staining indicated that proteoglycans were present, but very limited aggrecan staining was visible. Type II collagen was present throughout the matrix. In type II collagen hydrogels, Safranin-O staining was absent and aggrecan and type II collagen staining was sparse.

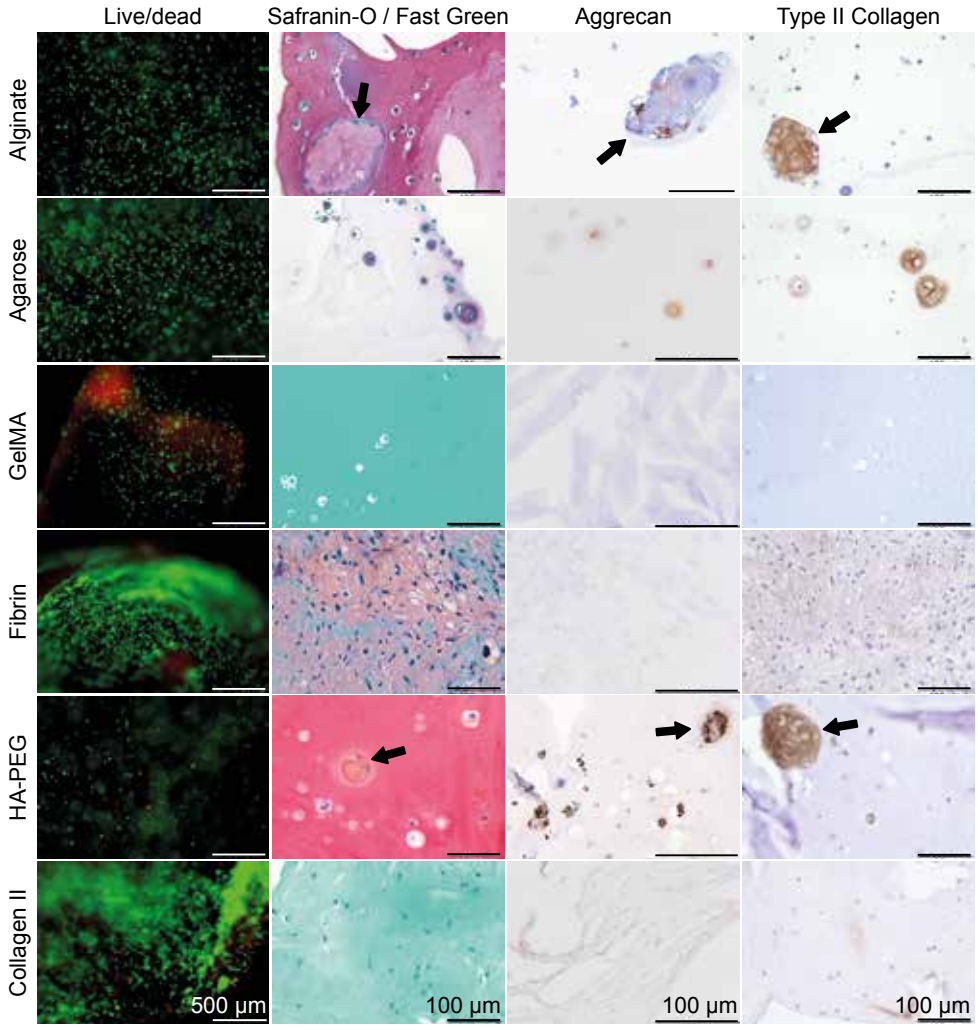


Figure 1 – Live/dead staining after 7 days of culture shows that viable cells outnumber dead cells in all hydrogel types. Histology for Safranin-O (red staining) and Fast Green (green staining), and immunohistochemistry for aggrecan and type II collagen (brown staining) after 28 days show that matrix was deposited in majority of the hydrogels. Matrix accumulated directly around the cells in most hydrogels, but in fibrin hydrogels, matrix was formed more dispersedly since hardly any hydrogel was left at the end of culture. In alginate and HA/PEG hydrogels, microscopic tissue-like structures were formed (see arrows), positive for Safranin-O, aggrecan, and type II collagen. Scale bar represents 500 µm in live/dead images and 100 µm in histology images. HA, hyaluronic acid; PEG, poly(ethylene glycol).

GAG and DNA content

After correction for the signal of cell-free hydrogels, cells in agarose and type II collagen hydrogels were shown to produce the highest amount of GAGs ($p \leq 0.014$, Figure 2), whereas fibrin and HA/PEG hydrogels contained more GAGs than alginate hydrogels ($p \leq 0.016$). In fibrin and type II collagen hydrogels, DNA content was higher than in alginate, agarose, and gelMA hydrogels ($p < 0.007$), and DNA content in HA/PEG was higher than in gelMA ($p = 0.01$). When GAG content was normalized to DNA, this was found to be highest for NP cells cultured in agarose hydrogels ($p < 0.001$ compared to alginate, fibrin, and HA/PEG, $p = 0.01$), although there was no significant difference with gelMA. More GAG/DNA was produced in type II collagen than in alginate or fibrin hydrogels ($p > 0.001$ and $p = 0.008$, respectively), and GAG/ DNA in HA/PEG was higher than in alginate ($p = 0.02$).

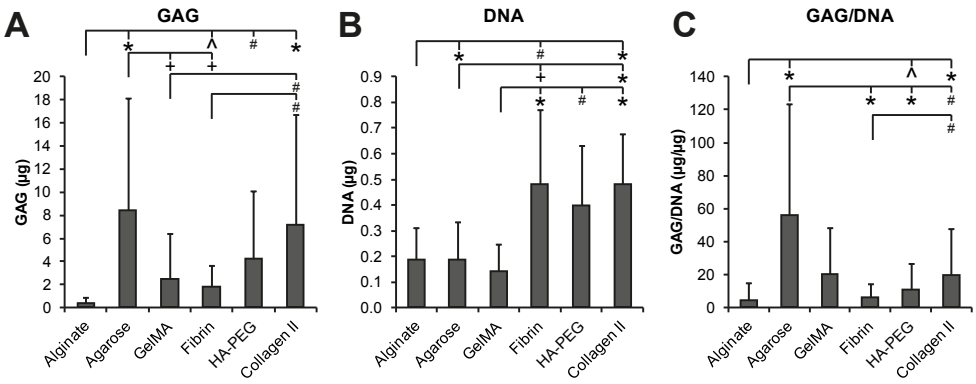


Figure 2 – NP cells of four different donors were cultured in six different types of hydrogels for 28 days (n = 3 per condition). All measurements were corrected with the background values of empty hydrogels. **A**: The highest GAG content was found in agarose hydrogels compared to alginate, gelMA, and fibrin, and in type II collagen hydrogels compared to alginate, gelMA, and fibrin. Fibrin and HA/PEG hydrogels contained more GAGs than alginate hydrogels. **B**: The highest amount of DNA was found in fibrin compared to alginate, agarose, and gelMA hydrogels, in type II collagen hydrogels compared to alginate, agarose, and gelMA, and in HA/PEG compared to gelMA. **C**: GAG expressed per DNA (mg/mg) was highest in agarose hydrogels compared to alginate, fibrin, HA/PEG, and type II collagen hydrogels. Higher GAG/DNA was measured in type II collagen compared to alginate and fibrin, and in HA/PEG compared to alginate. Significant differences are given as follows: $*p \leq 0.001$, $+ p \leq 0.005$, $\# p \leq 0.01$, $\wedge p \leq 0.025$. NP, nucleus pulposus; GAG, glycosaminoglycan; gelMA, gelatin methacryloyl.

Effect of serum in culture

Although serum is a common addition to cell culture, clinical application precludes the

use of animal-derived components such as serum. Therefore, the response of cells to the best-performing polysaccharide hydrogel (agarose) and the collagen-based hydrogels (gelMA and type II collagen) was also tested in the absence of serum.

GAG and DNA content

NP cells deposited higher amounts of GAGs in the presence of FBS than cells in the SF differentiation medium in agarose and gelMA hydrogels ($p \leq 0.001$, Figure 3). DNA content was higher for cells cultured in agarose and type II collagen hydrogels with the SF differentiation medium. Higher GAG/DNA was measured with FBS in all three hydrogels ($p \leq 0.001$). Total GAG production, that is, GAG content in the cultured hydrogels and GAGs released into the culture medium, was increased in the presence of FBS compared to the SF differentiation medium ($p < 0.001$ for all three hydrogels tested). Incorporation efficiency, determined as the percentage of total GAG deposited in the hydrogels, was higher in cells cultured in type II collagen hydrogels in the SF differentiation medium compared to the medium containing FBS ($p < 0.001$). This difference was not evident in agarose or gelMA hydrogels.

The effect of medium osmolality during NP cell expansion and differentiation in hydrogels

As agarose and type II collagen stimulated GAG deposition by NP cells most, both in FBS and serum free medium, these hydrogels were further employed to determine the effect of osmolality during expansion and redifferentiation of NP cells. Histology. NP cells isolated and expanded in 400 mOsm/kg showed more Safranin-O and type II collagen staining upon redifferentiation in both type II collagen and agarose hydrogels than cells isolated and expanded in 340 mOsm/kg (Figure 4). This Safranin-O and type II collagen staining pattern remained the same even after tuning osmolality during redifferentiation up to 500 mOsm/kg.

GAG and DNA content

Redifferentiation of NP cells that were isolated and expanded in high osmolality medium in agarose hydrogels resulted in higher GAG, DNA, and GAG/DNA compared to hydrogels with cells expanded in normal osmolality ($p < 0.001$, Figure 5A). Similar effects of osmolality in GAG and GAG/DNA production were found for cells cultured in type II collagen hydrogels ($p < 0.001$, Figure 5B). In both hydrogels, GAG production appeared to be independent of osmolality during redifferentiation. Results are also summarized in the Supplementary Table S1.

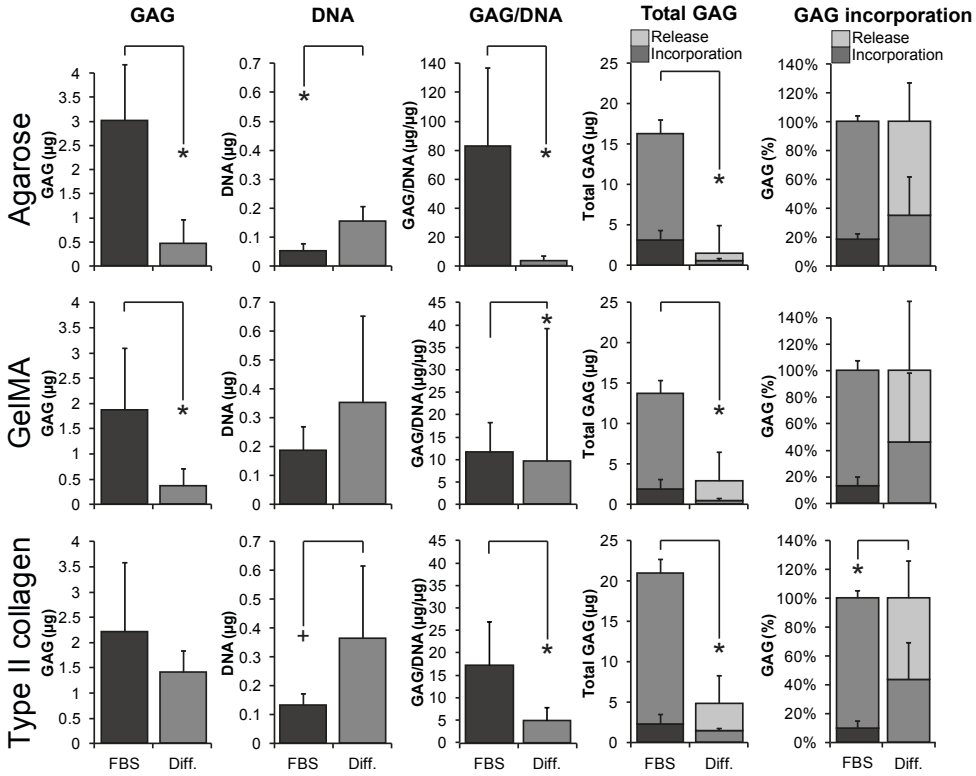
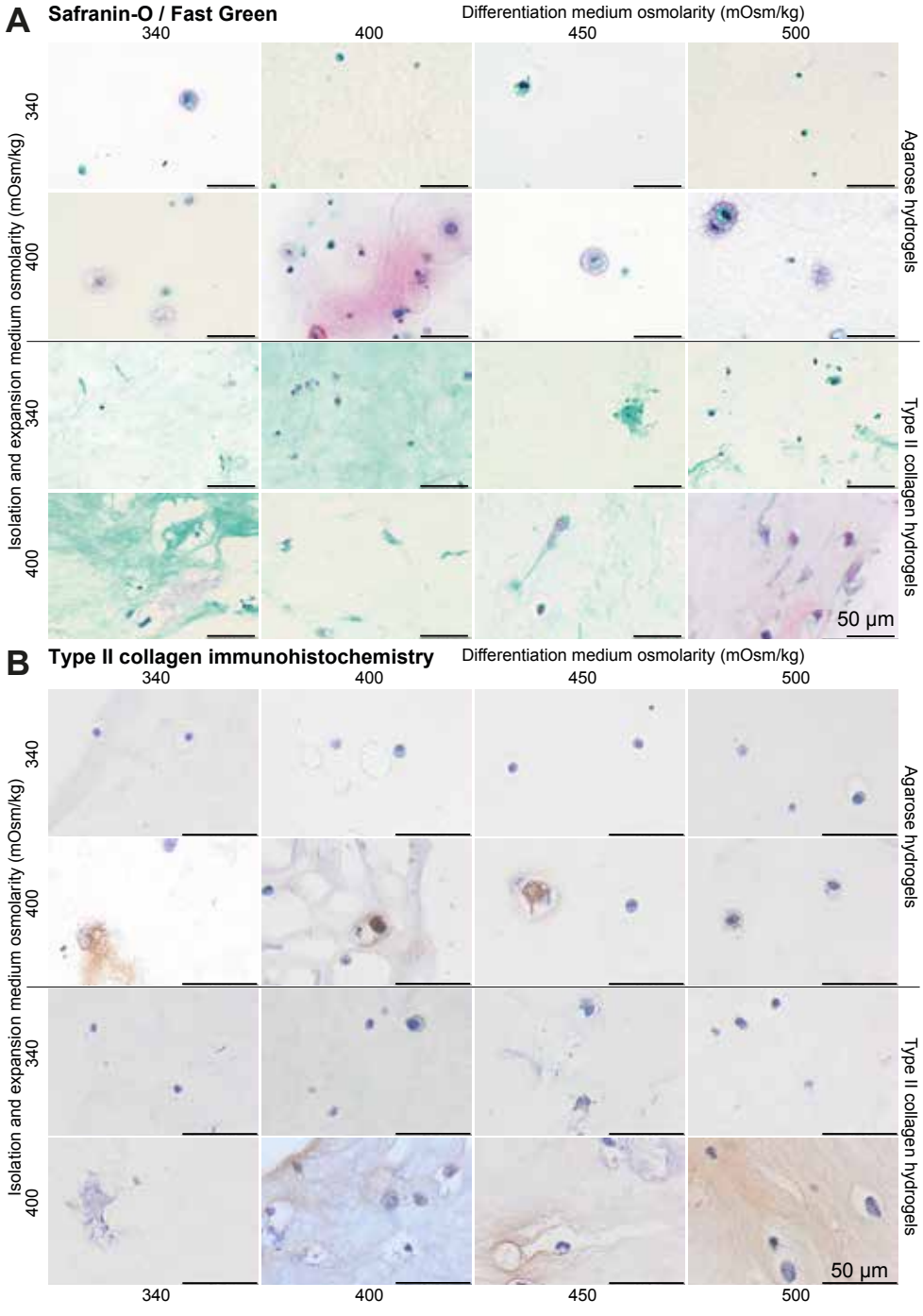


Figure 3 – NP cells of three donors were cultured in hydrogels (n = 5 per condition per donor) in medium containing FBS or SF differentiation medium (Diff.). In agarose and gelMA hydrogels, significantly higher GAG, GAG/DNA, and total GAG production was measured compared to SF differentiation medium. DNA content was higher in the absence of FBS for agarose, but no differences in DNA content were detected in gelMA hydrogels. In type II collagen hydrogels, DNA and incorporation efficiency were higher in SF differentiation medium compared to medium with FBS, but GAG/DNA and total GAG production were higher in the presence of FBS. Significant differences are given as follows: *p ≤ 0.001 and + p ≤ 0.005. FBS, fetal bovine serum; SF, serum free.

Figure 4 – NP cells isolated and expanded in either 340 or 400 mOsm/kg medium were cultured in 340–500 mOsm/kg SF differentiation medium. **A**: Histology of cells isolated and expanded in 400 mOsm/kg showed more Safranin-O-positive matrix in hydrogels. The osmolality of the differentiation medium did not alter this staining pattern. **B**: Type II collagen immunohistochemistry staining was present in hydrogels with cells isolated and expanded in 400 mOsm/kg medium, but not with cells isolated and expanded in 340 mOsm/kg medium. Scale bar represents 50 mm.


A
Safranin-O / Fast Green
Differentiation medium osmolarity (mOsm/kg)
340
400
450
500
Isolation and expansion medium osmolarity (mOsm/kg)
340
400
Agarose hydrogels
Type II collagen hydrogels
50 µm
B
Type II collagen immunohistochemistry
Differentiation medium osmolarity (mOsm/kg)
340
400
450
500
Isolation and expansion medium osmolarity (mOsm/kg)
340
400
Agarose hydrogels
Type II collagen hydrogels
50 µm

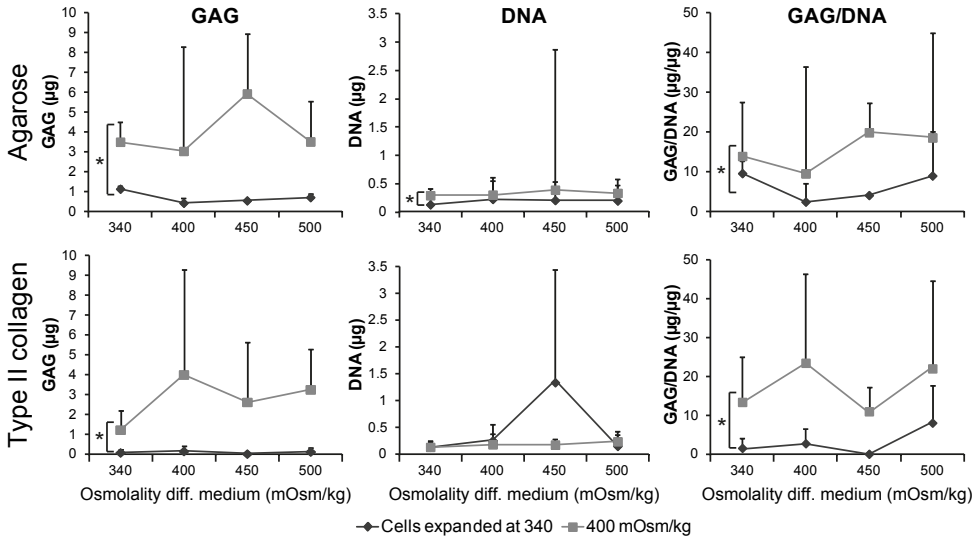


Figure 5 – NP cells from three donors isolated and expanded in either 340–400 mOsm/kg medium were cultured in hydrogels (n = 5 per condition) in SF differentiation medium with an osmolality range of 340–500 mOsm/kg. GAG, DNA, and GAG/ DNA content of the agarose hydrogels indicate that increased osmolality during isolation and expansion augmented the regenerative capacity of the NP cells. In type II collagen hydrogels, GAG and GAG/DNA were higher for cells isolated and expanded in 400 mOsm/kg medium compared to 340 mOsm/kg cells. Increasing osmolality during regeneration did not have any effect in either hydrogel. Significant differences are given as follows: *$p \leq 0.001$.

Discussion

In this study, six commonly used hydrogels, the presence of serum in culture medium, and osmolality of isolation, expansion, and culture medium were compared for redifferentiation and ECM production by NP cells. There was a clear difference in ECM producing capacity of human degenerated NP cells in hydrogels of different natural polymers. More matrix was deposited in agarose and type II collagen hydrogels, and when normalized for DNA content, the capacity of the NP cells to produce ECM was highest in agarose. The presence of FBS clearly affected cell behavior, resulting in augmented deposition of proteoglycans in all three hydrogels. However, in type II collagen hydrogels, incorporation efficiency of GAG was decreased compared to the SF differentiation medium. Regardless of the hydrogel employed, isolation and expansion in high osmolality medium strengthened the differentiation of NP cells, as indicated by the higher GAG and GAG/DNA in SF culture conditions compared to normal osmolality. The anabolic effect of increasing osmolarity during redifferentiation, however, was inconsistent.

The comparison of six commonly used hydrogels in this study points toward two hydrogels that are relatively effective for human NP cell-based ECM deposition. These are agarose and type II collagen hydrogels, as indicated by the production and deposition of proteoglycans, as markers of late-stage NP cell differentiation together with collagen II, and matrix proteins crucial for tissue functionality. Although a large number of studies have been done with biomaterials for NP repair, to the best of our knowledge, only two directly compared the response of NP cells to different hydrogel types. In the first, an immortalized human NP cell line produced more proteoglycans, even when normalized to cell number, in alginate than in atelocollagen hydrogels in the presence of FBS[38]. Porcine NP cells and mesenchymal stem cells (MSCs) separately cultured in SF medium were also shown to deposit more ECM in 1.5% alginate than cells in chitosan hydrogels[39]. However, none of these studies referred to above were based on primary human NP cells isolated from degenerated IVDs, and/or the hydrogels used to compare against were not included in this study. Aside from the culture environment and the type of polymer used, the cross-link density of the hydrogels, the polymer concentrations, and the resulting mesh size and stiffness may affect cell behavior[6]. Research on the effect of these properties of NP cell behavior is limited. However, alginate concentrations varying between 1% and 6% were shown not to affect gene expression levels of caprine NP cells[40], nor was matrix production by human NP cells affected in 1% versus 2% alginate or 1% versus 2% agarose[41]. Also, bovine NP cells produced similar amounts of GAGs in 3% versus 4% agarose[42]. Addition of HA to a type II collagen hydrogel decreased NP cell proliferation, but there were no differences between different HA concentrations, suggesting other causes than mere polymer concentration[43]. Cross-link density in a similar hydrogel influenced NP cell viability in the long term, but gene expression only in the short term[44]. Articular chondrocytes, however, did respond to increasing agarose concentration from 2% to 3% (w/v) with 30% increased GAG production[45], and GAG production by equine chondrocytes in gelMA hydrogels decreased by around 40% with increasing polymer concentration from 10% to 20% (w/v), again stressing the difference between NP cells and articular chondrocytes[47]. It remains to be determined how the concentration or cross-link density of type II collagen may further affect human NP cell behavior. A disadvantage of the collagen II gel is its water expelling behavior during cross-linking. This makes it difficult to estimate final polymer density and may result in reduced porosity, altering cell behavior. We have also not further verified whether exposure to different osmolarity affected hydration level, nor assessed hydration after equilibration, after cross-linking of these gels. However, as a collagenous matrix does not have a very high fixed charge density, osmotic pressure of the gel most likely was not changed. Moreover, no effect on viability was noted, nor was regeneration in collagen II gel affected by media of varying osmolarity, suggesting that these differences in hydration, in any case, did not have profound biological effects. The lack of Saf O staining, known to be insensitive to low proteoglycan levels[46], in contrast to the higher proteoglycan content as indicated by DMMB for sulfated GAGs, may suggest that in this gel, the deposited proteoglycans were distributed more evenly throughout the gel

compared to the agarose gels.

Another major limitation in standardizing NP regeneration research is the variation in composition and biologic effects between batches of FBS, a well-known issue, for example, in chondrogenic differentiation of adipose-derived MSCs[48] that will likely affect NP cells as well. FBS contains many (un)defined growth factors and nutrients that influence matrix production and deposition[49]. In our study, NP cells in hydrogels produced more GAGs in the presence of FBS compared to a more defined and clinically relevant SF differentiation medium, which is in line with a previous report[50]. Moreover, cell-based clinical applications, where cells are being expanded before transplantation, would require the use of chemically defined media because of the risks associated with the use of xenogeneic serum and the possible contaminations it might contain[48]. Serum deprivation may lead to more autophagy and lower ATP levels in murine NP cells in normoxic conditions[51]. It should be noted, however, that the murine NP cells were cultured in SF medium containing only DMEM, while in this study, a medium containing pyruvate, albumin, insulin, transferrin, selenium, and ascorbic acid was used to support NP cell redifferentiation.

Osmolality during isolation and expansion of NP cells was clearly shown to define the ECM producing capacity of redifferentiating human NP cells, independent of the hydrogel employed and osmolality during redifferentiation. The observation that NP cells produce more matrix in differentiation culture after expansion in high osmolality, even when brought back to normal osmolality, suggests that the phenotype of cells is irreversibly altered during isolation and expansion at low osmolality. These findings are especially important when employing NP cells for cell-based therapies, for example, herniated NP cells for transplantation into a degenerated NP[52]. Although this is not a common strategy, we suggest that culture osmolality should be considered for any cell type used for NP regeneration. Interestingly, increasing osmolality during redifferentiation culture of NP cells isolated and expanded in standard osmolality medium, did not affect matrix production. This is in line with previous studies where increasing medium osmolality did not result in increased matrix production in high-density cultures of human NP cells[17] and bovine NP cells in alginate[53]. Similar effects of osmolality during different stages of culture have been observed for chondrocytes. For healthy and osteoarthritic human chondrocytes, isolation and expansion at an osmolality of 380 mOsm/kg improved the cell phenotype compared to using hypo-osmotic medium of 280 mOsm/kg[34]. Bovine chondrocytes expanded at 400 mOsm/kg, cultured in agarose hydrogels, produced constructs with increased mechanical strength compared to cells expanded at 300 mOsm/kg, independent of differentiation osmolality[35], although GAG content was not different. In contrast, in articular chondrocyte 3D culture, increasing osmolarity during redifferentiation culture actually inhibited proteoglycan production[35,54]. This again underlines the notion that, although NP cells are "chondrocyte like," their distinct expression of different membrane markers, different composition of the native tissue[55], and different biological responses mean that they should be studied as a unique cell

type.

Conclusions

This study shows that the response of degenerated human NP cells to culture in a variety of natural hydrogels is different for each polymer. Overall, agarose and type II collagen hydrogels facilitate NP cells to produce high amounts of GAGs. Nondegrading agarose hydrogels may be suitable for studying NP redifferentiation, and degrading type II collagen hydrogels may be chosen for in vivo application, despite the partial dehydration found. Although redifferentiation of NP cells can be further improved, one of these hydrogels may be used in subsequent studies to compare against other candidate hydrogels, until a better reference is identified. Furthermore, while human NP cells do not thrive in the absence of FBS, their isolation and expansion in high osmotic medium can restore their level of matrix production. Moreover, the effect of expansion in high osmolarity merits further investigation. Possibly epigenetic changes, such as methylation and/or differential expression of non-coding RNA, such as miRNA, play a major role here. From a translational point of view, the responsiveness to growth factors after expansion in high osmolarity should be verified to see whether further enhancement of ECM production can be achieved.

Acknowledgments

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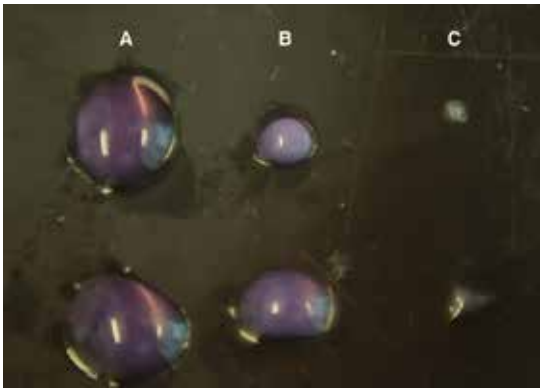
Supplementary data

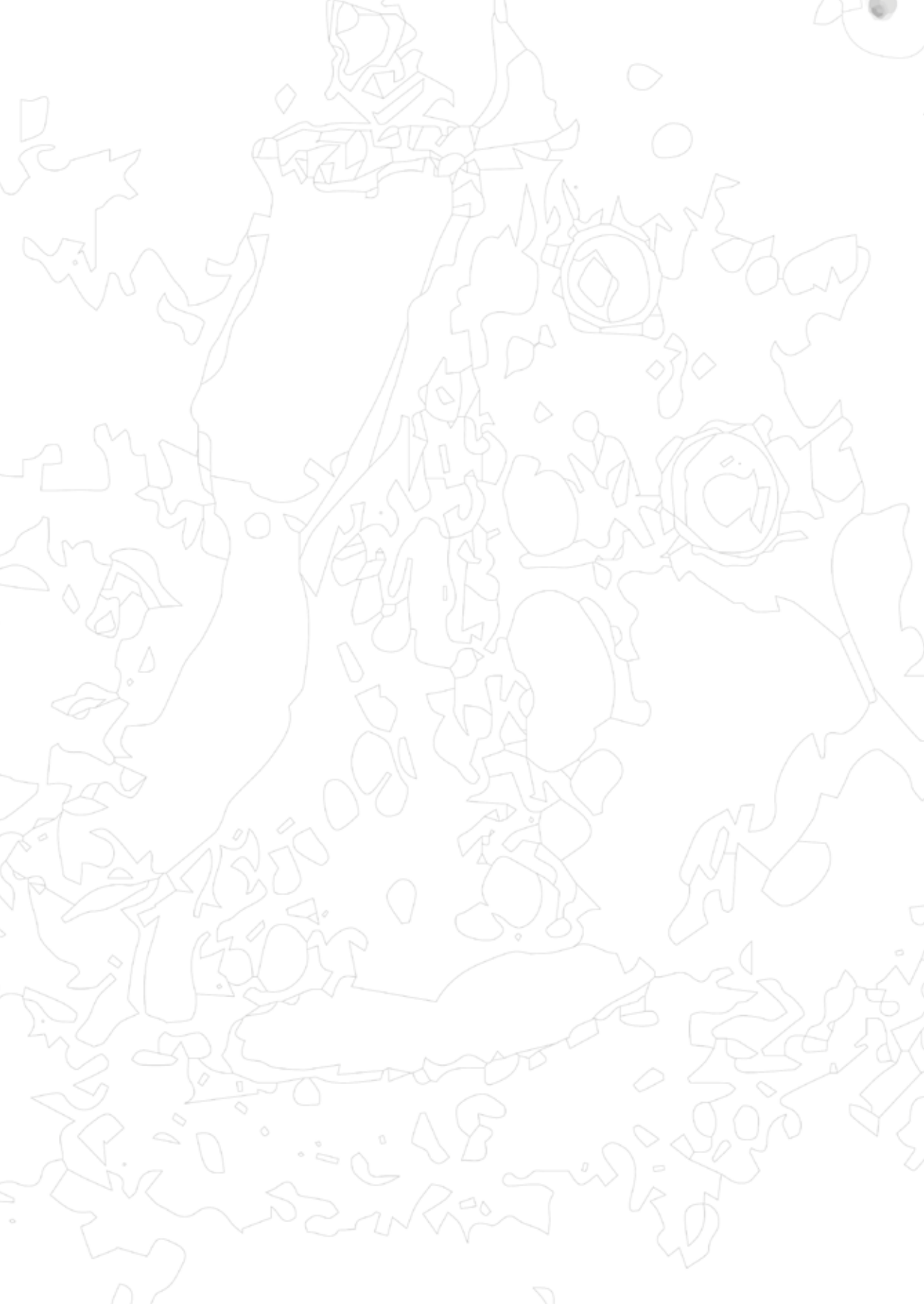


Supplementary figure S1 – Appearance of collagen gels after 28 days of culture; **A**: cell-free hydrogels in FBS-containing medium; **B**: hydrogels with cells in differentiation medium; **C**: hydrogels with cells in FBS-containing medium. Two gels per conditions are shown. FBS, fetal bovine serum.

Supplementary Table S1 – Summary of the Results Obtained with the Different Hydrogels, With and Without FBS.

Hydrogel	Stability & handling	GAG content	-FBS	DNA content	-FBS	GAG/DNA	-FBS	GAG incorp -FBS
Alginate	+ & +	+/-		+		+/-		ND
Agarose	+ & +	++	↓	+	↑	++	↓↓	=
GelMA	+ & +	+	↓	+	=	+	↓	=
Fibrin	- - & +	+		++		+/-		ND
HA/PEG	- & -	+		++		+		ND
Type II collagen	- & -	++	=	++	↑	+	↓	↑

HA, hyaluronic acid; PEG, poly(ethylene glycol); gelMA, gelatin methacryloyl; FBS, fetal bovine serum; GAG, glycosaminoglycan; ND, not determined; =, not different compared to presence of FBS; - -, very low; -, low; +, intermediate; ++, high.



Focal adhesion signaling affects regeneration by human nucleus pulposus cells in collagen- but not carbohydrate-based hydrogels

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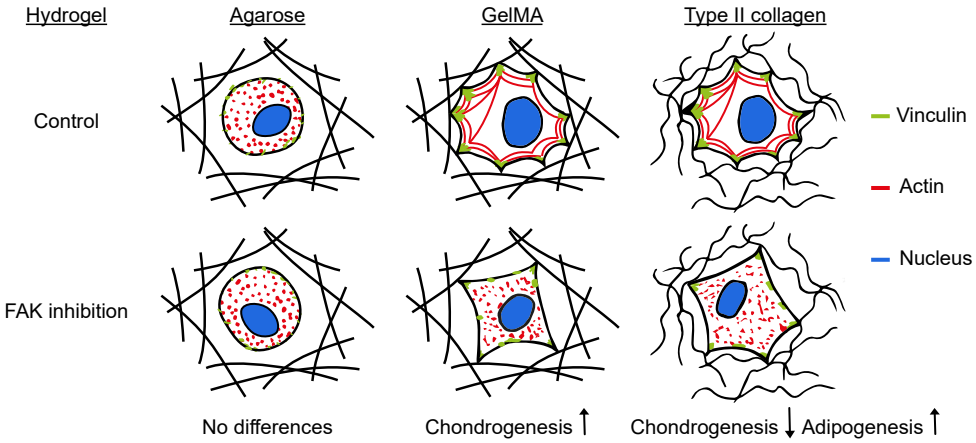
ACTA Biomaterialia, 2018

Chapter 4

Graphical abstract



Abstract

Hydrogel-based 3D cell cultures are an emerging strategy for the regeneration of cartilage. In an attempt to regenerate dysfunctional intervertebral discs, nucleus pulposus (NP) cells can be cultured in hydrogels of various kinds and physical properties. Stiffness sensing through focal adhesions is believed to direct chondrogenesis, but the mechanisms by which this works are largely unknown. In this study we compared focal adhesion formation and glycosaminoglycan (GAG) deposition by NP cells in a range of hydrogels. Using a focal adhesion kinase (FAK) inhibitor, we demonstrated that focal adhesion signaling is involved in the response of NP cells in hydrogels that contain integrin binding sites (i.e. methacrylated gelatin (gelMA) and type II collagen), but not in hydrogels deplete from integrin binding sites such as alginate and agarose, or CD44-binding hydrogels based on hyaluronic acid. As a result of FAK inhibition we observed enhanced proteoglycan production in gelMA, but decreased production in type II collagen hydrogels, which could be explained by alteration in cell fate as supported by the increase in the adipogenic marker peroxisome proliferator-activated receptor gamma (PPAR-γ). Furthermore, GAG deposition was inversely proportional to polymer concentration in integrin-binding gelMA, while no direct relationship was found for the non-integrin binding gels alginate and agarose. This corroborates our finding that focal adhesion formation plays an important role in NP cell response to its surrounding matrix.

Statement of Significance

Biomaterials are increasingly being investigated for regenerative medicine applications, including regeneration of the nucleus pulposus. Cells interact with their environment and are influenced by extracellular matrix or polymer properties. Insight in these interactions can improve regeneration and helps to understand degeneration processes. The role of focal adhesion formation in the regenerative response of nucleus pulposus cells is largely unknown. Therefore, the relation between materials, stiffness and focal adhesion formation is studied here.

Keywords

Nucleus pulposus, Hydrogel, Stiffness, Focal adhesion kinase, Regeneration

Introduction

Intervertebral disc (IVD) degeneration is one of the most common causes of chronic low back pain[1], which is a major problem worldwide[2]. Treatment strategies currently under development aim at regeneration of the nucleus pulposus (NP), the soft, central core of the IVD, where the first signs of degeneration become apparent[3]. A common regenerative strategy is the use of biomaterials[4,5], and specifically hydrogels, as cell carriers. Cells can be the endogenously present NP cells, but also NP cells isolated from the affected disc and retransplanted together with the gel. Widely used carriers include hydrogels of alginate, agarose, and collagen, with variable effects on NP-mediated regeneration, but their application is mainly trial- and error-based. Insight into hydrogel characteristics determining the NP cell response would greatly enhance future development and application of hydrogels for NP regeneration. Adequate mechanical properties of the hydrogel, an appropriate degradation rate to accommodate new tissue formation, and accessibility to nutrients and oxygen all provide for an environment which induces cell differentiation and matrix deposition. These properties and thus cell behavior are likely to be affected by hydrogel parameters such as the polymer's physicochemical properties (e.g. fixed charge density), polymer concentration and cross-link density (influencing stiffness, porosity, and diffusion of nutrients and oxygen)[4].

One important parameter is material stiffness[4]. Cells bind to their native extracellular matrix (ECM) and sense its stiffness by mechanotransduction through focal adhesions, the integrincontaining multi-protein structures linking the extracellular matrix to the actin cytoskeleton[6]. Focal adhesions provide information on the state of the pericellular matrix and allow the cell to respond to changes by adjusting the stiffness of the actin skeleton[7]. Immature focal adhesions, consisting of integrins binding to the ECM, vinculin, paxillin, and talin, recruit phosphorylated focal adhesion kinase (FAK)[8]. Via enhanced FAK phosphorylation through RhoA signaling, this leads to polymerization of globular actin (G-actin) into polymerized filamentous actin (F-actin)[9], and strengthening of the actin cytoskeleton[8]. It thereby influences cell morphology[10], which in turn regulates cell fate through Rho/ROCK signaling[11]. Rho/ROCK and actin polymerization ensure that YAP/TAZ transcriptional regulators translocate to the nucleus, thereby inhibiting adipogenic and chondrogenic differentiation and promoting osteogenic differentiation of adult mesenchymal stromal cells (MSCs)[12,13]. Thus this cascade regulates cell differentiation by relaying information on ECM properties to the cell nucleus[8]. As the NP is a cartilaginous tissue, chondrogenic differentiation is relevant for NP regeneration. Indeed, a decrease in substrate stiffness (which reduces focal adhesion and actin network strength) promoted chondrogenesis of porcine chondrocytes in monolayer on type I collagen-coated polyacrylamide hydrogels[14]. Early chondrogenesis of murine mesenchymal cells in micromass cultures was suppressed by FAK/Src phosphorylation[15], while inhibition of focal adhesions could stimulate chondrogenic redifferentiation and decrease proliferation of porcine and human chondrocytes in monolayer or alginate beads[16].

However, many of these (re)differentiation studies have been performed in 2D monolayer and hence are of little relevance for the *in vivo* situation. Therefore, more and more studies on redifferentiation of cartilaginous cells are focusing on 3D environments, such as tissues and hydrogels[17–19]. A 3D environment seems to promote redifferentiation: porcine and human chondrocytes after monolayer expansion dedifferentiate and exhibit decreased type II collagen gene expression, which could be recovered in alginate beads[16]. In the IVD environment, mechanotransduction has been shown to be integrin-dependent in NP cells in monolayer[20–22] and in 3D[23]. Although it has already been shown in monolayer that strain affects the cytoskeleton and actin stress fibers of bovine NP cells[24], and matrix stiffness affected phenotype and differentiation of porcine NP cells[19,21,25], the mechanisms behind this are not yet fully elucidated, especially in 3D. Noteworthy, porcine NPs contain notochordal cells, which have a distinctly different morphology from human mature and degenerated NP cells. As such, the role of focal adhesions in stiffness sensing by human degenerated NP cells and NP-cell mediated regeneration is unknown.

In this study, we investigate the role of substrate stiffness and focal adhesion formation in regeneration by human degenerated NP cells in 3D culture. To this end, frequently used polysaccharide-based gels such as agarose and alginate were chosen, in addition to hydrogels based on collagen, including methacrylated gelatin (gelMA)[4]. In order to explore how chondrogenesis by degenerated NP cells is influenced by the stiffness of the ECM, the effect of varying concentrations of hydrogel polymer on chondrogenic regeneration was measured. In order to determine whether a differential response of NP cells is dependent on focal adhesion formation, functional studies were performed in the presence of a FAK inhibitor. To this end the polysaccharide-based hydrogels naturally devoid of integrin-binding motifs, including a hyaluronic acid-based hydrogel which binds the CD44 receptor, were compared to the collagen-based gels to determine the necessity of the presence of these motifs.

Materials & methods

Culture

Cell isolation and expansion

Human IVDs were collected during post-mortem examination from patients deceased at the University Medical Center Utrecht. Redundant IVD tissue was used anonymously according to the regulations of the Local Medical Ethical Committee (METC) (number 12–364), and hence without information on existing spine pathology. From 9 donors (age 44–67, mean age 52), IVDs macroscopically scored as Thompson grade III, NP tissue was

visually identified and dissected. Thereafter, cells were isolated using 14 U/ml pronase (Roche, Basel, Switzerland) + 110 U/ml DNAse (Sigma, St. Louis, MO) for 1 h followed by 13.7 U/ml collagenase (Roche) + 110 U/ml DNAse overnight. NP cells were expanded by seeding at 3000 cells/cm^2 [26] using DMEM (31885, Gibco, Life Technologies, Carlsbad, CA) + 10% heat-inactivated fetal bovine serum (HyClone, Life Technologies) + 2% antibiotics mixture (final concentration 200 U/ml penicillin, 20 kU/ml streptomycin, 50 mg/L amphotericin-B and 50 mg/L gentamycin (Lonza, Basel, Switzerland)) and 10 ng/ml recombinant human basic fibroblast growth factor (bFGF) (R&D Systems, Minneapolis, MA). During the second passage, anti-microbial agents were reduced to 1% penicillin and streptomycin (100 U/ml + 100 lg/ml, pen/strep, Gibco). NP cells were expanded for 2 passages until use in hydrogel cultures.

Hydrogel preparation

Three hydrogels with different stiffness and adjustable polymer concentration were prepared to determine whether stiffness affects matrix deposition by NP cells (see Table 1). Alginate (medium viscosity, Sigma) was mixed with the NP cell suspension and the solution was added dropwise into a sterile 102 mM $CaCl_2$ solution to form beads. The beads were allowed to cross-link for 10 min. Alginate hydrogels were recrosslinked weekly (by incubation with CaCl2) for stability. Agarose (low electroendoosmosis (EEO), Sigma) hydrogels were prepared by autoclaving and the polymer solution was maintained at 45°C until use. The temperature was lowered to 39°C before mixing in the NP cell suspension, after which gelation was allowed to occur at room temperature. Gelatin-methacryloyl (gelMA) was prepared as described before[27] by incubation of gelatin from porcine skin type A (Sigma) with methacrylic anhydride for 1 h at 60°C, followed by dialysis and freeze-drying. Irgacure 2959 (0.05%, BASF, Ludwigshafen, Germany) was added to the gelMA solution and mixed with the NP cell suspension. Hydrogels were prepared by injection into a mold[28] and UV-cross-linking for 15 min at 365 nm at an intensity of 2.6 mW/cm^2. To investigate the effect of hydrogel stiffness on proteoglycan production by NP cells, the polymer concentration was reduced to 1% (w/v) for alginate and agarose hydrogels and to 5% (w/v) for gelMA hydrogels. Hyaluronic acid/poly(ethylene glycol) (HA-PEG, HyStem, Esibio, Alameda, CA, USA) solution was mixed with cross-link solution and left to cross-link at 37°C for 30 min according to the manufacturer's instructions. Type II collagen hydrogels (3D collagen culture kit, EMD Millipore, Billerica, MA, USA) were prepared by neutralizing the pH of the acidic 6 mM collagen solution in acetate buffer. Cells were mixed into this solution and were left to cross-link at 37°C for 1 h. All hydrogels contained 2 * 10^6 cells/ml, based on previous studies on human NP cells[29–31], and were approximately 50 ll each.

Table 1 – Polymers, concentrations, and cross-linking methods of the different hydrogels.

Hydrogel	Concentration (% (w/v))		Cross-linking
Alginate	1%	2%	Ca^{2+} ions
Agarose	1%	2%	Thermal gelation
GelMA	5%	10%	UV-cross-links
HA-PEG		1%	Thiol-based cross-linking of HA and PEG
Type II collagen		0.1%	pH-dependent gelation

Hydrogel culture

To assess the effect of different polymer concentrations, the cell-laden alginate (1 and 2% (w/v)), agarose (1 and 2% (w/v)), and gelMA (5 and 10% (w/v)) hydrogels were cultured for 28 days in DMEM + 10% FBS + 0.4 nM ascorbic acid-2-phosphate + 1% pen/ strep. The role of focal adhesion formation in chondrogenic matrix production was studied by the use of the low molecular weight (284 Da) FAK inhibitor FAK-i-14, thereby inhibiting the formation of focal adhesions. To this end cell-containing hydrogels were cultured for 18 days in a serum-free differentiation medium (DMEM + 2% insulin-transferrin-selenium- ethanolamine (ITS-X, Gibco) + 2% ascorbic acid-2-phosphate (ASAP, Sigma) + 2% human serum albumin (HSA, Sanquin, Amsterdam, The Netherlands) + 1% pen/ strep). Inhibition of FAK activity as early event in the integring binding cascade, was accomplished by adding 1, 5 or 10 μM 1,2,4,5-benzenetetraamine tetrahydrochloride (FAK-i-14), which has an IC50 of 1 μM [32] (Tocris, Bristol, UK) and was previously used at 10 μM in a chondrocyte-based study[33]. As vehicle control dimethyl sulfoxide (DMSO, Merck) was added to the differentiation medium. Lactate dehydrogenase (LDH) was measured in the culture medium on the second day of culture with a Cytotoxicity Detection Kit (Roche). In all experiments, empty hydrogels without cells were cultured and taken along as controls in all analyses.

Analyses

Dynamic mechanical analysis

Hydrogels without cells (n = 5 per polymer) were prepared as described above and incubated for 1 day in differentiation medium. Dynamic mechanical analysis (DMA) was performed on a DMA Q800 (TA Instruments, New Castle, DE, USA) at room temperature with 0.001 N continuous preload. Stress was induced at a rate of 1 N min^{-1} to a maximum of 18 N on all samples (n = 5). The Young's modulus (E) is defined as the slope of stress (σ) to deformation (ε).

Biochemistry

Hydrogel-cell constructs were incubated overnight at 60°C in a papain digestion buffer (250 µg/ml papain (Sigma), 1.57 mg/ml L-cysteine (Sigma)). GAG content was measured with a 1,9- dimethyl-methylene blue (DMMB) assay with pH adjusted to 1.0 because of interference of alginate and gelMA hydrogels at pH 3.0. DNA content was determined with a Picogreen assay (Life Technologies, Carlsbad, CA).

Vinculin/actin immunohistochemistry

For the vinculin/actin cytoskeleton staining (FAK100 kit, Millipore), hydrogels were fixed for a maximum of 16 h in 4% buffered formalin. Cells cultured on glass coverslips coated with type I collagen for 24 h served as controls. Hydrogels were cut in approximately 0.5 mm slices with a scalpel, permeabilized with 0.2% Triton-X, blocked in PBS + 5% bovine serum albumin (PBS-BSA), washed, and incubated with 2 ng/ml primary α-vinculin antibody for 1 h at room temperature. After washing, the hydrogels and controls were simultaneously incubated with 10 µg/ml secondary antibody α-mouse-Alexa 488 (A11029, Invitrogen) and 120 ng/ml TRITC-conjugated Phalloidin for 1 h at RT. Nuclei were stained with 0.2 µg/mL DAPI for 10 min and thoroughly washed in PBS + 0.05% Tween (PBS-T). Hydrogels and controls were stored in PBS at 4°C until visualization with a DMi8 confocal microscope with an SP8- X white light laser and a 63/1.3 NA objective (Leica Microsystems, Wetzlar, Germany). Z-stack images are represented here as maximum intensity projections. Intensity of the confocal images was enhanced in Photoshop (Adobe, San Jose, CA, USA) and levels were adjusted for each image separately for descriptive analysis.

Extracellular matrix histology and immunohistochemistry

For a qualitative impression of extracellular matrix production, after 18 days of culture, hydrogels were fixed in 4% buffered formalin, dehydrated and embedded in paraffin. A Safranin-O/Fast Green staining was performed by incubating 5 µm sections with Mayer's hematoxylin (Merck) for 5 min, washing in tap water for 10 min and staining with 0.4% w/v Fast Green (Merck) during 4 min. After washing in 1% acetic acid, the sections were stained for 5 min in 0.125% (w/v) Safranin-O (Merck), followed by dehydration and mounting. For aggrecan immunohistochemistry, antigen retrieval was performed by incubating in citrate buffer at 96°C for 20 min and cooling to room temperature (RT) for an additional 20 min. For types I and II collagen, antigen retrieval consisted of incubation with pronase (1 mg/ml in PBS, Roche) and hyaluronidase (10 mg/ ml in PBS, Sigma) (both at 37°C for 30 min). Immunostaining protocols were the same after the antigen retrieval. Washing in between steps was performed with PBS-T. Sections were blocked with PBS-BSA for 30 min at RT and incubated overnight at 4°C with the primary antibody diluted

in PBS-BSA (aggrecan (diluted 1:150, 6-B-4 (Novus Biologicals, Littleton, CO, USA), type I collagen (2 mg/ml, EPR7785 (abcam, Cambridge, UK), or type II collagen (0.4 µg/ml, DSHB II-II6B3 (DSHB, Iowa City, IA)). The EnVision kit (α-mouse for aggrecan and type II collagen, α-rabbit for type I collagen, Dako, Glostrup, Denmark) was used for visualization according to the manufacturer's protocol. Microscopy pictures were taken with a BX43 upright microscope and CellSens software (Olympus, Tokyo, Japan).

Chondrogenic, osteogenic and adipogenic gene expression

After 18 days of culture, hydrogels were snap frozen at 80°C. For RNA isolation hydrogels were cut into small pieces and TRIzol reagent (Thermo Scientific) was added. The lysate was then homogenized with an Ultra-turrax T10 homogenizer (IKA, Staufen, Germany) and purified with Direct-zol™ columns (Zymo, Irvine, CA, USA). 100 % ethanol was added to the lysate and centrifuged over the Zymo-Spin columns and treated with DNAse I for 15 min. The columns were then washed with pre-wash and wash buffer and RNA was eluted with RNAse-free water. Quality and quantity were checked using a Nanodrop (Thermo Scientific). A High-capacity cDNA kit (Life Technologies) was used to synthesize complementary DNA from RNA on an iCycler Thermal Cycler (Bio-Rad, Hercules, CA, USA). In order to determine towards which lineage NP cells redifferentiated after expansion, a panel of genes was determined: aggrecan (ACAN), type I, type II and type X collagen (Col1A1, Col2A1, ColX), matrix metalloproteinase 13 (MMP13, a matrix degrading enzyme), osteopontin (SPP1, an osteogenic marker), and peroxisome proliferator-activated receptor gamma (PPAR-γ, an adipogenic marker). All genes of interest were normalized to the geometric mean of the housekeeping genes 18S and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Sequences and details of all genes are shown in Table 2. RT-qPCR was performed on a LightCycler 96 (Roche) using SYBR Green (Bio-Rad).

Table 2 – List of employed primers for RT-qPCR.

Gene	Forward 5'-3'	Reverse 5'-3'	Product size (bp)	Annealing Temp. (°C)
18S	GTAACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG	151	57
GAPDH	ATGGGGAAGGTGAAGGTCG	TAAAAGCAGCCCTGGTGACC	70	60
ACAN	CAACTACCCGGCCATCC	GATGGCTCTGTAATGGAACAC	160	56
Col1A1	TCCAACGAGATCGAGATCC	AAGCCGAATTCCTGGTCT	191	57
Col2A1	AGGGCCAGGATGTCCGGCA	GGGTCCCAGGTTCTCCATCT	195	57
ColX	CACTACCCAACACCAAGACA	CTGGTTTCCCTACAGCTGAT	225	56
MMP13	GGAGCATGGCGACTTCTAC	GAGTGCTCCAGGGTCCTT	208	56
SPP1	CATCTCAGAAGCAGAATCTCC	CCATAAACCACACTATCACCTC	355	56
PPAR-γ	CTGAATGTGAAGCCCATTGAA	GTGGAAGAAGGGAAATGTTGG	275	57

Statistical analyses

All statistical analyses were carried out with SPSS statistics 21 software (IBM, North Castle, NY, USA). Kruskall-Wallis tests were performed for GAG and DNA data, followed by post hoc multiple Mann-Whitney tests with Benjamini-Hochberg corrections in Excel (Microsoft, Redmond, WA, USA). In addition, a Spearman's correlation test was performed for cells in hydrogels cultured with a FAK inhibitor. Residuals of the qPCR data were normally distributed according to Levene's statistic, so a one-way ANOVA was performed. All data are displayed as the mean ± the standard deviation.

Results

Mechanical analysis

The stiffness of cell-free hydrogels was measured after one day of incubation in culture medium at 37°C (Figure 1). Young's moduli of agarose and gelMA hydrogels were highest (27 ± 12 and 11 ± 1.5 kPa, respectively), followed by alginate and HA-PEG hydrogels (4 kPa). The type II collagen hydrogel was softest, with a Young's modulus of 2 ± 1 kPa. The high variation in measurements of agarose hydrogels might be explained by their shape, as they were formed in a U-bottom 96-wells plate, resulting in a hemispheric shape. Alginate, HA-PEG, and type II collagen hydrogels were more flattened after 1 day of culture, and gelMA hydrogels were cast as perfect discs.

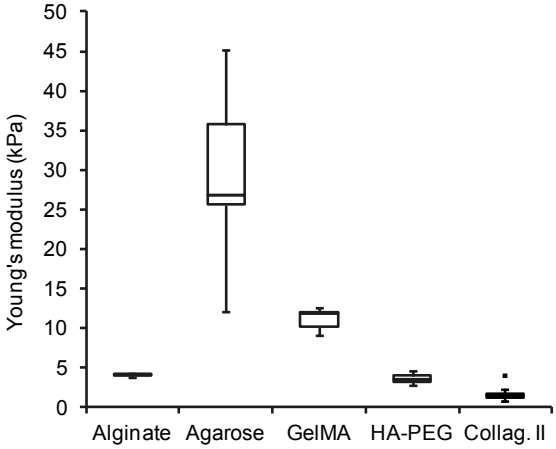


Figure 1 – Compression testing shows the highest Young's modulus for agarose hydrogels, followed by gelMA, and alginate and HA-PEG, and the lowest was measured for type II collagen hydrogels.

The three hydrogels allowing for adaptation of their polymer concentration were used to study the response of incorporated cells to halving the regular polymer concentrations (from 2% (w/ v) to 1% (w/v) for alginate and agarose and from 10% (w/v) to 5% (w/v) for gelMA). Figure 2 shows that GAG deposition and GAG corrected for DNA in gelMA hydrogels were higher in the 5% hydrogels compared to the 10% hydrogels ($p = .002$), but no significant differences were measured in alginate or agarose hydrogels. All donors demonstrated a similar response in the same direction although the magnitude of change varied. This was observed in general for all parameters measured.

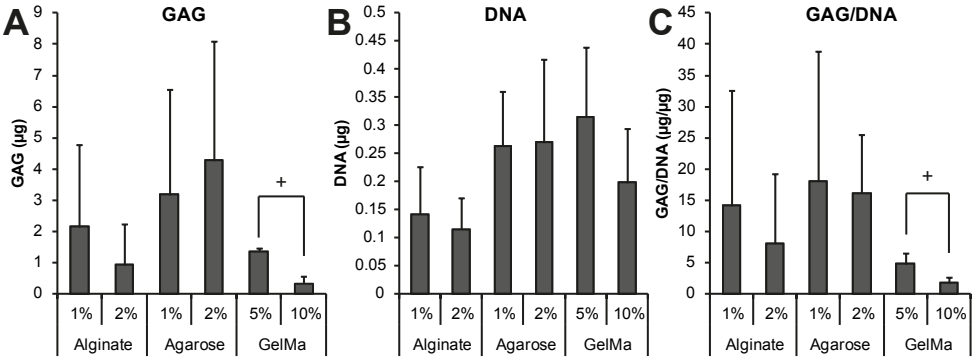


Figure 2 – Nucleus pulposus cells from human degenerated discs from 4 donors (n = 5 gels per condition per donor) were cultured in hydrogels of different concentrations. **A**: GelMA hydrogels of 5% (w/v) contained more GAGs than those of 10% (w/v). **B**: No differences in levels of DNA were found. **C**: GAG corrected for DNA was higher in 5% (w/v) gelMA hydrogels than in 10% (w/v) gelMa hydrogels. Significant differences are given as follows: + $p \leq .005$.

Focal adhesion formation

Cell morphology and focal adhesion formation were imaged by staining for vinculin and actin (Figure 3). Confocal microscopy revealed the existence of focal adhesions in NP cells in monolayer culture on type I collagen-coated glass coverslips. The NP cells on glass generally had a stellate shape, and the F-actin cytoskeleton formed clear stress fibers with visible and elongated focal adhesions at their ends. In 3D culture in agarose, round NP cells were observed and vinculin staining showed a patchy membrane-associated staining, but without clear association with the actin cytoskeleton, as only G-actin was detected. In gelMA hydrogels, the majority of cells displayed a similar morphology as in agarose with G-actin diffusely present in the cytoplasm, although in some cells (Supplementary Figure S1) F-actin fibers in cytoplasmic processes were present. Bright focal staining of vinculin was present throughout the cytoplasm although occasionally vinculin was also present at the cell membrane. In type II collagen hydrogels, however, NP cells were more elongated or even spindle-shaped and showed F-actin stress fibers extending into different processes of the cell, with focal adhesions at their ends, although not as elongated and mature as in 2D.

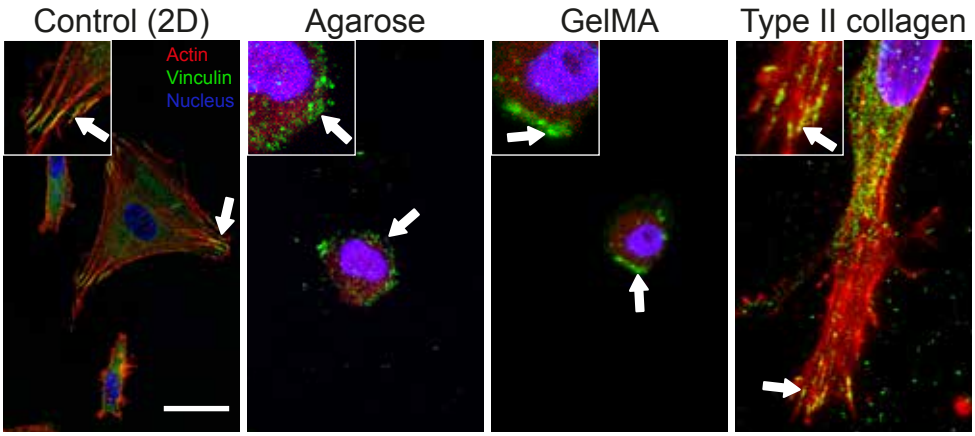


Figure 3 – Confocal microscopy of human degenerated nucleus pulposus cells cultured on type I collagen-coated glass coverslips and in several hydrogels. Red staining is actin, green staining is vinculin, and blue staining is the cell nucleus. Arrows indicate focal adhesions. Z-stack images are represented here as maximum intensity projections. See Supplementary data for rotation movie of a 3D representation of an NP cell in a type II collagen hydrogel. Scale bar represents 10 μm, magnification is the same in all images. Images are representative for 2 donors.

Inhibition of focal adhesion kinase

Cytotoxicity

No differences in LDH production were measured on day 2 between cells cultured with the different concentrations of FAK-i-14 or control medium in any of the hydrogels (data not shown), showing the inhibitor was not cytotoxic.

DNA and GAG content

Serum was omitted from the medium to prevent interference with focal adhesion. Consequently, GAG levels were lower in serum-free medium than when serum was present (Figure 4 compared to Figure 2). The GAG content of alginate hydrogels cultured with 5 mM FAK-i-14 was higher than with 10 mM FAK-i-14 ($p = .004$, Figure 4A). No differences were measured for cells in agarose hydrogels. In gelMA hydrogels, the GAG content increased with increasing concentration of FAK-i-14, as measured by a weak but significant correlation (Spearman coefficient 0.286, $p = .011$). No effects of FAK inhibition were detected for cells cultured in HA-PEG hydrogels. In type II collagen hydrogels, a weak but significant negative correlation was found between GAG content with FAK-i-14 concentration (Spearman coefficient 0.265, $p = .024$). No differences in DNA content were detected (Figure 4B). GAG/DNA was higher in alginate hydrogels cultured with

5 μM FAK-i-14 than with 10 μM FAK-i-14 (p = .004), but no correlation with inhibitor concentration was found (Figure 4C). No differences in GAG/DNA were detected for NP cells in agarose, gelMA, and HA-PEG. GAG corrected for DNA was higher for NP cells in type II collagen hydrogels cultured without FAK-i-14 than with 10 μM, and a moderate negative correlation was found with increasing FAK-i-14 concentration and GAG/DNA in type II collagen hydrogels (Spearman coefficient -0.354, p = .002).

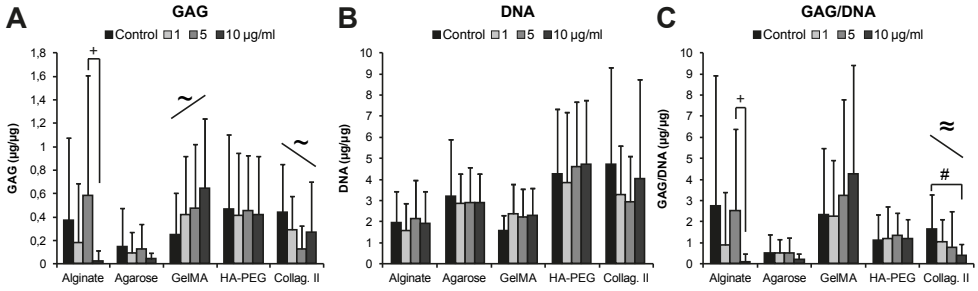


Figure 4 – Human degenerated nucleus pulposus cells from 5 donors cultured in several hydrogels in the presence of focal adhesion kinase inhibitor 14 (FAK-i-14) at different concentrations (1, 5 or 10 **μ**M)(n = 5 gels per condition per donor). Control contains the carrier (DMSO)). **A**: A positive correlation was found between GAG content and FAK-i14 concentration for gelMA and a negative correlation for type II collagen hydrogels. **B**: No differences in DNA content were found. **C**: A negative correlation was found for GAG/DNA in type II collagen hydrogels with increasing concentration of the FAK inhibitor. Significant differences are given as follows: + p ≤ .005, # p ≤ .01, ≈ Spearman coefficient 0.354, p = .002, ~ Spearman coefficient ± 0.3 p < .025.

Vinculin and actin localization with FAK inhibition

NP cells were cultured in agarose, gelMA and type II collagen hydrogels in the presence or absence of the FAK inhibitor. In agarose, inhibition of FAK did not seem to influence the pattern of vinculin localization or actin polymerization (Figure 5). In the presence of FAK-i-14, all NP cells in gelMA hydrogels were round and there was a reduced diffuse cytosolic non-polymerized actin staining. Some focal vinculin staining was present at the cell membrane, but at a clearly lower intensity. Cells in type II collagen changed from being elongated and stretched to rounded cells in the presence of FAK-i-14 and diffuse low-intensity staining for actin without stress fibers was present in the cytoplasm. Also in these hydrogels vinculin staining was greatly reduced.

No visible staining for collagens or proteoglycans

Histology and immunohistochemistry did not show any positive staining for Safranin-O, type II, or type I collagen (data not shown; n = 2 gels per condition, at 4 donors). This is most likely due to the short culturing period of 18 days in serum-free culture medium.

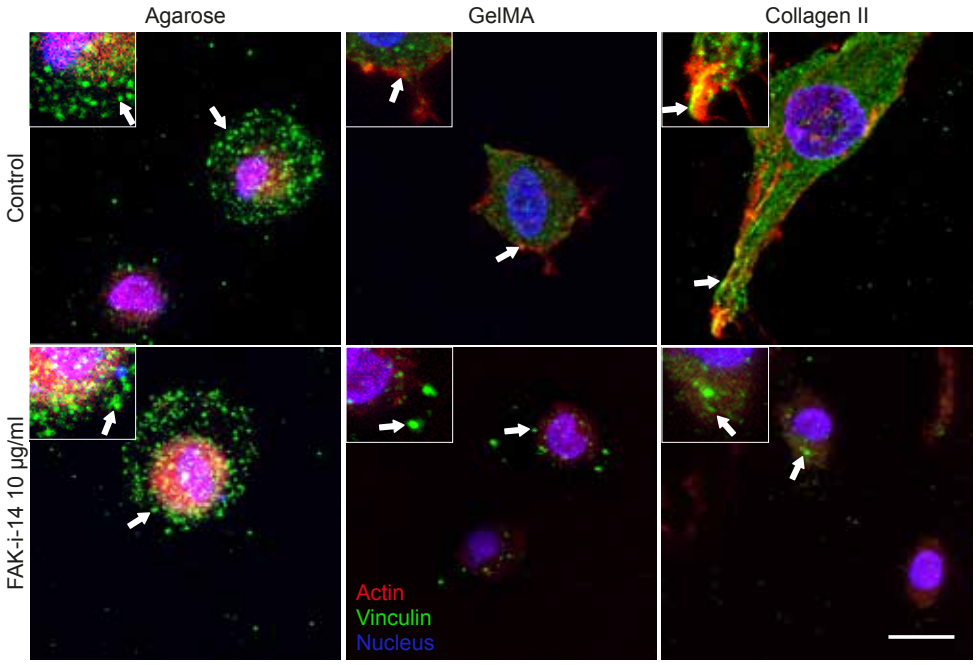


Figure 5 – Confocal images of cells in agarose, gelMA, and type II collagen hydrogels that were cultured in the presence of 10 **µ**M FAK-i-14, and compared to controls. Cells were stained for vinculin (green), actin (red), and the cell nucleus (blue). White arrows indicate focal adhesions. Z-stack images are represented here as maximum intensity projections. No actin or vinculin was present in the nuclei; any apparent staining comes from adjacent structures that were superimposed. Scale bar indicates 10 **µ**m. Images are representative for two donors.

Gene expression in response to FAK inhibition

In order to explain the differential effect of FAK inhibition on NP cell fate in gelMA versus type II collagen hydrogels, gene expression was measured after 18 days of culture in gelMA or type II collagen hydrogels in the presence or absence of 10 µM FAK-i-14 (Figure 6A). Type X collagen expression could not be detected in either hydrogel, while gene expression of the osteogenic marker osteopontin (SPP1) was completely inhibited in cells cultured in the presence of FAK-i-14. In controls without FAK inhibition, expression of chondrogenic marker aggrecan, hypertrophic marker MMP13, osteogenic marker osteocalcin (SPP1), and adipogenic marker PPAR-γ was 15-fold, 11-fold, 9-fold, and 32-fold higher in gelMA hydrogels compared to type II collagen hydrogels ($p = .001$, $p = .003$, $p = .016$, and $p = .001$, respectively, see Figure 6A). Gene expression profiles of cells cultured in gelMA remained unchanged in the presence of FAK-i-14 (Figure 6B). However, in cells in type II collagen hydrogels, FAK-i-14 increased the gene expression of type II collagen (4.6-fold, $p = .05$), MMP13 (6-fold, $p = .023$) and PPAR-γ (30-fold, $p < .001$), whereas gene expression of aggrecan and type I collagen remained unchanged (Figure 6C).

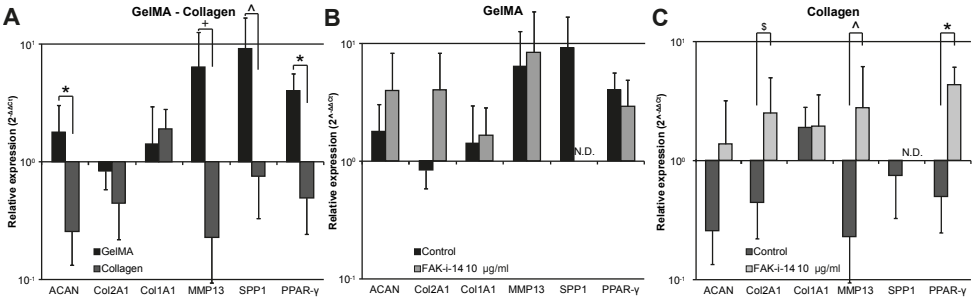


Figure 6 – Gene expression of human degenerated nucleus pulposus cells (4 different donors) cultured in gelMA or type II collagen hydrogels for 18 days in the absence or presence of FAK-i-14, relative to housekeeping genes, are displayed on a logarithmic y-axis. Gene expression of type X collagen could not be detected. (n = 5 gels per condition per donor) **A**: In the absence of FAK-i-14, gene expression levels of aggrecan, MMP13, osteocalcin (SPP1), and adipogenic marker PPAR-**γ** were higher in gelMA compared to type II collagen hydrogels. **B**: No differences in gene expression were detected in gelMA hydrogels. **C**: Gene expression levels for type II collagen, MMP13, and PPAR-**γ** of cells in type II collagen hydrogels were higher in the presence of the FAK inhibitor compared to control. Significant differences are given as follows: * $p \leq .001$, + $p \leq .005$, ^ $p \leq .025$, \$ $p \leq .05$; N.D.: not detectable with RT-qPCR. ACAN = aggrecan, Col1A1 = type I collagen, Col2A1 = type II collagen, ColX = type X collagen, MMP13 = matrix metalloproteinase 13, SPP1 = osteopontin, PPAR-**γ** = peroxisome proliferator-activated receptor gamma.

Discussion

The current study shows that stiffness sensing and regeneration capacity of NP cells isolated from degenerated human discs is mediated, at least in part, by focal adhesion signaling. NP cells formed some kind of focal adhesions in all environments tested, but with different morphologies and distributions and distinctly different from 2D NP cell culture. NP cells responded to a reduction in gelMA hydrogel polymer concentration by increasing matrix production. This did not occur in the polysaccharide-based alginate or agarose hydrogels. Regardless of the differences in cell morphology, only in the gelatin- and collagen-based hydrogels, both characterized by integrin-bindings motifs, focal adhesion signaling appeared to play a role in extracellular matrix production. This was augmented with increasing concentration of a focal adhesion kinase inhibitor in gelMA but inhibited in type II collagen hydrogels. Differential gene expression of aggrecan, MMP13, osteopontin (SPP1), and PPAR-γ indicate a more chondrogenic phenotype in gelMA compared to type II collagen hydrogels, while in the presence of FAK inhibitor NP cells in type II collagen hydrogels seemed to enter the adipogenic lineage as demonstrated by an increase in PPAR-γ gene expression.

A decrease in polymer concentration influenced matrix production only in integrin motif-containing hydrogels. The chondrogenic effect of decreasing polymer concentration in gelMA hydrogels, in contrast to a lack of effects for alginate and agarose, was in line

with previous data. Decreasing amounts of GAGs were produced by chondrocytes in hydrogels with increasing gelMA concentration[18,34], while varying alginate concentration did not affect GAG production by chondrocytes, NP, or AF cells[35]. However, it is difficult to compare hydrogel stiffness between studies because of confounders such as biomechanical loading and confinement, cross-linking methods and sample shape. Generally, hydrogel stiffness is concentration-dependent and power law-dependendent for agarose and gelMA[27,36–43]. Of course at some point, polymer concentration will likely also inhibit matrix production through nutrient and oxygen deprivation, or changes in fixed charge density[44], although high concentrations of PEG (20% (w/w)) still enabled matrix production by bovine chondrocytes[45,46]. The variations in agarose concentration used in the current study were shown previously not to affect the diffusion coefficient[47], while in alginate[47] and GelMA[48] this was halved upon doubling the polymer concentration in the range investigated here. Doubling GelMA concentration also resulted in a 50% decrease of glioblastoma cell growth[48]. However, in the current study, NP extracellular matrix production nor cell content were affected by varying alginate concentration, nor was cell content altered by changing GelMA density, so at the concentrations used, it is unlikely that the effects seen with GelMA were attributable to a decrease in nutrient provision. Most likely NP cells, originating from a tissue low in oxygen and nutrients, are much less sensitive to nutrient deprivation than fast growing tumor cells.

Focal adhesion signaling appeared to be involved in the response of NP cells to polymer concentration in hydrogels that contain integrin binding sites. MSCs have been shown adapt their differentiation pathway in response to matrix stiffness, with low stiffness leading to adipogenic (2 kPa), higher to chondrogenic (20 kPa), and even higher stiffness leading to osteogenic differentiation (30–40 kPa)[49]. Stem cell fate appears to be mediated, among others, by a cascade involving adhesion signaling, subsequent FAK activation, actin filament bundling and thereby translocation of YAP/TAZ to the nucleus, which induces osteogenesis and inhibits chondrogenesis and adipogenesis[50] and as such can only differentiate between an osteogenic versus non-osteogenic state. In the current study, despite the fact that NP cells are already in the chondrogenic lineage, they appeared to respond in a similar manner to hydrogels of different stiffness. Previously, Arg-Gly-Asp (RGD)-binding integrins were shown to be involved in mechanosensing in healthy, but not degenerated NP cells[23]. Here, also in degenerated NP cells stiffness sensing is suggested to be mediated by collagen-binding integrins which can be modulated by FAK inhibition. However, the extensive actin stress fibers formed in collagen II gels were in contrast with the low stiffness of the hydrogel and concomitant low expression of the osteogenic marker osteopontin, compared to gelMA. This may be related to the observation that after focal adhesion formation, F-actin networks can play other roles than cytoskeleton tensioning[51]. Osteopontin was completely inhibited in the presence of a FAK inhibitor in both gelMA and type II collagen. However, in the much softer type II collagen hydrogel FAK inhibition resulted in a reduction of chondrogenesis and enhanced adipogenic gene expression, which is in accordance to its stiffness already predisposing more towards the adipogenic lineage[49]. Assuming

that FAK was inhibited fully, this suggests that also other mechanisms were at play in the response of the NP cells to their surroundings in the gelMA versus type II collagen hydrogels. In addition to differences in matrix stiffness, different collagens are known to bind to different (combinations of) integrins[52], possibly explaining why the response of NP cells in the two hydrogels was different. In line with this concept, differentiation of MSCs was shown to also be influenced by specific integrin combinations[53]. Adipose-derived MSCs respond differently to type I and type II collagen and show more NP gene expression in response to type II collagen. However, chondrocytes in gelMA hydrogels of lower stiffness (around 2–3 kPa) showed a more fibroblastic morphology and high cell proliferation, suggesting dedifferentiation[54]. Here it should be noted that gene expression data should be interpreted with caution and may only be used to provide an explanation rather than proof for a particular phenomenon. First of all, gene expression does not always coincide with protein production, as we have shown before for the response of NP cells to osmolarity[26]. This is in fact also shown here by an absence of a change in aggrecan mRNA levels in gelMA gels upon FAK inhibition, while at the protein level aggrecan production was clearly enhanced in the gelMA gels. Moreover, the co-expression of transcription factors for different lineages have been shown before for undifferentiated human degenerate NP-derived cells, where exposure to differentiation media was required for final differentiation[55]. Most likely, the final balance with other pathways in cells will determine differentiation at the protein level. This may also explain why in the current study PPAR-γ mRNA was detected in gelMA, even at a similar level to those in collagen with FAK inhibitors, despite the higher proteoglycan production in the former, further cautioning against overinterpretation of mRNA expression data.

To what extent cells sense substrate stiffness directly or via the total number of binding sites, which varies with polymer concentration, remains the subject of heavy debate[49,56]. As porosity increases with reducing polymer concentration and stiffness, it is difficult to distinguish between stiffness sensing and extracellular matrix tethering by integrin binding[49,56]. It was suggested that the amount of anchor points of extracellular matrix molecules to the underlying polymer, but not its stiffness, seemed a major factor in determining the cell response[56]. This seems to be further supported by the observation that redifferentiation of porcine chondrocytes in 3D in agarose hydrogels was shown not to depend on hydrogel stiffness but rather on the number of focal adhesions mediated by addition of RGD[57].

Surprisingly, vinculin patches were also observed with confocal microscopy in NP cells in agarose hydrogels, while agarose by itself does not contain any cell binding motifs. The fact that no apparent differences were seen between the absence and presence of a FAK inhibitor and no actin polymerization was evident, suggests that these adhesions in agarose have formed in the absence of FAK activity. Indeed, it was shown that FAK activity is not required for focal adhesion formation[58], nor does it affect the molecular composition of the adhesion complexes[59]. Nevertheless, its activity is required for focal adhesion formation-mediated cell behavior such as migration[59]. FAK activity mediates

focal adhesion protein phosphorylation and thereby activation of actin linkage to focal adhesions and subsequent signaling. Hence the activation stage and conformation of focal adhesion complex components rather than their quantity determines signaling[60,61]. The absence of actin polymerization in agarose further supports the absence of FAK activity in these gels. The lack of effect of changes in agarose concentration, and hence hydrogel stiffness, may be related to the absence of serum in our cultures. Serum is known to contain various integrin binding molecules, including laminin and fibronectin, which may mediate tethering to the surrounding hydrogel[62]. In addition, it enhances cell-based production of ECM[30,63]. This also explains the observation that in the current study only limited matrix production was observed, as serum nor TGF-β was added to enhance this[64]. As such, stiffness sensing in alginate, agarose, and HA-PEG hydrogels may occur only when cells have produced integrin-binding matrix[65]. In line with this thought, bovine chondrocytes cultured in agarose hydrogels respond to mechanical loading with increased matrix synthesis rates only after the initial deposition of extracellular matrix[66,67]. However, to what extent this is relevant for clinical application, is unclear, as the presence of serum proteins will be very limited in the largely avascular IVD[68].

Finally, it should be noted that the use of hydrogels for NP regeneration still will require a solution to their current incapacity to restore biomechanical functionality[69]. The biomechanical properties of the gels used here were also below those required for optimal IVD function[70]. In addition, the cells used may be suboptimal. Autologous NP cells from the affected degenerated disc may be the most feasible option now, and as we showed previously donor age did not affect cell behavior in terms of proliferation[26], this suggests applicability in patients of all ages. However, novel developments such as iPS technology may in the future provide for a more regenerative cell type[71].

Conclusion

NP cell behavior in 3D in hydrogels bearing integrin-binding motifs is regulated, to a large extent, by focal adhesions, supported by mechanistic studies in the presence of a FAK inhibitor. The direction of the effect, however, may differ, possibly depending on initial stiffness or differences in integrin binding between gelatin (type I collagen)-based and type II collagen-based hydrogels. Deeper insight into hydrogel carrier-related aspects governing NP cell behavior may enhance effective NP cell-mediated tissue regeneration *in vivo*.

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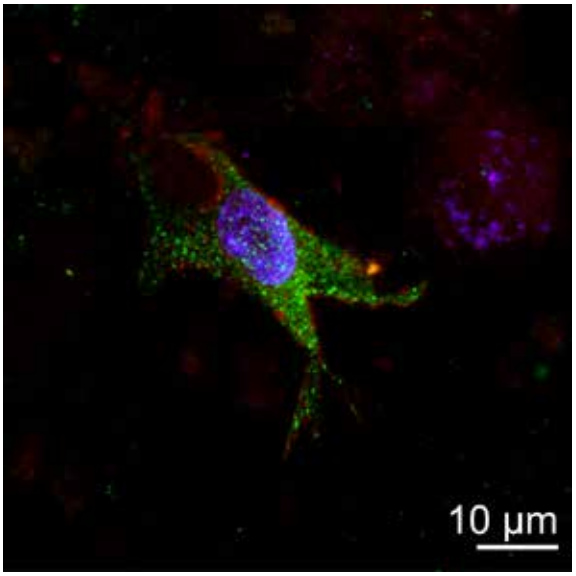
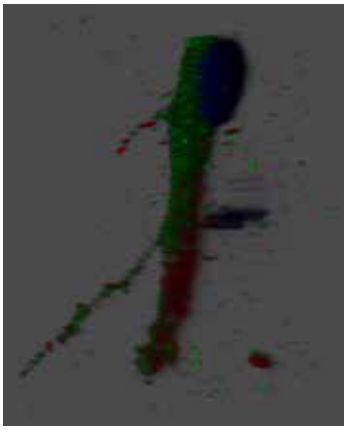
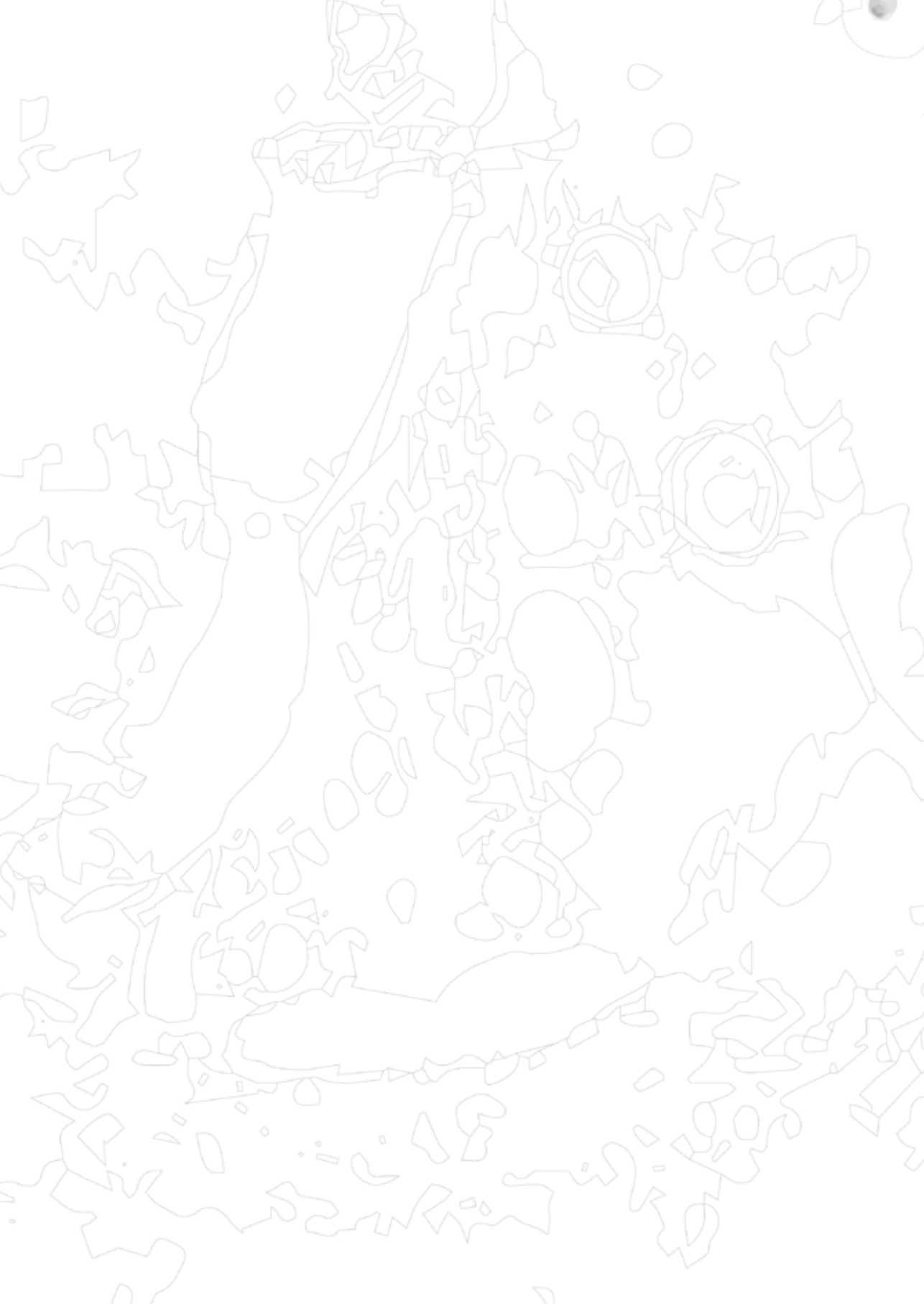
Supplementary data



Figuur S1 – NP cell in gelMA hydrogel with actin fibers extending into processes of stellate cells. Z-stack images are represented here as maximum intensity projections.



Figuur S2 – Movie of rotation of a 3D representation of an NP cell in a type II collagen hydrogel.



Bone Morphogenetic Proteins for Nucleus Pulposus Regeneration

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In preparation

Chapter 5

Abstract

The cells of the nucleus pulposus (NP) can be stimulated to regenerate by addition of growth factors or mesenchymal stromal cells (MSCs). Bone morphogenetic proteins (BMPs) are among the most promising growth factors for NP regeneration. Although BMP2 and BMP7 have been used most frequently, others have also shown potential for NP regeneration. Heterodimers have been shown to be more potent than homodimers, but it is not known whether combinations of the homodimers would perform equally well. In this study we compared BMP2, BMP4, BMP6, and BMP7, combinations and heterodimers for regeneration by human NP cells alone and complemented with MSCs. The BMPs investigated induced variable matrix deposition by NP cells, but BMP4 was the most potent. Only the heterodimers BMP2/6H and BMP2/7H were more potent than their respective homodimer combinations. In co-cultures of NP cells and MSCs, compared to pellets based on the same number of NP cells without addition of MSCs, the MSCs did not enhance matrix deposition, although total GAG production was higher in co-cultures. Co-cultures also showed a stronger response to BMP4 than to BMP7, similarly as observed with NP cells. In these co-cultures, the heterodimer BMP4/7H was more potent than the homodimer combination, in contrast to the NP cell cultures. The current results indicate that BMP4 might have the highest potential for regeneration of the intervertebral disc and can be applied together with MSCs. Moreover, the added value of BMP heterodimers over their respective homodimer BMP combinations depends on the BMP combination applied and the cells used.

Introduction

Low back pain, a considerable problem in today's society[1], is associated with degeneration of the intervertebral disc (IVD)[2]. The first signs of IVD degeneration are visible in the nucleus pulposus (NP)[3], where loss of glycosaminoglycan (GAG) content leads to loss of water content and ultimately pain and decreased mobility. Current treatments of chronic low back pain, conservative or surgical, do not address the underlying cause of degeneration. Growth factors are likely candidates to achieve biologic repair[4]. Among these growth factors are the bone morphogenetic proteins (BMPs) of the transforming growth factor-β (TGF-β) superfamily, which are commonly known for their involvement in bone formation. Since their discovery in the 1960s they have been found to play important roles throughout the entire body, including formation and maintenance of cartilaginous tissues[5]. BMP2 stimulates growth plate chondrocyte proliferation and hypertrophy to accelerate longitudinal bone growth[6] and has been shown to be important in postnatal cartilage development and maintenance[7]. BMP4 can induce and accelerate chondrogenic differentiation[8-9] and its co-expression with BMP2 is essential for osteogenesis[10]. BMP6 accelerates hypertrophic differentiation of chick chondrocytes[11-12]. BMP7 is a cartilage repair factor, enhancing anabolic and reducing catabolic responses in cartilage and IVD[13]. Hence, the chondrogenic capacity of many BMPs is interesting for regeneration of the cartilaginous NP. Noteworthy, BMP heterodimers were suggested to be more potent than homodimers, not only in bone formation, but also in IVD regeneration[14-18]. However, it is not clear whether these effects are limited to heterodimers only, or can be achieved by costimulation by the separate monomers, as this has been studied only once in bone formation[14].

For IVD regeneration mainly BMP2 and BMP7 have been investigated, most likely because they are clinically available[19]. In rabbit models of induced IVD degeneration, injection of BMP7 into the epidural space or NPs relocated to the epidural space was shown to be safe[20] and dosages of 2 µg[21] or 100 µg[22] BMP7 per disc had regenerative effects. However, in a spontaneous degeneration model in beagle dogs, injection of dosages up to 250 µg per disc, did not induce regeneration but resulted in extensive extradiscal bone formation[23]. Other BMPs, such as BMP3, -4, -5,-, -10, -11, -12, -13, -14, and BMP15, have been shown to be effective in regeneration of the NP *in vitro* and *in vivo* in pre-clinical models[24-25], but it is not clear whether they have similar effects in human IVD tissue.

One reason why BMPs may fail to induce NP regeneration may reside in the limited number and/or capacity of degenerated NP cells to adequately respond to growth factor stimulation. To this end, mesenchymal stromal cells (MSCs) are clinically being employed for NP regeneration, as NP cell collection from non-extruded discs for isolation and expansion induces (further) disc degeneration[26] and might not be a feasible clinical

approach. The regenerative effects of MSCs are thought to be mediated by trophic effects and/or their differentiation toward NP cells[27-31]. From a clinical perspective, BMPs and MSCs combined could further augment IVD regeneration. In this scenario, BMPs are expected to both stimulate the resident NPs to proliferate and produce matrix and facilitate MSCs to enter the chondrogenic lineage[32]. However, it is unclear whether BMP-based NP regeneration will be augmented by the presence of MSCs[33].

In the current study, we compared different BMP homodimers, heterodimers, and their respective homodimer combinations for their potency of inducing GAG formation by degenerated human NP cells *in vitro*. Furthermore, we explored the effect of the two best performing BMPs, i.e. BMP4 and BMP7, in co-cultures of NP cells and MSCs as a first step towards understanding how clinical applications of BMPs alone or in combination with MSC transplantation may augment IVD regeneration.

Materials and Methods

Cell culture

Cell isolation

Intact IVDs of 11 donors (age 47 to 68, mean age 53) were obtained as part of the standard postmortem procedure, as approved by the medical ethical committee of the University Medical Center Utrecht (METC nr. 12-364). The vertebrae were removed along the endplates and the tissue was graded for degeneration according to the Thompson classification[34]. Only Thompson grade III IVDs were used in this study. NP tissue was conservatively separated from annulus fibrosus tissue and cells were isolated using 1 hr incubation with 14 U/ml pronase (Roche, Basel, Switzerland) and 110 U/ml DNAse (Sigma, St. Louis, MO, USA), followed by overnight incubation with 13.7 kU/ml collagenase (Roche) and 110 U/ml DNAse, all diluted in Dulbecco's Modified Eagle Medium (DMEM, low glucose, pyruvate, L-glutamine, 31885, Gibco, Carlsbad, CA, USA). NP cells were then plated and expanded with DMEM + 10% heat inactivated fetal bovine serum (FBS) (HyClone, Logan, UT, USA) + 200 U/ml penicillin, 200 µg/ml streptomycin, 50 mg/l amphotericin-B and 50 mg/L gentamycin (Lonza, Basel, Switzerland)) and 10 ng/ml recombinant human basic fibroblast growth factor (bFGF, with BSA as carrier, R&D Systems, Minneapolis, MA) for 2 passages before use in regeneration cultures. MSCs were isolated from bone marrow of a 69-year old female undergoing arthroplasty (METC number 08-001) by centrifugation on Ficoll-paque PLUS (GE Healthcare, Little Chalfont, UK) and expanded in alpha MEM (α-MEM, Gibco) + 10% FBS + 1% ascorbic acid-2-phosphate (ASAP, Sigma) + 100 U/ml penicillin + 100 µg/ml streptomycin (pen/strep, Gibco) + 1 ng/ml bFGF. After three days the MSC cultures were washed to remove non-adherent cells.

MSC multipotency assays

Multipotency of the isolated MSCs was assessed by adipogenic, osteogenic, and chondrogenic differentiation[35]. For adipogenic and osteogenic differentiation, the cells were plated in 12 wells plates at 6000 cells/cm^2 and grown until confluency. Then adipogenic medium (α-MEM + 10% FBS + pen/strep + 1 μM dexamethasone (Sigma) + 0.5 mM IBMX (3-isobutyl-1-methylxanthine, Sigma, St. Louis, MO) + 0.2 mM indomethacin (Sigma) + 1.72 μM insulin) or osteogenic medium (α-MEM + 10% FBS + 0.2 nM ASAP + pen/strep + 10 mM β-glycerophosphate (Sigma) + 10 nM dexamethasone (Sigma)) was added to the cultures. 250,000 cells were pelleted and cultured with chondrogenic medium (DMEM + 0.2 nM ASAP + 1% ITS-Premix (Fisher Scientific, Waltham, MA, USA) + pen/strep + 0.1 μM dexamethasone + 0.4 nM transforming growth factor beta 1 (TGF-β1, with BSA as carrier, R&D Systems). After 21 days, Oil-Red-O staining was performed to assess adipogenesis. After 7 days, alkaline phosphatase (ALP) produced by the osteogenically differentiated MSCs was visualized with fuchsin (Fuchsin+ kit, K0625, DAKO, Glostrup, Denmark). Chondrogenically differentiated pellets were fixed with 4% buffered formalin, embedded in paraffin and immunohistochemistry for type II collagen was performed as described below.

BMPs in NP differentiation culture

For differentiation, NP cells of 4 donors were seeded on 0.12 cm^2 type II collagen-coated polycarbonate film (PCF) 0.4 μm transwell filters (Merck Millipore, Billerica, MA, USA) at passage 2 at 1*10^6 cells/cm^2 with differentiation culture medium (DMEM + 2% insulin-transferrin-selenium-ethanolamine (Gibco) + 0.4 nM ASAP + 2% human serum albumin (Sanquin, Amsterdam, The Netherlands) + pen/strep. BMP2, BMP4, BMP6, BMP7, the heterodimers BMP2/6H, BMP2/7H, and BMP4/7H (all with BSA as carrier, R&D Systems) were added to the culture medium at 4 nM, and combinations of BMP2+6, BMP2+7, and BMP4+7 homodimers were added at 2 nM per homodimer. As positive control 0.4 and 4 nM TGF-β1 (10 and 100 ng/ml, respectively) was included. Based on the matrix production in the comparison, BMP4 and BMP7 were also applied to 4 donors at a concentration of 0.04, 0.4, 0.2, and 4 nM. Cultures were performed for 28 days in a humidified incubator at 37°C and 5% $C0_2$. Medium was changed 3 times per week and stored at -80°C until further analysis. Culture medium was collected during the entire culture and stored at -80°C for GAG and PIICP analysis.

MSC and NP cell co-culture

As MSCs did not thrive in the transwell culture system, co-cultures were performed in pellet culture. Pellets of a single MSC donor with 4 NP cell donors were formed by

centrifugating U-bottom 96 wells plates containing cells suspensions in culture medium at 1750 rpm for 2 minutes. Medium was changed every 3 days. Different ratios of NP cells to MSCs were investigated, including 0%, 5%, 10%, 20%, 50%, or 100% NP cells (Table 1) with a total number of $0.25*10^6$ cells. Control pellets were prepared with the same numbers of NP cells without the addition of MSCs. Since no differences were found between NP cell ratios, subsequent co-culture experiments with BMPs were done with the 50:50 NP:MSC cell pellets, which are also frequently used in literature[27-31, 33]. Pellets were formed with MSCs and NP cells (50:50), and cultured in the presence of 0.4 nM BMP4, BMP7, BMP4+7 (0.2nM each), BMP4/7H, or TGF-β.

Table 1 – Pellets were made with different amounts of MSCs and NP cells. ($*10^6$ cells per pellet)

	NP	MSC	NP (%)
1		0.25	0
2	0.0125	0.2375	5
3	0.025	0.225	10
4	0.05	0.2	20
5	0.125	0.125	50
6	0.25		100
7	0.0125		100
8	0.025		100
9	0.05		100
10	0.125		100

Histology

2 cultured constructs or pellets per donor and treatment group were fixed for two days with 10% buffered formalin (J.T.Baker, Avantor Performance Materials, Center Valley, PA), dehydrated though a graded series of alcohol and embedded in paraffin. Sections of 5 µm were stained with 0.125% Safranin-O for GAGs and 0.4% Fast Green for collagens.

Immunohistochemistry

Immunohistochemistry for aggrecan, type I, and type II collagen were performed after 0.03% hydrogen peroxidase activity blocking and antigen retrieval with pronase (1 mg/ml, Sigma) and subsequent hyaluronidase (10 mg/ml, Sigma) incubation (for type I and II collagen) or cooking in citrate buffer (10 mM sodium citrate, pH 6.0, for aggrecan). After antigen retrieval, protocols were the same for all antibodies. Primary antibodies for type I collagen (2 µg/ml, rabbit, EPR7785, Abcam, Cambridge, UK), type II collagen (0.4 µg/ml, mouse, DSHB II-II6B3, DSHB, Iowa City, IA, USA), or aggrecan (1:150, mouse,

6-B-4, Abcam), were diluted in PBS + 5% BSA and incubated overnight at 4°C, after which the species-specific Envision kits were used for visualization (DAKO). Sections were counterstained with Mayer's haematoxylin solution and dehydrated and mounted with Depex. Microscopy pictures were taken with an Olympus BX51 upright microscope and the Cell^F software (Olympus, Tokyo, Japan).

Biochemistry: tissue GAG and DNA content

3-5 cultured constructs or pellets per donor and treatment group were digested in digestion buffer (250 µg/ml papain and 1.57 mg/ml L-cysteine (Sigma)). A Picogreen assay (Life Technologies, Carlsbad, CA) was used to determine DNA content against a λ-DNA standard curve. Fluorescence was measured in a PolarStar Optima fluorescence microplate reader (Isogen Life Science) at 485 nm excitation and 530 nm emission. Glycosaminoglycan (GAG) content of digested samples or medium was measured with a 1,9-dimethyl-methylene blue (DMMB, Sigma) assay and quantified against a chondroitin-6-sulfate standard curve. The optical density (OD) was determined at 525 nm and reference wavelength 595 nm with a VersaMax microplate reader and SoftMax Pro software (Molecular Devices).

Procollagen II ELISA

The carboxyterminal propeptide of type II collagen (PIICP) content in culture medium is directly related to the type II collagen production during culture[12]. We have previously shown that in week 2 most PIICP is produced and is representative for the total amount formed during culture[36]. The PIICP was measured on 3 medium samples per donor and treatment group using an enzyme-linked immunosorbent assay (ELISA, Cloud-Clone Corp, Houston, TX). Samples and standard diluted in culture medium were incubated for 2 hours, after which incubations with detection antibodies were performed for 1 hour and, after washing, 30 minutes subsequently. Then the plate was washed and incubated with 3,3',5,5'-tetramethylbenzidine (TMB) substrate solution for 20-25 minutes. After addition of the stop solution the plate was measured at 450 nm on a Versamax microplate reader.

Statistics

Statistics were performed using SPSS software (IBM, Armonk, NY). For the normally distributed procollagen ELISA data, univariate ANOVA with randomized block design with post-hoc Tukey *t*-test was performed. Otherwise, non-parametric Kruskall-Wallis with *post hoc* multiple Mann-Whitney comparisons were performed with Benjamini-Hochberg correction for multiple testing. Outliers detected with the 5*sigma rule (values

outside the mean ± 5 * standard deviation range) were removed. Data is displayed as the mean ± SD.

Results

The effects of BMPs on degenerated human NP cells

BMP treatment resulted in deposition of GAGs by NP cells cultured on transwell filters, as indicated by positive Safranin-O staining (Figure 1). The lowest intensity staining was seen with cells cultured with 4 nM TGF-β. Type II collagen was also deposited, mostly visible throughout the neotissue (Figure 1), although only patches of positive staining were seen occasionally in the presence of 4 nM TGF-β. Overall, proteoglycan-containing extracellular matrix was visible in the presence of any BMP as well as 0.4 nM TGF-β, but cells cultured in the presence of 4 nM TGF-β were packed together with very little extracellular matrix in between them.

The presence of type I collagen in all neotissue indicates that matrix formation was not exclusively chondrogenic (Figure 2). Type I collagen was diffusely present throughout all cultured neotissues, although the intensity was highest in the presence of BMP2+6 and lowest with BMP2/7H. In the presence of 4 nM TGF-β neotissue was primarily positive for type I collagen.

The regenerative effects of BMP homodimers were compared (Figure 3). Cells cultured in the presence of BMP4 produced the highest amounts of GAGs, significant compared to BMP2 and BMP6 ($p \leq 0.001$), but not BMP7. BMP2 produced the lowest amounts of GAG, GAG/DNA, and total GAG content, defined as the GAG content of the construct and GAG released into the medium ($p \leq 0.005$). Compared to the 0.4 nM TGF-β control, cells cultured with BMP4 produced more GAG and GAG/DNA and had higher incorporation efficiency ($p < 0.001$). Cells cultured in the presence of BMP2 contained lower amounts of GAGs, DNA, and total GAG than cells with 0.4 nM TGF-β ($p \leq 0.01$). Procollagen II (PIICP) measured in culture medium of week 2 as a measure of type II collagen production, was higher with BMP7 than with BMP2, BMP4, and BMP4+7 ($p \leq 0.015$, Figure 3F), but otherwise no differences were noted.

The heterodimers BMP2/6H, BMP2/7H, and BMP4/7H at 4 nM were compared to their respective homodimer combinations cultured at 2 nM each to achieve a final concentration of 4 nM in order to determine whether heterodimers have a better regenerative effect (Figure 3). The heterodimer BMP2/6H performed better in inducing GAG production ($p = 0.002$) but produced less type II collagen than BMP2+6. This was in line with the more intense Safranin-O stained matrix in BMP2/6H compared with BMP2+6, but type II collagen staining was also more intense. GAG/DNA and type I collagen deposition did not differ between BMP2/6H and BMP2+6. BMP2/7H treatment resulted in significantly higher GAG production and DNA content compared to BMP2+7 ($p < 0.001$). However,

there were no differences at the GAG/DNA level. Safranin-O staining seemed to be more intense in BMP2/7H compared to BMP2+7 while there was no difference in collagen type II staining patterns between the heterodimer and the combined homodimers. Notably, less collagen type I seemed to be deposited in BMP2/7H compared to BMP2+7.

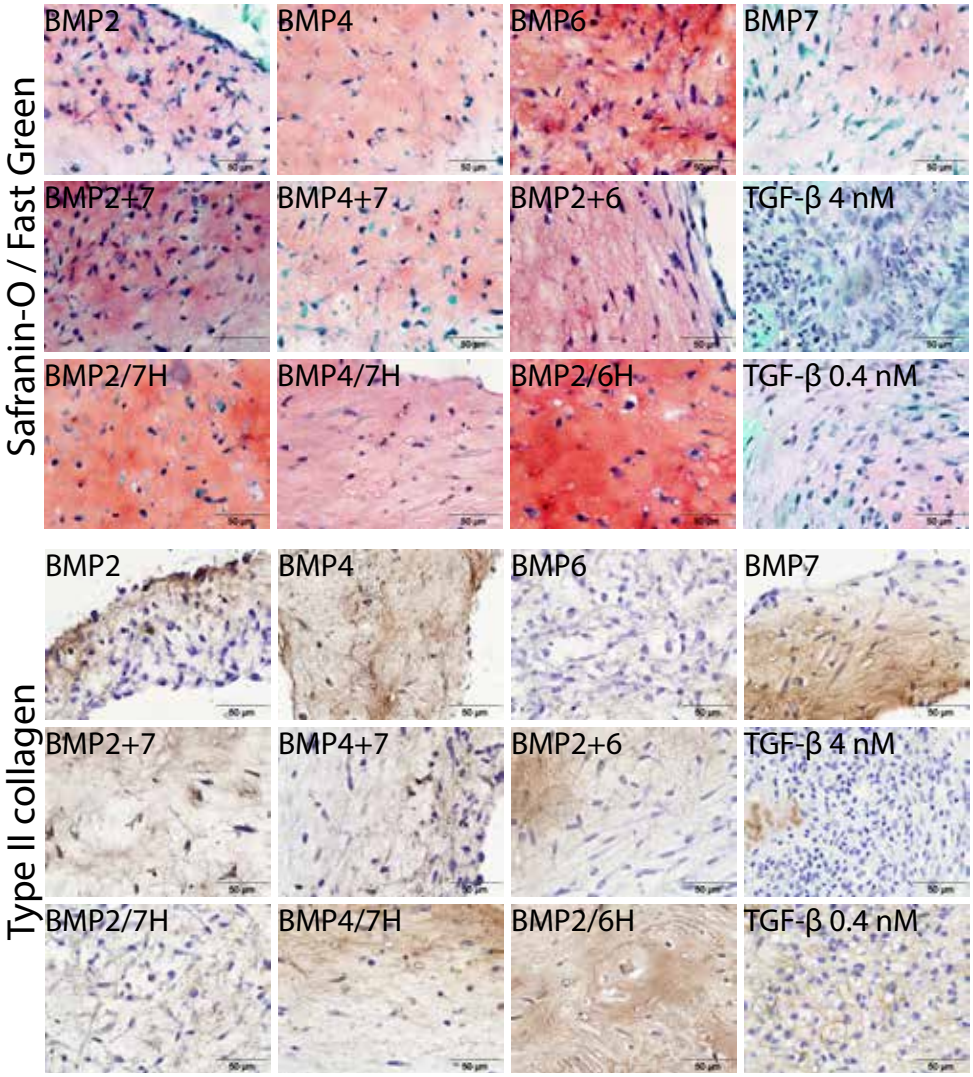


Figure 1 – Human degenerated nucleus pulposus cells were cultured on type II collagen-coated transwell inserts in the presence of different BMPs alone, in combination, or heterodimer (H) with final concentration 4 nM. Safranin-O/Fast green/Hematoxilin staining shows that GAGs were deposited in the presence of all BMPs, although the staining was less intense for BMP7. Cells in the presence of controls with 0.4 or 4 nM TGF-β produced hypercellular tissues with limited ECM. Immunohistochemistry shows that type II collagen was deposited in all neotissues, but only localized staining was seen occasionally in the presence of 4 nM TGF-β.

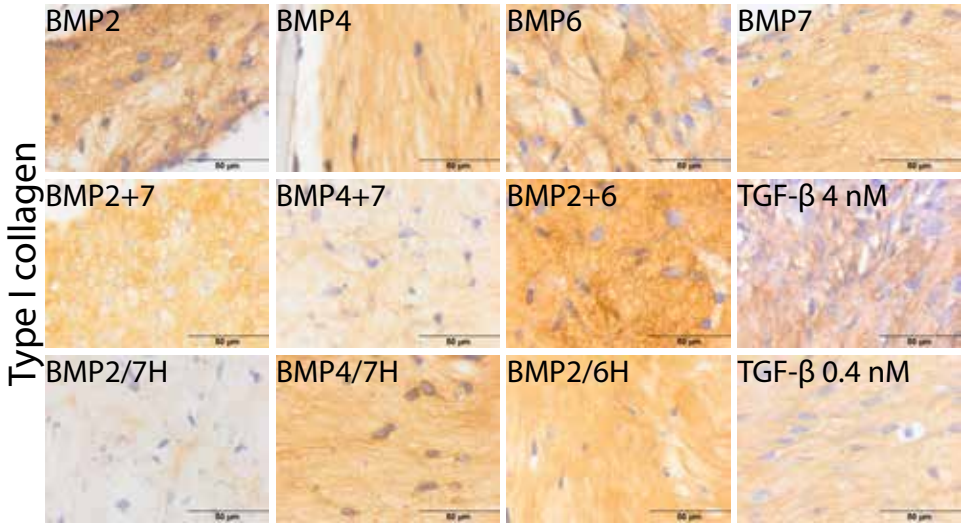


Figure 2 – Human degenerated nucleus pulposus cells cultured in transwell filter coated with type II collagen in the presence of 4 nM BMPs (alone, in combination, or heterdimers (H)) or 0.4 or 4 nM TGF-β were positive for type I collagen visualized by immunohistochemistry. The intensity was higher for BMP2+6 and very faint for BMP2/7H.

No differences were found between BMP4/7 H and BMP4+7 in GAG, DNA, GAG/DNA, or incorporation efficiency, or in the Safranin-O or type II collagen staining pattern. BMP4/7H treated constructs stained more intense for type I collagen compared with BMP4+7 (Figures 1 and 2). Although heterodimers BMP2/6H and BMP2/7H induced higher GAG and DNA content and total GAG than their homodimer combinations, GAG/DNA levels were not different. Any increase in matrix deposition thus seemed to have been caused by a higher cell number. Matrix production with heterodimer BMP4/7H was not different from BMP4+7.

Dose dependency of BMP4 and BMP7 stimulation

As BMP4, BMP4+7, and BMP4/7H appeared to be particularly efficient in inducing GAG production by NP cells, dose dependency was determined with NP cells from 4 donors cultured on transwell inserts at concentrations between 0.04 and 4 nM. BMP7 was included as control for the homodimer combination and heterodimer BMP4/7H. The highest GAG deposition was measured in the presence of 2 nM BMP4. Any concentration of BMP4 induced more GAG production than the BMP-free control ($p<0.001$), while 0.4, 2 and 4 nM BMP4 induced more GAG production than 0.04 nM ($p<0.001$). 4 nM BMP7 induced more GAG production than control, 0.04, and 0.4 nM ($p\leq0.001$) and 2 nM BMP7 ($p=0.009$). The increased GAG production by both BMP4 and BMP7 could largely be attributed to the increase in DNA content. Normalized per DNA, GAG deposition was highest for 0.4 nM BMP4, significant compared to control, 0.04, and 4 nM BMP4 ($p\leq0.005$).

In the presence of 4 nM BMP7, GAG/DNA was higher than with control, 0.04, 0.4, or 2 nM BMP7 (p≤0.009). No differences in total GAG production were measured between the BMPs or the concentrations used, but variability was high. GAG incorporation was most efficient for 2 nM and 4 nM BMP4 compared to control, 0.04, and 0.4 nM BMP4 (p≤0.028). The highest GAG incorporation efficiency was measured in the presence of 4 nM BMP7 (p≤0.003).

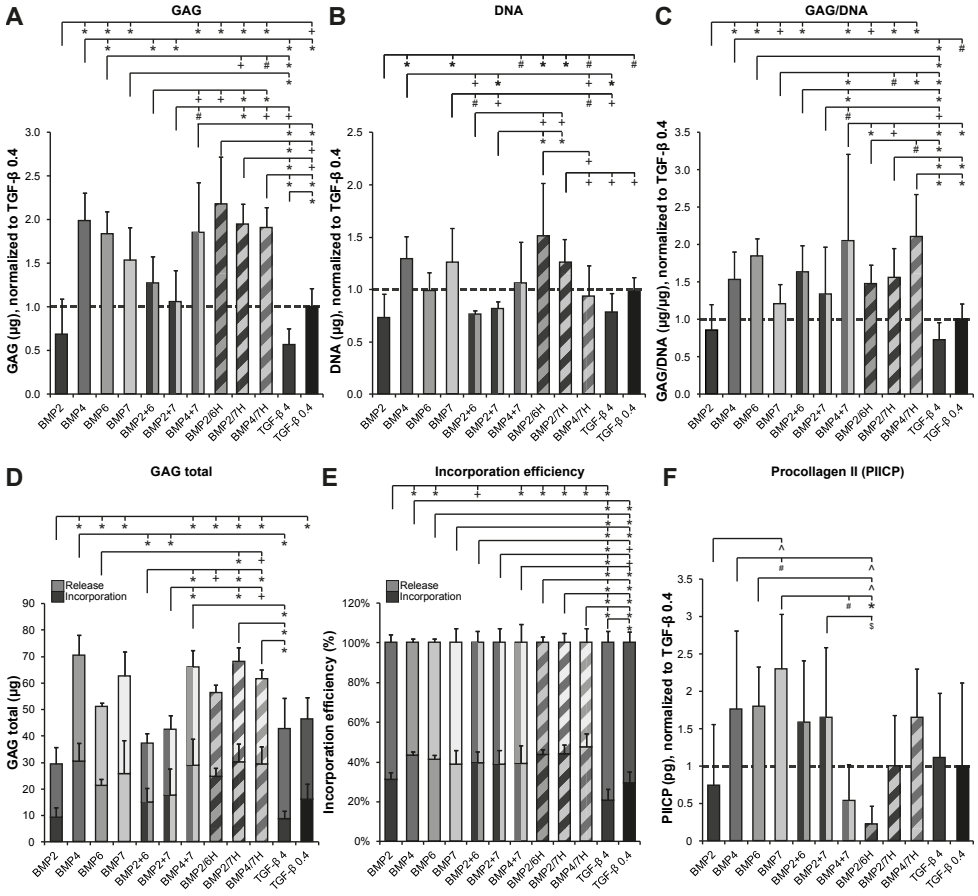


Figure 3 – GAG production by human degenerate nucleus pulposus cells from 4 donors were cultured on filters coated with type II collagen and treated with different BMPs (4 nM total concentration, also with combinations of homodimers and heterodimers (H)) and compared to TGF-β controls (4 and 0.4 nM, equal to 100 and 10 ng/ml). GAG and DNA content, GAG/DNA, and procollagen type II content (PIICP) are displayed relative to 0.4 nM TGF-**β** treated NP cells, which were set at 1 (indicated by the dashed line). All data are n=3 per donor per condition. **A**: GAG content was highest in differentiation cultures with BMP4 and BMP2/6H and lowest for 4 nM TGF-β and BMP2. **B**: DNA content was highest when cells were cultured with BMP2/6H, BMP4, BMP2/7H, and BMP7. **C**: The amount of GAG corrected for DNA was highest in BMP4/7H, followed by BMP4+7, BMP6, and BMP4. **D**: Total GAG production, including GAGs released into the medium and contained in the neotissue, was highest in BMP4, BMP2/7H and BMP4+7. **E**: Incorporation efficiency was highest in cells cultured with BMP4/7H. F: Procollagen II (PIICP) production in week 2 was higher by NP cells cultured with BMP7 than with BMP2, BMP4, and BMP4+7. Significant differences are indicates as follows: * p≤0.001, + p ≤0.005, # p≤0.01, ^ p≤0.025, $ p≤0.05.

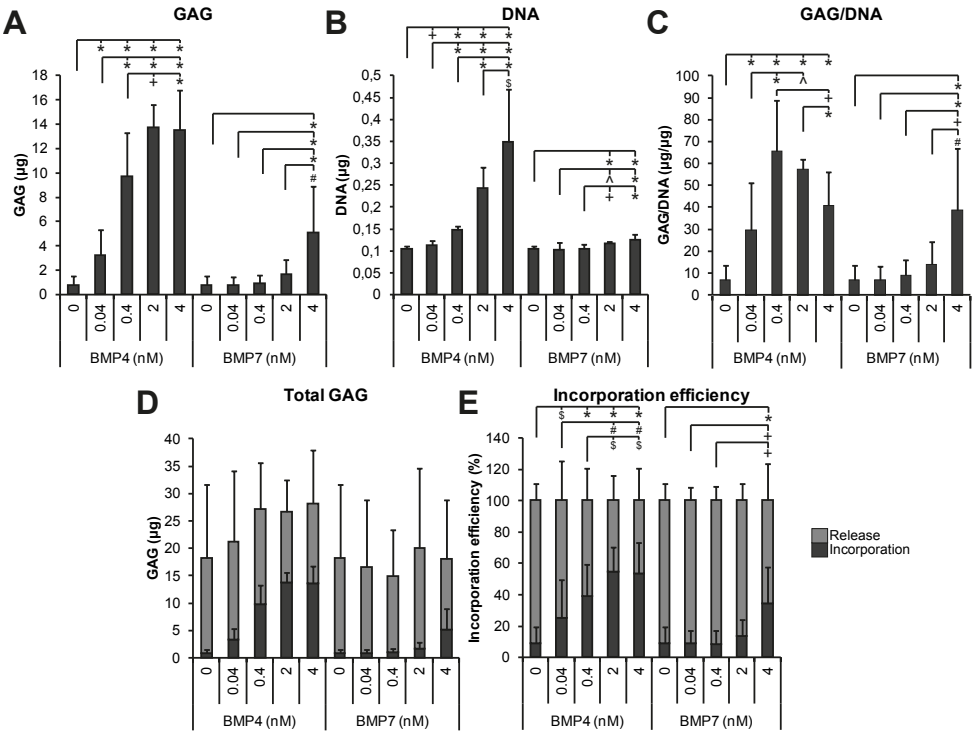


Figure 4 – Dose response of human degenerated nucleus pulposus cells of 4 donors (n=3 per donor per condition) to different concentrations of BMP4 and BMP7 was measured at the biochemical level. **A**: GAG production increased with increasing BMP4 or BMP7 concentration. **B**: Higher DNA content was measured with increasing BMP4 and BMP7 concentration. The effect of BMP7 was less pronounced than BMP4. **C**: GAG/DNA was highest for 0.4 nM BMP4. In the presence of 4 nM BMP7, GAG/DNA was higher than with control or lower concentrations of BMP7. **D**: No differences were measured for the total amount of GAG, including GAGs released into the medium. **E**: Incorporation efficiency was highest for 2 nM BMP4 and 4 nM BMP4. Higher incorporation efficiency was measured in the presence of 4 nM BMP7 compared to control and lower concentrations of BMP7. Significant differences are given as follows: * p≤0.001, + p ≤0.005, # p≤0.01, ^ p≤0.025, $ p≤0.05.

MSC multipotency

Multipotency assays for adipogenic and osteogenic differentiation of the MSCs were performed in a 12-wells plate with adipogenic or osteogenic medium, and chondrogenic differentiation was achieved in pellet cultures with chondrogenic medium. Adipogenic diffentiation was confirmed by an Oil-Red-O staining, ALP staining with fuchsin was positive, indicating osteogenic differentiation, and chondrogenesis was shown by deposition of type II collagen in the extracellular matrix (Figure 5).

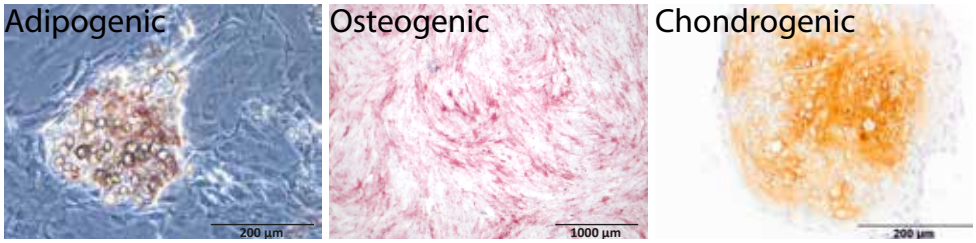


Figure 5 – Multipotency assays were performed to ascertain the tri-potency of the human MSCs used in this study. Red staining by Oil-Red-O indicated lipid formation after adipogenic differentiation of MSCs. Alkaline phosphatase was stained in pink by Fuchsin, indicating that osteogenic differentiation was successful. Chondrogenesis was confirmed by type II collagen deposition in pellets.

The effect of co-culture ratio of NP and MSCs

Co-cultures with different NP:MSC ratios were compared to find an optimal cell ratio for matrix production. Co-culture pellets contained $0.25*10^6$ cells in total. As controls for the added value of MSCs, pellets with the same number of NP cells without MSCs were taken along, and thus contained a variable total number of cells. No Safranin-O staining was visible in any of the co-culture pellets, but it was present in pellets of only NP cells (Figure 7). A faint immunostaining for type II collagen was seen in all pellets, while type II collagen was absent in pellets containing only MSCs. Type I collagen was present in all pellets, albeit in different amounts. This did not seem to be related to NP cell number or NP:MSC ratio. However, type I collagen staining did seem more intense in NP cells only pellets.

GAG deposition was higher in pellets with $0.25*10^6$ NP cells alone than in co-cultures, NP cells alone controls, and MSCs alone ($p \leq 0.001$, Figure 7A). DNA content was higher in pellets of $0.25*10^6$ NP cells than in co-culture pellets with $0.125*10^6$ NP cells (50:50 NP:MSC) and $0.025*10^6$ NP cells (10:90 NP:MSC), and than all other NP alone control pellets ($p \leq 0.007$, Figure 7B). Pellets of $0.25*10^6$ NP cells alone produced more GAG/DNA than all other pellets ($p \leq 0.003$, Figure 7C). Total GAG production including GAGs released into the medium was higher in pellets with $0.25*10^6$ NP cells alone than in all other pellets, except 0.125 NP cells co-cultures (50:50 NP:MSC) and MSC only pellets ($p \leq 0.025$, Figure 7D) and higher in all co-culture pellets compared to their NP alone controls ($p < 0.001$). Also the incorporation efficiency was highest in NP cells alone compared to all other pellets except $0.125*10^6$ NP cells alone (Figure 7E). In the presence of MSCs, no differences were found between any of the NP:MSC ratios, except for a slightly higher total GAG production in the $0.125*10^6$ NP (50:50 NP:MSC ratio) cell pellets ($p \leq 0.021$). Therefore, for follow up experiments, we chose to use the 50:50 NP:MSC ratio, which has been used frequently in the literature as well[27-31, 33].

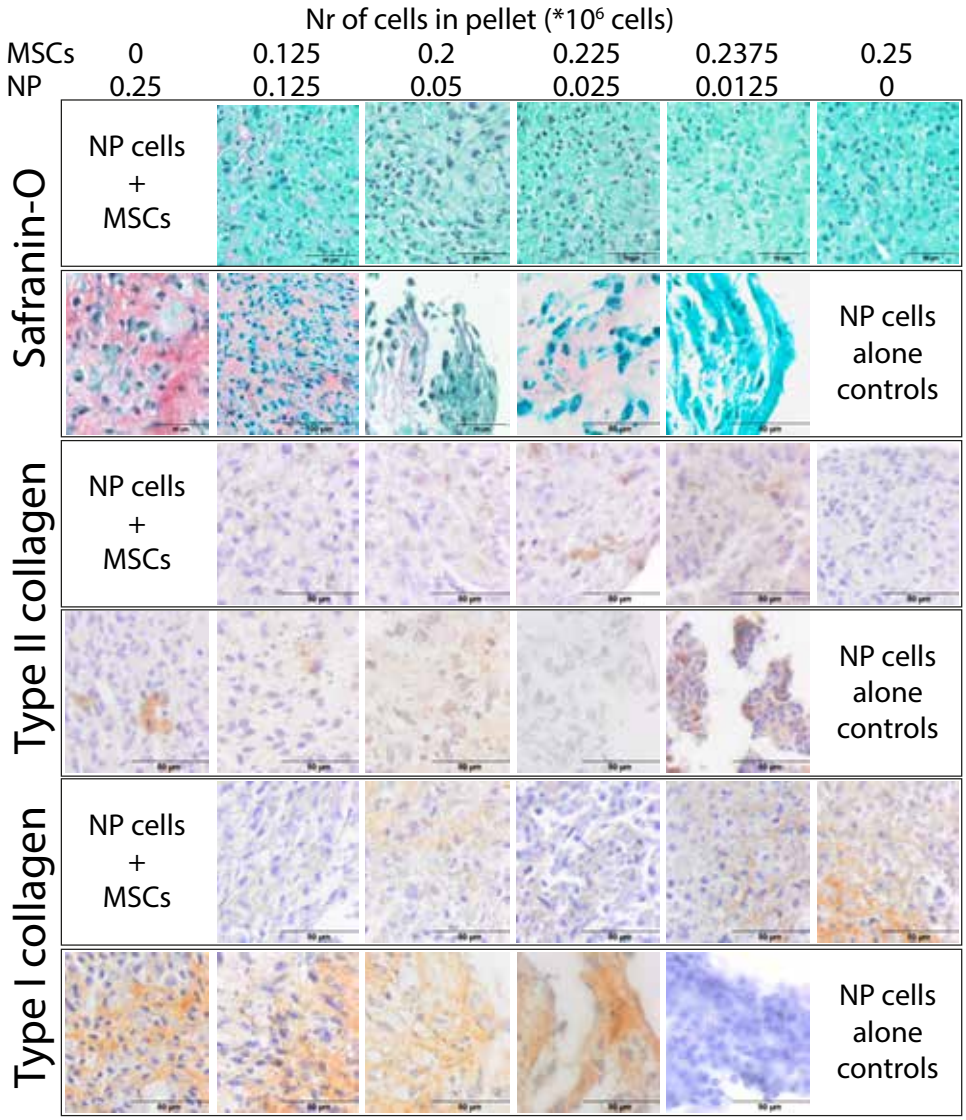


Figure 6 – Human degenerated nucleus pulposus cells were co-cultured with human MSCs in pellets in chondrogenic medium without the addition of growth factors. Pellets contained a total number of 0.25*10^6 cells, with a varying ratio of NP:MSC cells. Pellets with 0.25*10^6 NP cells means NP cells alone, 0 NP cells indicates MSCs alone. Histology shows Safranin-O staining in pellets of NP cells alone (lower panel), but not in co-cultured NP cells and MSCs in pellets (upper pannel). Limited type II collagen was present in the pellets containing NP cells (lower panel) and was entirely absent from MSCs alone pellets. Type I collagen was variably present in all NP:MSC pellets without any apparent relation to NP cell amount in the pellets (upper panel), but staining was more intense in NP cells alone controls (lower panel).

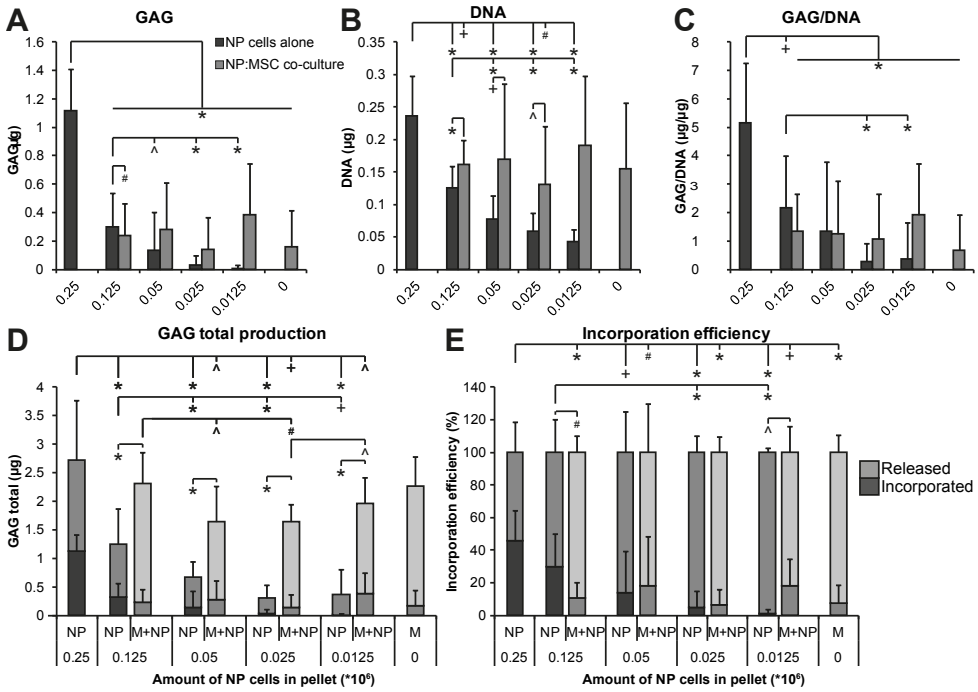


Figure 7 – Human degenerated nucleus pulposus cells (3 donors, n=3 per donor per condition) were co-cultured with human MSCs from a single donor in pellets in chondrogenic medium without the addition of growth factors. Pellets contained a total number of $0.25*10^6$ cells, with a varying ratio of NP:MSC cells. Pellets with $0.25*10^6$ NP cells means NP cells alone, 0 NP cells indicates MSCs alone. **A**: The highest GAG content was measured in pellets with $0.25*10^6$ NP cells alone. **B**: A small difference in DNA content was measured in pellets of $0.25*10^6$ NP cells compared to 50:50 NP:MSC pellets with $0.125*10^6$ NP cells. **C**: Pellets of $0.25*10^6$ NP cells alone contained more GAG/DNA than all other pellets. **D**: Small differences were measured in total GAG production including GAGs released into the medium. **E**: In pellets with $0.25*10^6$ NP cells alone incorporation efficiency was higher than any of the other pellets. Significant differences are given as follows: * p≤0.001, + p ≤0.005, ^ p≤0.025, # p≤0.01.

BMPs with NP cells co-cultured with MSCs

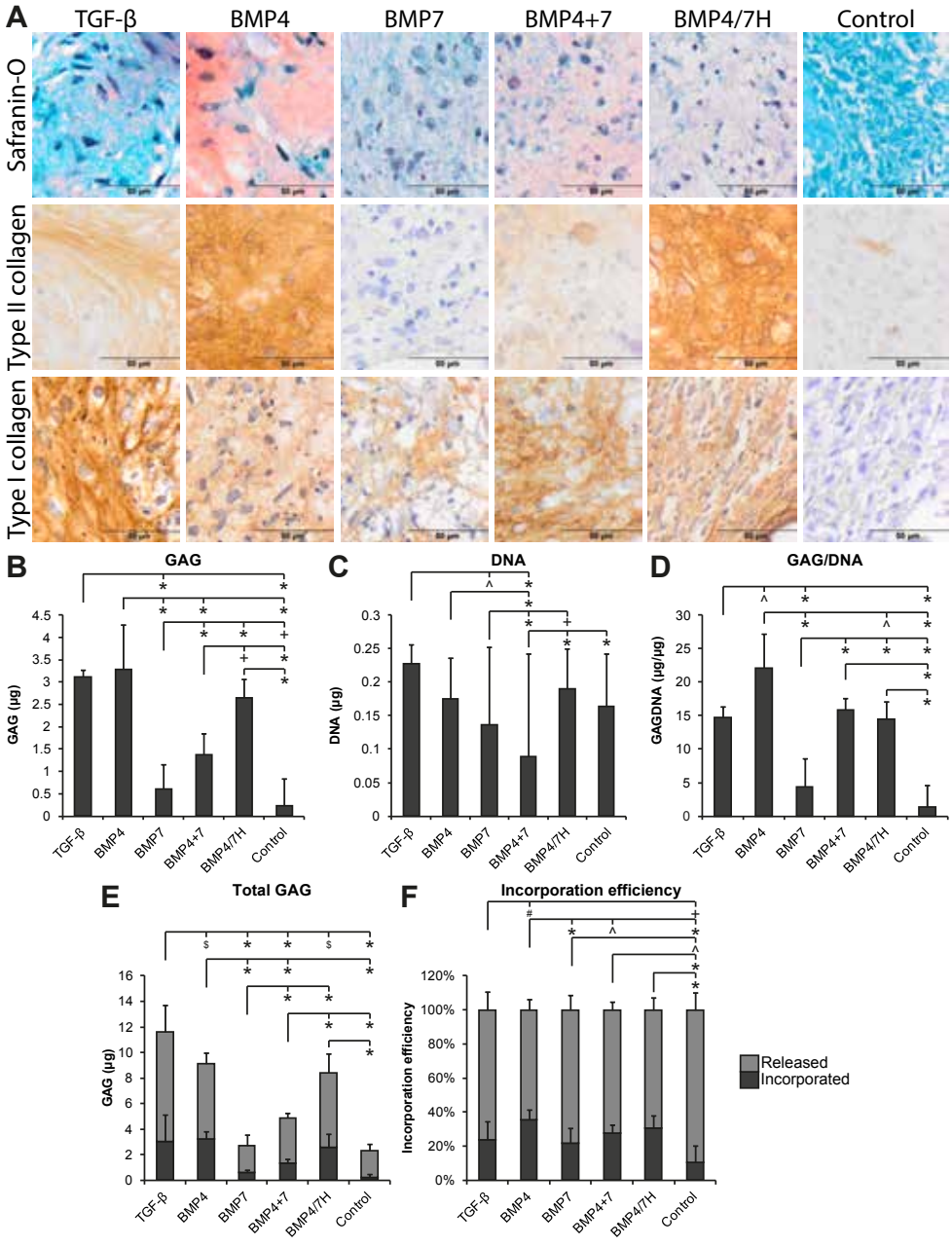
NP cells co-cultured with MSCs in a 50:50 ratio in pellets in chondrogenic medium alone and in the presence of BMP4+7 expressed only limited type II collagen. In the presence of 0.4 nM TGF-β, BMP4, or BMP7, the combination of homodimers and the heterodimer the co-culture pellets show positive staining for various matrix components in varying intensities, such as proteoglycans, indicated by Safranin-O staining, and type II and type I collagen, as indicated by immunohistochemistry (Figure 8A). The most intense Safranin-O staining was observed in pellets cultured with BMP4 followed by the combination of the homodimers of BMP4 and BMP7, while the most intense type II collagen staining was seen in the presence of BMP4 and heterodimer BMP4/7H. Pellets cultured with TGF-β showed the most intense type I collagen staining, with only limited Safranin-O and type II collagen staining.

Figure 8 – Matrix production of 50:50 NP:MSC pellet co-cultures in the presence of 0.4 nM BMPs, combination of homodimers, or heterodimer (H) was compared to 0.4 nM TGF-β or control (basal chondrogenic medium without growth factor supplementation). **A**: Safranin-O staining was present in all growth factor treated MSC+NP pellets but not in controls. The most intense staining was seen in pellets cultured with BMP4. Type II collagen was present in all pellets except for controls. Most intense staining was seen in BMP4 and BMP4/7H treated pellets. The most intense type I collagen staining was visible in pellets cultured with TGF-β; it was also present when cultured with the various BMPs. Only control pellets were negative for type I collagen. **B**: GAG content was highest for BMP4, TGF-β, and BMP4/7H. **C**: DNA content was higher for pellets cultured with TGF-β compared to BMP7 and BMP4+7. **D**: GAG corrected for DNA was higher for all growth factors compared to control. E: Total GAG production, determined as GAGs in the tissue as well as released into the medium, was higher for TGF-β, BMP4, BMP4+7, and BMP4/7H compared to BMP7 and control. F: Incorporation efficiency, calculated as the percentage of GAG incorporated into the tissue of the total GAG, was highest for BMP4. Significant differences are given as follows: * $p \leq 0.001$, + $p \leq 0.005$, # $p \leq 0.01$, ^ $p \leq 0.025$.

Co-culture of NP:MSC in a 50:50 ratio in the presence of growth factors resulted in significantly higher matrix deposition compared to basal chondrogenic medium. GAG content was highest in pellets cultured with BMP4 compared to BMP7, BMP4+7, and basal chondrogenic medium ($p<0.001$, Figure 8B). DNA content was higher for pellets cultured with TGF-β compared to BMP7 and BMP4+7 ($p \leq 0.018$, Figure 8C). GAG corrected for DNA was higher in the presence of all growth factors compared to basal chondrogenic medium ($p<0.001$) and highest for BMP4 compared to TGF-β, BMP7, BMP4/7H, and basal chondrogenic medium ($p \leq 0.024$, Figure 8D). More GAG/DNA was also measured for pellets cultured with TGF-β, BMP4+7 and BMP4/7H compared to BMP7 ($p<0.001$). Total GAG production was higher for TGF-β, BMP4, BMP4+7, and BMP4/7H compared to BMP7 and basal chondrogenic medium ($p<0.001$), and highest for TGF-β compared to BMP4, BMP7, BMP4+7, BMP4/7H and basal chondrogenic medium ($p \leq 0.033$, Figure 8E). GAG incorporation efficiency was highest for BMP4 compared to TGF-β, BMP7, and BMP4+7 ($p \leq 0.012$). GAG, DNA, GAG/DNA, and total GAG were higher for the heterodimer BMP4/7H compared to the combination of homodimers.

Discussion

In the current study it was shown that all BMPs, their homodimer combinations and heterodimers induced proteoglycan formation and deposition of proteoglycans, type I and type II collagen by human degenerated NP cells. In particular, BMP4, BMP4+7, BMP6 and the heterodimers BMP2/7H and BMP4/7H showed high potential for matrix deposition by NP cells based on the higher amounts of proteoglycans deposited, also corrected for cell number, compared to BMP2 and its combinations, BMP7, or TGF-β. Furthermore, GAG incorporation was most efficient for NP cell monoculture in transwells treated with BMP4+7, BMP2/7H, and BMP4/7H. Altogether, these findings indicate that heterodimers of BMPs can display enhanced regenerative effects compared to their respective combination of homodimers, but the effects were frequently not different from their most potent homodimers alone. Notably, coaddition of MSCs to NP cells in


A
TGF-β
BMP4
BMP7
BMP4+7
BMP4/7H
Control
Safranin-O
Type II collagen
Type I collagen
B
GAG
GAG (µg)
C
DNA
DNA (µg)
D
GAG/DNA
GAGDNA (µg/µg)
E
Total GAG
GAG (µg)
F
Incorporation efficiency
Incorporation efficiency
Released
Incorporated

the absence of growth factors did not augment matrix deposition, although total GAG production was increased. Co-cultures of NP and MSCs to which BMPs were added showed that most regeneration was accomplished with BMP4.

The present study shows that of the BMPS tested here, BMP4 is a more suitable candidate for NP regeneration, although BMP2 and BMP7 have been thus far the usual candidates employed in regenerative treatment strategies of the degenerated disc[20-24]. Our results seem to contrast with a previous study in which bovine NP cells in monolayer were transduced with vectors for various BMPs (BMP2, -3, -4, -5, -7, -8, -10, -11, -12, -13, -14, and -15 and SOX9)[37]. BMP2 and 7 were most effective in inducing GAG production, followed by BMP4. BMP4 and -14 induced the highest type II collagen content. However, while the physiology of healthy bovine NP cells does not necessarily resemble human NP cells from degenerated discs[38], it also remains to be determined whether the concentrations of the various BMPs produced by the transfected bovine NP cells were equal and hence can provide solid evidence on the differences in biologic potency of the BMPs studied. the difference in effects may be explained by the notion that BMP2 and BMP7 operate via different pathways, where BMP7 might be more potent than BMP2 in human degenerated NP cells[39]. Similar differences might be true for BMP4 and BMP7, as has been shown in human MSCs[40]. In the present study BMP4 was revealed to have an optimal effect on matrix production by NP cells already around 0.4 to 2 nM (equivalent to 5.6 and 28 ng/ml), whereas BMP7 did not appear to reach its maximum effect yet at 4 nM (equivalent to 63.2 ng/ml). Altogether these findings indicate that BMP4 is a better candidate for future NP regenerative strategies.

Notably, while BMP4/7H compared to the homodimer combination BMP4+7 did not exhibit higher regenerative effects on degenerated NP cells in monoculture, it exhibited an additive matrix anabolic and proliferative effect on NP cells co-cultured with MSCs, as shown previously for bone regeneration[14]. This discrepancy might be attributed to cell type-specific signaling, although the mechanism behind possible enhanced potency of heterodimers is not clear. The potency of the BMP2/7H heterodimer was confirmed in bone formation in CHO cells and rats[15], and in mini-pigs[17], although here the combination of separate homodimers was not included. In cartilaginous tissues, the effects of heterodimers have been studied to a limited extent only[18, 41] also without comparison to (a combination of) the homodimers. As such, the present study provides clear evidence that heterodimers can be more regenerative than the respective homodimer combinations, both in culture with NP cells alone and co-cultures of NP cells with MSCs. The increased matrix deposition was mainly attributed to an increase of cell number, suggesting that differentiation per se was not affected. Heterodimers were shown to have different receptor binding properties than homodimers, and thereby enhance Smad signaling more than homodimer receptor binding[42-44] and might have reduced affinity for antagonists compared to homodimers[44-45]. The stability of the BMP-

to-receptor-binding seems to be higher for heterodimers compared to homodimers, which may provide another explanation[42]. Moreover, in Drosophila, enhanced receptor binding was found to give differential effects that could not be explained by altered down-stream signaling alone[46]. In co-cultures of NP cells with MSCs, however, BMP4 still induced more matrix formation than the heterodimer BMP4/7H.

In the current study it seemed that MSCs did not have an additive effect on the tissue regeneration potential of NP cells in the absence of growth factors, although total GAG production was higher. MSCs are currently being used to prevent NP degeneration or enhance NP regeneration *in vitro*[27-31] and in animal models[29, 47] and have been shown to be safe in clinical trials reported[48] and ongoing (for example, NCT01290367). In line with the current findings, MSCs did not augment matrix production when co-cultured with canine NP cells compared to NP cells alone, even in the presence of BMP2[33]. The authors hypothesize an important role for oxygen levels and species differences between canine and human cells for the discrepancy with previous observations, where increased matrix production of human degenerated NP cells in the presence of MSCs was observed[28-30]. Even more so, MSC donor variability may play an important role in MSC-based regenerative treatment strategies. Given the high amounts of proteoglycans released into the medium in co-cultures, it is possible these might be retained in the confined IVD *in vivo* as small fragments have been found in IVD tissue[49]. Nonetheless, BMP4 seemed to have a stimulatory effect on extracellular matrix production on NP:MSC co-cultures that was comparable to NP cells alone. As BMP proteins are expensive and limited by short half-lives[50], MSCs could be employed as carriers for BMPs. Possibly, BMP production by transfected MSCs can induce regeneration by NP cells and implantation of these MSCs might be a very interesting treatment strategy[51-56].

Conclusion

Although BMP2 and BMP7 have been clinically approved, they are not the most potent inducers of regeneration by human degenerated nucleus pulposus cells. Instead, BMP4 emerges from this study as the most potent inducer of regeneration by NP cells and NP cells combined with MSCs. Heterodimers were found to be more potent than the combination of homodimers, but showed a clear cell- or culture-dependency.

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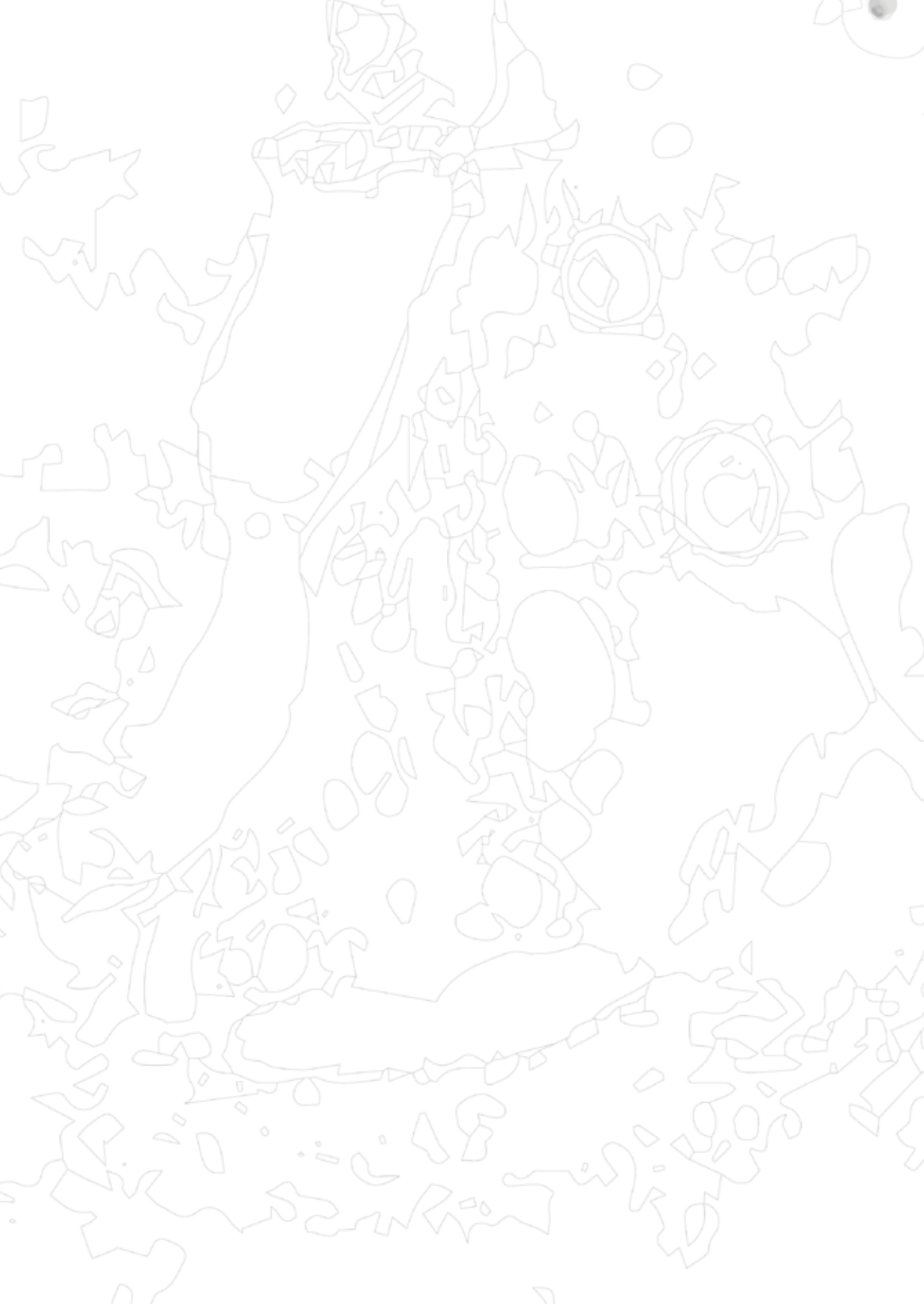

Summary and general discussion

Chapter 6

Summary

In this thesis, we aimed to understand local and external, chemical, and physical cues in nucleus pulposus (NP) regeneration as research tools and therapeutic applications in regeneration strategies.

In *chapter 2*, NP cells were exposed to hyperosmotic culture medium during redifferentiation in transwell filter cultures. Gene expression of type II collagen and especially aggrecan increased with increasing osmolality using NaCl or sucrose, but not with urea. However, no differences between osmolalities were measured in matrix production after 28 days in the presence of NaCl, while this could not be explained by increased expression of matrix degrading enzymes or decreased expression of collagen cross-linking enzymes. Both urea and sucrose inhibited matrix formation, regardless of osmolality. When NP cells were cultured in hydrogels in *chapter 3* to assess whether the effect of osmolality on NP cells was influenced by culture in hydrogels, exposure to hyperosmotic differentiation medium did not influence matrix production either. A higher osmolality during isolation and expansion, however, did augment proteoglycan production, both absolute and corrected for DNA, indicating alteration of the NP cell phenotype prior to redifferentiation culture.

A common addition to culture media for expansion or differentiation is fetal bovine serum (FBS). In *chapter 3*, NP cells were cultured in hydrogels in medium containing FBS or in a serum-free (SF), chemically defined differentiation medium. In agarose and gelMA hydrogels, matrix production and proliferation were higher in the presence of FBS compared to SF differentiation medium. In type II collagen hydrogels, matrix production was also higher with FBS, but the incorporation efficiency was higher in SF differentiation medium.

Hydrogels are important tools in NP tissue regeneration as cell carriers. However, no reference hydrogel was as yet identified to compare novel hydrogels against. Six commonly used hydrogels, alginate, agarose, gelatin methacryloyl (gelMA), fibrin, hyaluronic acid – poly(ethylene glycol) (HA-PEG), and type II collagen, were compared for regeneration by NP cells in *chapter 3*. Although all hydrogels supported NP cell viability, in terms of GAG production agarose performed best, followed by type II collagen hydrogels. Because of the postulated role of hydrogel stiffness, the mechanism of NP cell response to polymer concentration and the role of adhesion formation was further investigated in *chapter 4*. NP cells in gelMA, which is composed of gelatin derived from type I collagen and contains integrin binding sites, responded to a decrease in polymer concentration with an increase in GAG production, but not cells in alginate or agarose hydrogels. With confocal microscopy, elongated focal adhesions were observed connected to actin fibers in NP cells cultured in 2D or in type II collagen hydrogels. Immature focal adhesions were found in NP cells in agarose hydrogels. In gelMA hydrogels, both morphologies were found. When a focal adhesion kinase inhibitor was added to NP cells cultured in different

hydrogels, it only affected NP cells in collagen-based hydrogels including gelMA and type II collagen. Although focal adhesions were detected in agarose hydrogels as well, cells in agarose did not respond to changes in inhibitor concentration.

Although bone morphogenetic protein (BMP)2 and BMP7 are most frequently used for stimulating NP regeneration, in *chapter 5* it is shown that they may not be the best candidates for human NP regeneration. BMP4, the heterodimer BMP4/7H, and the combination of BMP4+7, but also BMP6 and the heterodimer BMP2/6H, are more potent inducers of matrix production by NP cells. In a dose response curve, the superior potency of BMP4 was confirmed compared to BMP7 and also in co-cultures of NP cells with mesenchymal stromal cells or mesenchymal stem cells (MSCs), BMP4 induced the highest GAG deposition.

General discussion

Regeneration of the intervertebral disc

Regenerative medicine is a potentially powerful tool to achieve tissue repair. For regeneration of the IVD, and in particular the NP, several strategies can be employed. Together, cell therapy, biomaterials, and growth factors have the potential to achieve regeneration of the IVD[1]. Optimization of the treatment strategy needs to be conducted in several *in vitro* systems before safety and efficacy can be proven in pre-clinical animal studies. When the treatment still holds promise, clinical trials can commence, which may lead to eventual implementation of the therapy in clinical practice. Several of the challenges for NP regeneration are addressed below.

Disc milieu and influence on nucleus pulposus cells

As the IVD is the largest avascular structure in the body, oxygen and nutrient transport relies mainly on diffusion[2-3]. Especially the NP cells in the middle of the disc experience hypoxia and low nutrient concentrations[4]. This combination of hypoxia, low glucose, and low pH levels influences cell viability, phenotype, metabolism, and proteoglycan production[5-8]. During degeneration, oxygen and nutrient levels decrease even further, whereas acidity of the disc increases due to accumulation of waste products like lactic acid[3,9]. The high proteoglycan content in healthy IVDs causes high hydrostatic and osmotic pressures between 450 and 550 mOsm/kg in the tissue[10-11], and even though it decreases with degeneration, it still remains high compared to other tissues[12]. Moreover, the NP is constantly subjected to load, even at rest[13]. Upon isolation, the NP cells are suddenly exposed to high levels of oxygen, pH, and, with the addition of serum, nutrients, but osmotic values decrease. Furthermore, loading is not present in expansion culture or in most regeneration cultures. Altogether, these factors can influence the NP cell phenotype[5]. Therefore, it is important to study how these factors affect the human NP cell phenotype and regenerative capacity for successful therapeutic strategies.

The role of osmolality

NP cell volume has been shown to increase by up to 20% by a sudden drop in osmolality[14] or decrease with high osmolality, depending on the osmolyte[15], from which the cell's mechanobiology and downstream signaling might not fully recover as also shown for chondrocytes[15-17]. *In vitro* cultures of NP cells are often not carried out at osmolality similar to the *in vivo* situation, which might influence the cell response to the experimental conditions. Indeed a limited number of studies, including our own in

chapter 2, show that expression of matrix genes such as type II collagen and aggrecan are upregulated in response to increased medium osmolality during regeneration cultures[7,18-19]. Despite this increase at the gene expression level, several studies, including *chapters 2 and 3* in this thesis, fail to show an increase in matrix production at the protein level[7,20-21]. A discrepancy between messenger RNA (mRNA) levels and protein production frequently occurs, with sometimes large effects on mRNA levels being translated into smaller or absent effects on protein production[22-24]. This discrepancy can be based on mRNA processing, nuclear export and protein degradation, with in mammalian cells a correlation less than 0.5 between mRNA and protein levels[25]. This further emphasizes the limitations of *in vitro* studies of employing cells alone, even in a 3D-envrionment, and the importance of quantitative and qualitative protein analysis in translational research.

Expansion of chondrogenic cells under high osmolality does seem to enhance regeneration at the protein level, both for NP cells (*chapter 3*) and chondrocytes[26-27], independent of the osmolality during actual regeneration. Apparently, keeping osmolality close to the native value is beneficial for chondrocyte-like cells[26-27] and facilitates maintenance of their chondrogenic phenotype, as described before for bovine NP explants[18]. As the native osmolality of cartilage is high, between 350 and 450 mOsm/kg depending on location, very similar data have been obtained for chondrocytes[17,21,26-30], which is not surprising considering that the environments of NP cells and chondrocytes are similar in many ways. Strikingly, NP cells expanded at high osmolality showed a more regenerative phenotype even upon regeneration at lower osmolality, suggesting epigenetic effects of expansion under high osmolality. Osmotic stress has been proposed to also underlie epigenetic regulation in other cell types. In fibroblasts, hypertonic stress can regulate deacetylation of histones that switch genes on or off and thereby possibly promote apoptosis[31], whereas hypotonic stress might activate transcription inhibitors in order to protect the cell's survival mechanisms[32]. Apart from the role of miRNA expression in IVD degeneration[33-35], epigenetic mechanisms have not yet been widely studied for the IVD[36]. Further support that a similar mechanism is at play in NP cells, is that cells from herniated disc tissue were not capable of adequate regeneration when compared to NP cells from normal NP tissue[37]. Herniation leads to NP tissue swelling by unlimited absorption of water from the body and hence the herniated NP cells are exposed to hypoosmotic shock. Interestingly, a direct response consists of the production of inflammatory factors by these NP cells[38-39], such as interleukin (IL)-6 and prostaglandin E_2 (PGE_2) with clear matrix catabolic effects. This phenomenon was also observed in other cells such as human intestinal epithelial cells, where especially hypoosmotic stress induced production of inflammatory factors IL-6 and IL-8[40]. In mouse fibroblasts and NP cells, similar effects were noted in response to hyperosmotic shock[41-42]. No catabolic response in terms of enhanced matrix degradation was noted in our studies, but this could have been due to either the origin of the NP cells, i.e. from degenerated and hence intrinsically inflamed discs, or because of their dedifferentiation during expansion.

How cells respond to osmolality is still partially unclear. It has been postulated that

rather than responding to static osmolality as such, cells respond to osmolality changes. TonEBP(tonicity enhancer-binding protein) has been shown to be upregulated directly after exposure to hyperosmotic medium and is involved in cell response to osmolality, such as cell volume regulation and protein expression[30,43-47]. β1,3-Glucuronosyltransferase-I (GlcAT-I) is a regulator of GAG production and was shown to be regulated by TonEBP[48]. Changes in osmolality in cartilaginous tissues typically are generated by tissue loading, which creates hydrostatic pressure and an efflux of water, thereby temporarily increasing the osmotic pressure. During the day, the osmolality of the NP changes due to loading and unloading rather than being static[17,49]. Hydrostatic pressure of 1 MPa applied for 20 seconds increased GAG production in the NP, although it decreased with pressures above 5 MPa, indicating an optimum for this effect[50]. Vice versa, osmolality influences the response of NP cells to hydrostatic pressure[19]. These responses may be mediated by the transient receptor potential vanilloid 4 (TRPV4) channel, which has been shown to be both an osmotic and mechanical sensor in chondrocytes[17,51-52]. A change in osmolality induces a temporary increase in intracellular Ca^{2+} influx through this channel, resulting in an increase in chondrogenic gene expression[53]. As intermittent compressive loading has been shown to enhance chondrogenesis by stimulating transforming growth factor beta (TGF-β) expression in bovine cartilage[54] and in leporine bone marrow-derived MSCs in agarose culture[55], endogenously produced TGF-β may be the factor responsible for the enhanced chondrogenesis found upon temporary increases in osmolality. Noteworthy, these studies did not explore the effect of loading on other members of the TGF-β superfamily. For NP cell-mediated regeneration, these mechanisms have not been studied extensively, but osmotic and mechanical loading are closely related and essential for NP cell functioning, they will likely involve similar processes[56]. In addition, biomechanical loading is also suggested to have additive effects on the response to growth factors such as TGF-β[57]. However, in this thesis we have not separately addressed the biomechanical component of the NP cell milieu.

When studying the role of osmolality *in vitro*, it should be borne in mind that the osmolyte that is used may have effects unrelated to osmolality. Apart from effects on parameters such as cell viability (*chapter 2*), also matrix production may be regulated independently of osmolality. The frequently used osmolyte NaCl is known to also exert effects through Na/K pumps. The activity of membrane transport pathways, like Na/K pumps and Na/K/2Cl transporters, is decreased under high hydrostatic pressure in chondrocytes[28]. This means that the concentrations of Na and K inside the cell cannot be adjusted during osmolality changes in the tissue when under hydrostatic pressure. However, in bovine chondrocytes no differences were found whether NaCl or two osmolytes that cannot enter the cell were used to increase osmolality: sucrose or N-methyl-d-glucamine chloride (NMDG-Cl), a bulky alternative to NaCl that cannot pass most known channels[26,58], suggesting that the enhanced chondrogenic capacity was purely osmolality-related. However, as the effects of other osmolytes, including sucrose, in our NP culture system differed from those of NaCl, at least part of the response may have been mediated through these channels.

Although NP cells seem to respond to high osmolality during expansion, it is not known to what extent this would also be the case for other cells. In particular MSCs, considered as possible therapeutics in IVD degeneration, may respond entirely differently. Indeed, exposure to high osmolality during differentiation decreased proliferation and ECM gene expression in rat disc-derived MSCs and human adipose-derived MSCs[59-61], although this may also be partially dependent on the culture system used[61]. Whether this response is different during, and could be altered by prior expansion of MSCs at high osmolality, may be worthwhile investigating. Indeed, osmolyte-dependent osmolarity was shown to affect human adipose MSC capability for angiogenesis and increased osmolality increased their chondrogenic capacity[62]. Altogether, for redifferentiation of NP cells, adjusting the osmolality does not seem to be needed, but it is highly important that disc osmolality levels are maintained during isolation and expansion. Moreover, any other cell types considered for regeneration should be pre-conditioned *in vitro* to prepare them for the harsh environment they are about to face.

Role of cell-matrix interaction

Both for *in vivo* application and *in vitro* studies, the native environment should be mimicked as much as possible. Most cell types in 3D cultures show phenotypes that are closer to their native state than 2D cell cultures on stiff glass or plastics[63]. The interaction of chondrocyte-like cells such as NP cells with their ECM or the biomaterial in which they are cultured provides important information to the cells[64-70]. As the ECM of the NP exists mostly of collagen, elastin, and proteoglycans[71], interactions mediated by integrins and the CD44 receptor are the most important ones for NP cells[64-67,72]. The signals relayed through these interactions have an effect on the cell phenotype. Most of the research on the cell-matrix interaction has been done in 2D, studying migration and differentiation behavior of various cell types[73-75]. However, *in vivo*, these interactions occur in 3 dimensions and studies in 3D culture systems would provide a better understanding of these processes *in vivo*[67,69,76]. Moreover, this information can be used towards designing therapeutic approaches for regeneration[77]. Hydrogels are optimal candidates in this respect. According to the definition of a hydrogel: a network of polymers that imbibes and retains water[78], this exactly characterizes the healthy NP. It displays properties of a fluid as well as properties of a solid[79]. Although a lot of research for NP regeneration is performed with hydrogels[77], no reference hydrogel has been defined that would facilitate effective regeneration and provide a standard for comparison of newly developed hydrogels. In *chapter 3* we aimed to define a first standard and found that NP cells in agarose hydrogels showed the highest regeneration capability, closely followed by type II collagen hydrogels.

Hydrogel properties, such as polymer characteristics and concentration, porosity, cross-link density, and fixed charge density, may affect NP-mediated regeneration in many

ways. One important aspect may, in addition, be their biomechanical properties. Matrix stiffness has been shown to influence the cell fate differentiation path[73] and could also influence NP cells. Indeed focal adhesions were found to be present in NP cells, also in 3D environments, as we showed in *chapter 4* and NP cells were shown to express a wide and partially cell specific pattern of integrins[65]. Focal adhesions and integrin binding sites in hydrogels influence the behavior of immature NP cells with respect to their phenotype/ differentiation stage and GAG production (*chapter 4*)[65,69-70], as was shown previously for chondrocyte differentiation[80-83]. In *chapter 4*, inhibition of focal adhesion formation in type I collagen-based gelatin hydrogels increased chondrogenesis, but in type II collagen hydrogels chondrogenesis was decreased and adipogenesis was increased. In carbohydrate-based hydrogels, no effects were found, despite the presence of focal adhesions. Like in MSCs, in NP cells, different integrin binding to different collagens may induce different differentiation pathways[84-85]. Although carbohydrate-based hydrogels may not affect NP cell behavior through their biomechanical properties, this does not exclude a regenerative response. For example, in *chapter 3* in this thesis, we found the highest matrix deposition in agarose hydrogels. In this case, the absence of binding motifs may have induced a rounded cell shape, which in turn has been shown to enhance chondrogenic differentiation[86]. Moreover, although this was not explored in the current thesis, after deposition of ECM by the regenerating cells, stiffness sensing in carbohydrate-based gels may still occur[17,87].

Cell therapy for IVD regeneration

Implantation of cells can aid in regeneration of the NP, as the resident cells are few in number in IVDs (about 5000 cells/mm3)2. In a clinical trial, autologous cells isolated from human herniated disc material have been implanted after expansion and showed improvement of the affected IVD, both in MRI evaluation and reduced pain perception88. However, cells from herniated tissue showed reduced regeneration capacity compared to control NP cells37, while harvesting non-herniated NP cells can cause (further) degeneration of the IVD, as it induces trauma to both the annulus fibrosus (AF) and NP tissue89. Although allogeneic NP cells could be considered, the low NP cell number also poses a challenge as these would also need to be expanded in the laboratory to obtain sufficient numbers for treatment90-91 and optimal expansion conditions for NP cells have not been defined yet.

Currently, MSCs are being investigated as an alternative cell source for IVD regeneration. Allogeneic MSCs are increasingly available and more evidence emerges indicating they are safe to use clinically and intradiscal application results in improved quality of life[92-99]. Whether this is due to MSC-based regeneration, is unclear. *In vitro*, several studies have seen an anabolic effect of human MSCs[100-104], however, in *chapter 5* we failed to reproduce this effect of MSCs on matrix deposition in co-culture, although matrix production as

such was clearly enhanced. A similar lack of tissue regeneration was also seen in co-cultures of canine NP cells with MSCs *in vitro* as well as *in vivo* upon transplantation of MSCs[105-106]. It is hard to say what causes these differences, as the experiments mentioned earlier were conducted under similar conditions as those described in *chapter 5*. The fact that MSC populations are heterogeneous and vary greatly between donors, isolation protocols, and separate expansion events (even within the same donor) might account for (at least part of) the differences observed[107]. To overcome donor variability and ensure clinical efficacy, the appropriate donor(s) need to be identified and expanded to generate a reliable MSC-based off the shelf product. To this end, *in vitro* predictive parameters are required that coincide with *in vivo* efficacy, a challenge faced in many areas of research[108]. Application of MSCs together with BMPs seems feasible; co-cultures of MSCs and NP cells in the presence of BMPs in *chapter 5* show that BMP4 greatly enhances regeneration in these co-cultures.

Regeneration by growth factors

Growth factors can achieve anabolic responses by NP cells and thereby facilitate biologic repair[109-110]. Growth factors are polypeptides that have biologic functions throughout the body, where they direct cell proliferation, differentiation, matrix production or breakdown, or production of other growth factors[111]. Many growth factors are involved in chondrogenic differentiation and have been extensively studied for the IVD. The TGF-β superfamily contains many members and has important roles in chondrogenesis and maintenance of chondrogenic tissues[112]. Well-known members of this superfamily are the bone morphogenetic proteins (BMPs), which are particularly interesting in chondrogenesis. Several BMPs have been shown to be promising for disc repair[113], although research is mainly focused on BMP2 and BMP7 because they have been approved for clinical use[114]. Few direct comparisons have been carried out[113,115]. BMPs are usually produced as homodimers, but heterodimers were suggested to be more potent than their homodimers in bone[116-119], cartilage[117], and for chondrogenic differentiation of MSCs[120], possibly due to their differential receptor binding and reduced affinity for antagonists[121-122]. Heterodimers have been used for NP regeneration[123-124], but very few compared the heterodimers to homodimers[125]. Since the first study in bone in 1995[116], few direct comparisons have been made between the heterodimers and their combined homodimers and only in bone[119,126-127]. These scientific gaps were explored in *chapter 5*. We found that BMP2, BMP4, BMP6, and BMP7 have varying capacity for induction of GAG production by NP cells in a high-density culture on transwell filters, and BMP4 the highest. Heterodimers in some, but not all, instances seemed to perform better than the combination of the involved homodimers, dependent on whether NP cells or NP cells in combination with MSCs were studied, but were never more potent than the most potent homodimer of that combination on its own.

Although further research is warranted, it seems already that BMP2 and BMP7 are not among the most potent growth factors for IVD regeneration. Many more BMPs exist with matrix anabolic effects that have not been directly compared for NP regeneration in particular[113,115]. Moreover, BMPs operate via an extensive and complicated pathway[128] and different BMPs bind to different BMP-receptor combinations, which in turn initiate different intracellular signaling cascades and elicit divergent responses[129] and thereby different potency for NP regeneration[130]. Controversy exists around the clinical use of growth factors. BMP2, BMP7, and BMP14 or GDF5 for fusion of the vertebrae and other off-label applications[131] were shown to entail the risk of adverse events. This is most likely due to the high concentrations that are generally employed[132-133] in view of the rapid clearance to the circulation and the general instability of growth factors. Also, intradiscal application of growth factors of the BMP-family is challenged by dose-dependent effects of the growth factors determining cell fate, proliferation, and matrix production. For example, 1 µg/ml BMP2 in the medium induced ossification of the AF in an explant culture[134] and injection of several dosages of BMP7 into the degenerated NPs of dogs induced extradiscal bone formation without appreciable IVD regeneration[132].

To overcome problems associated with overdosing with a bolus injection of a free growth factor, strategies for different means of delivery are being developed[109-110,135]. To this end, biomaterials are gaining popularity as they can be used to tailor local delivery[136]. However, BMP2/7H covalently linked to a hydrogel did induce regeneration *in vitro*[133] but failed to induce regeneration *in vivo* in a goat model[125]. Dosage and short half-life problems of growth factor-based treatments can also be overcome by gene therapy[135,137-139], as growth factors would be continuously produced by live cells[135]. Safety concerns have limited the use of viral vectors for clinical applications so far[140], although the immune privileged status of the IVD may provide a safe environment for gene therapy[109]. *In vivo* intradiscal application of an adenovirus-mediated TGF-β gene transfer has indeed shown enhanced expression of the transgene and enhanced proteoglycan production in rabbits[141]. Although non-viral methods hold promise, usually viral transfection yields higher efficiencies[142]. Given that MSCs are already widely used in NP regeneration *in vivo*, they could also serve as factories to produce BMPs intradiscally, implanted after genetic modification *in vitro*. This strategy is already being employed with lentiviral or retroviral approaches for severe and lethal diseases[135], and if safety can be demonstrated in the IVD, it is a promising strategy for IVD regeneration[143-145]. Viral infection of NP cells[142,146-147] or MSCs[148] gives stable transfection with high efficiencies (around 70%) and has been shown to be effective for NP regeneration. However, it is unclear whether BMP-based NP regeneration will be augmented by the presence of MSCs[149] or whether MSCs will undergo hypertrophic differentiation, a well-documented challenge within the field of joint cartilage regeneration[150]. To this end, having in mind the fact the NP native tissue is hypoxic, hypertrophic differentiation of MSCs may not be an issue. Multiple studies have shown that that hypertrophy of chondrogenically differentiated MSCs occurs more readily at normoxia than at hypoxia[151-152], although this might also depend on the intrinsic chondrogenic capacity of the MSC population.

Because of safety concerns with viral gene delivery[140], a plethora of different techniques and methods for non-viral alternatives has emerged[153-154]. Electroporation is a successful method for transfection of NP cells[155] and has been shown to be effective for human MSC transfection, as well[156]. Transfection by polyplex micelles with a heme oxygenase plasmid delivered *in vivo* has been shown to be feasible and induced regeneration in a rat stab model of degeneration[157]. A relatively new technique involves gene editing with CRISPR/Cas technology, originally identified as a bacterial defense mechanism against invading bacteriophages, which allows genome modification by precise targeting of specific genes[158]. To date, this technique has not yet been employed in IVD research.

Concluding remarks

This thesis focused on elucidating important factors in the regeneration of the NP through osmolality, cell-tissue interaction and BMPs. Maintaining the osmolality in the disc might prevent (further) degeneration, and it is crucial to intervene at an early degeneration stage. If NP cells are to be used, high osmolality is an important condition during expansion. However, osmolality on its own appears to be insufficient during NP regeneration. NP homeostasis is an interplay between high osmolality, hypoxia, glucose levels, pH[5-6], and loading[7] and all these factors should be taken into account when aiming for IVD regeneration.

A first step towards a reference hydrogel for human NP regeneration research has been taken. Agarose appeared to induce the highest regenerative capacity of the embedded NP cells and might serve as a reference in vitro, but it does not degrade and might thus pose a problem for therapeutic applications. Type II collagen hydrogels are possibly better options for implantation. The response of the resident NP cells and the implanted cells to the polymers used in the hydrogel should be carefully considered, as mechanical properties and the binding capacity might have a substantial influence on the outcome. Still, other relevant characteristics of the hydrogels are important and might elicit unexpected responses, and the roles of all hydrogel properties should be researched in more detail, including biomechanical loading.

Regeneration of the NP can be achieved by growth factors such as BMPs, as they have shown promising results *in vitro*. BMP4 seems to be the most potent, although more detailed studies should be performed with all members of the BMP family. It does seem useful to look beyond "the usual suspects", especially when it comes to BMPs or hydrogels. Delivery of these growth factors is an important and controversial issue, but MSCs as growth factor factories could offer a promising alternative.

Overall, the combination of several approaches might be the most promising for targeting IVD degeneration[159].

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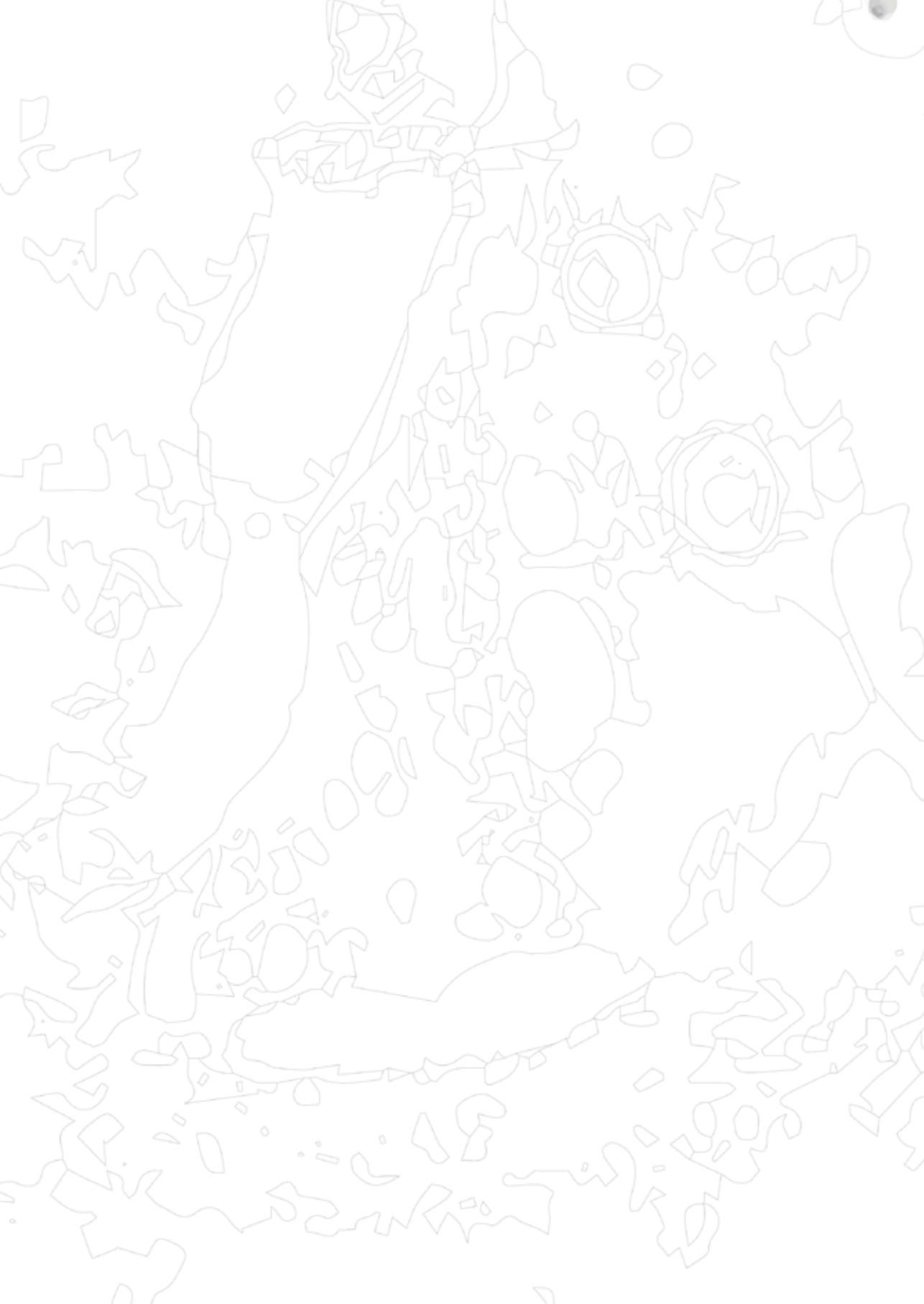

Addenda

List of abbreviations

2D	Two-dimensional
3D	Three-dimensional
ACAN	Aggrecan
AF	Annulus fibrosus
ALP	Alkaline phosphatase
ANOVA	Analysis of variance
ASAP	Ascorbic acid-2-phosphate
bFGF	Basic fibroblast growth factor
BMP	Bone morphogenetic protein
BSA	Bovine serum albumin
CHO	Chinese hamster ovary
Col1A1	Type I collagen
Col2A1	Type II collagen
ColX	Type X collagen
DMA	Dynamic mechanical analysis
DMEM	Dulbecco's modified Eagle medium
DMMB	1,9-dimethyl-methylene blue
DMSO	Dimethyl sulfoxide
ECM	Extracellular matrix
ELISA	enzyme-linked immunosorbent assay
ENaC	Endothelial sodium channel
F-actin	Filamentous actin
FAK	Focal adhesion kinase
FAK-i-14	1,2,4,5-benzenetetraamine tetrahydrochloride
FBS	Fetal bovine serum
G-actin	Globular actin
GAG	Glycosaminoglycans
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
geIMA	Gelatin-methacryloyl
GlcAT-I	β1,3-Glucuronosyltransferase-I
HA	Hyaluronic acid
HA-PEG	Hyaluronic acid/poly(ethylene glycol)
HSA	Human serum albumin

IBMX	3-isobutyl-1-methylxanthine
IL	Interleukin
ITS-X	Insulin + transferrin + selenium + ethanolamine
IVD	Intervertebral disc
LDH	Lactate dehydrogenase
LOX	Lysyl oxidase
LOXL	Lysyl oxidase like
METC	Medical ethical committee
MMP	Matrix metalloproteinase
mRNA	Messenger RNA
MSC	Mesenchymal stromal cell
NP	Nucleus pulposus
OD	Optical density
p	Statistical significance
PBS	Phosphate buffered saline
PBS-T	PBS + Tween
PCF	Polycarbonate film
PEG	Poly(ethylene glycol)
Pen/strep	Penicillin + streptomycin
PGE_2	Prostaglandin E_2
PIICP	carboxyterminal propeptide of type II collagen
PLOD	Procollagen lysyl hydroxylase
PPAR-γ	Peroxisome proliferator-activated receptor gamma
qPCR	Quantitative polymerase chain reaction
RGD	Arg-Gly-Asp
RT-qPCR	Quantitative real-time polymerase chain reaction
SF	Serum-free
SPP1	Osteopontin
TGF-β	Transforming growth factor beta
TMB	3,3',5,5'-tetramethylbenzidine
TonEBP	Tonicity enhancer binding protein
TRPV4	Transient receptor potential vanilloid 4
α-MEM	Alpha modification of minimum essential medium

Nederlandse samenvatting

De tussenwervelschijf

Lage rugpijn is wereldwijd een van de voornaamste oorzaken van invaliditeit. De medische zorg voor chronisch lage rugpijnpatiënten en de grote kans op arbeidsongeschiktheid kosten de Nederlandse samenleving miljarden euro's per jaar. Chronische lage rugpijn wordt vaak veroorzaakt door slijtage, ook wel degeneratie genoemd, van de tussenwervelschijf. Het begint meestal in het zachte midden van de tussenwervelschijf: de nucleus pulposus (NP). De NP bestaat voornamelijk uit proteoglycanen, negatief geladen eiwitten die het grootste deel van het weefsel vormen, en type II collageen en heeft een hoog watergehalte. De NP wordt rondom begrensd door een fibreuze ring, de annulus fibrosus (AF), en boven en onder de tussenwervelschijf zitten twee kraakbeeneindplaten die de tussenwervelschijf verbinden met de ruggenwervels. De tussenwervelschijf zorgt ervoor dat de ruggengraat flexibel is en de bewegingskrachten kan opvangen. In een gezonde tussenwervelschijf zijn zenuwen en bloedvaten alleen in de buitenste laag van de AF aanwezig. Eén van de grote veranderingen die optreden tijdens degeneratie van de NP is de afname van de concentratie proteoglycanen,. Deze afname verlaagt de hoeveelheid water die normaal vastgehouden wordt in de NP en daarmee ook de druk die de tussenwervelschijf kan weerstaan. Tegelijkertijd worden ook de eindplaten beschadigd, waardoor minder voedingsstoffen de NP kunnen bereiken. Ook vertoont de AF scheurtjes, waardoor zenuwen en bloedvaten kunnen ingroeien. Dit leidt tot verandering van het weefsel en pijn.

De huidige behandeling voor patiënten met lage rugpijn bestaat meestal eerst uit fysiotherapie en sport. Wanneer dit niet voldoende blijkt, is opereren veelal het enige alternatief. Hierbij worden de omliggende wervels gefuseerd, of de tussenwervelschijf wordt vervangen door een kunst-tussenwervelschijf. Beide procedures zijn erg ingrijpend en ongeveer even weinig effectief. Fusie van de wervels kan leiden tot versnelde slijtage van de omliggende tussenwervelschijven. Lange-termijn gegevens over de uitkomsten van kunst-tussenwervelschijven zijn maar beperkt aanwezig. Bovendien zijn deze behandelingen geen oplossing voor het onderliggende probleem, namelijk degeneratie. Regeneratieve geneeskunde richt zich op herstel van het weefsel en zou het probleem wel oplossen. Regeneratie zou kunnen worden bereikt met het implanteren van biomaterialen, cellen of groeifactoren, of een combinatie hiervan. Hiermee zou het lichaam gestimuleerd worden om zelf het 'zieke' weefsel te herstellen en de pijn te verminderen.

Het doel van dit proefschrift was om de lokale en externe chemische en fysische signalen die een rol spelen bij NP regeneratie te begrijpen. Hieronder vallen osmolariteit, de driedimensionale omgeving die hydrogels kunnen vormen, interacties met die omgeving en groeifactoren. Deze kennis zou kunnen worden gebruikt in laboratoria om NP regeneratie te bestuderen. Dit kan helpen bij het ontwikkelen van een therapie om

regeneratie van de beschadigde NP te bewerkstelligen.

Cellen: de fabrieken van het lichaam

Cellen produceren alle weefsels in het lichaam. Gespecialiseerde cellen zoals bijvoorbeeld aanwezig in de NP, produceren de componenten van het weefsel en zijn daarom uitermate geschikt om de NP te repareren. Het oogsten van deze cellen uit de NP van een tussenwervelschijf van de patiënt zelf veroorzaakt (verdere) degeneratie van de tussenwervelschijf, dus dit maakt het gebruik van eigen cellen onwenselijk. Bij een hernia komt er een stukje NP buiten de AF, en cellen weghalen uit dit weefsel zou dat omzeilen, maar een probleem is dat de cellen uit een hernia wellicht niet meer de optimale capaciteit voor regeneratie hebben. Een alternatief voor NP cellen van de patiënt of een donor is het gebruik van stamcellen die kunnen differentiëren naar NP cellen.

Een cel is onderdeel van een weefsel of orgaan. Een cel krijgt continu signalen van het omliggende weefsel en uit de rest van het lichaam, bijvoorbeeld via hormonen en groeifactoren. Hoe de omgeving van een cel invloed heeft op hoe de cel zich gedraagt hebben we bestudeerd in dit proefschrift.

Componenten van het kweekmedium

In de tussenwervelschijf zijn proteoglycanen en zouten aanwezig die zorgen voor een omgeving met een hoge osmolariteit. Om te onderzoeken of osmolariteit belangrijk is in het gedrag van NP-cellen, zijn in hoofdstuk 2 NP-cellen van een versleten tussenwervelschijf gedurende regeneratie van NP weefsel blootgesteld aan kweekmedium van hoge osmolariteit. Deze hoge osmolariteit werd bereikt door het toevoegen van een osmolyt, een stof die de osmolariteit van een oplossing beïnvloedt, aan kweekmedium. Drie verschillende osmolyten in verschillende hoeveelheden (verschillende osmolariteiten) werden vergeleken: zout, suiker en ureum. Voor deze drie behandelingen werd de genexpressie en eiwitproductie van de NP-cellen gemeten en vergeleken met standaard kweekmedium met lage osmolariteit. Genexpressie en weefselproductie zijn belangrijk omdat ze een maat zijn voor het functioneren van een NP-cel en het herstel van het weefsel. De genexpressie van type II collageen en vooral proteoglycanen was toegenomen in de behandelingen met zout (NaCl) of suiker (sucrose), maar niet in de cellen behandeld met ureum. Wanneer NaCl werd gebruikt, werden echter geen verschillen gevonden in weefselproductie tussen verschillende osmolariteiten. De osmolyten ureum en sucrose zorgden voor sterk verminderde weefselproductie onafhankelijk van de osmolariteit.

Wanneer de osmolariteit werd verhoogd tijdens isolatie en opkweken van de cellen

was er wel degelijk invloed op de uiteindelijke weefselproductie. De cellen die werden blootgesteld aan een hoge osmolariteit produceerden meer weefsel dan de cellen die in lage osmolariteit werden vermenigvuldigd. Mogelijk induceert blootstelling aan lage osmolariteit een verandering in deze cellen die niet meer ongedaan gemaakt wordt als de osmolariteit weer wordt verhoogd. Dit is een belangrijke aanwijzing dat osmolariteit een cruciale rol speelt bij de werking van deze cellen en het behoud van gezond weefsel.

Een gebruikelijke component van kweekmedium is kalfsserum, dat een onbekende mix aan stoffen bevat die weefselgroei stimuleren. Omdat er ook ziektekiemen aanwezig zouden kunnen zijn, is serum niet geschikt voor gebruik in patiënten. In hoofdstuk 3 hebben we de invloed van serum onderzocht op weefselproductie van NP cellen. NP-cellen werden gekweekt in medium met serum en weefselproductie werd vergeleken met cellen gekweekt in een gedefeniëerd medium zonder serum. De productie van weefsel en het aantal aanwezige cellen in het gekweekte weefsel waren hoger in medium met serum. In sommige gevallen was de kwaliteit van het weefsel hoger zonder serum.

Driedimensionale omgeving

De gezonde NP bestaat uit een omgeving met veel water en eiwitten die het weefsel vormen en lijkt hierdoor op een hydrogel. Er zit relatief veel ruimte tussen de NP-cellen. Hydrogelen worden veel gebruikt in NP-weefselregeneratie om de omgeving in het lichaam na te bootsen, maar het is niet duidelijk aan welke eigenschappen een dergelijke hydrogel moet voldoen. Er worden veel verschillende soorten hydrogelen gebruikt in de literatuur en hierdoor is het moeilijk om de resultaten van verschillende onderzoeken te vergelijken. In hoofdstuk 3 is een vergelijking gemaakt tussen zes veelgebruikte hydrogelen: alginaat, agarose, gemethacryleerde gelatine (gelMA), fibrine, hyaluronzuur gecombineerd met poly(ethyleenglycol) (HA-PEG) en type II collageen. De NP-cellen bleven in alle hydrogelen in leven, maar de weefselproductie door de cellen was het hoogst in agarose, gevolgd door type II collageen.

Waarschijnlijk speelt de stijfheid, onder andere bepaald door polymeerconcentratie, van de hydrogelen een rol bij weefselproductie, zoals aangetoond voor stamcellen. Daarom hebben we in hoofdstuk 4 onderzocht wat het mechanisme is achter de reactie van NP-cellen op polymeerconcentratie en focale adhesievorming (een type verbinding van cellen met het weefsel) en welke invloed dit heeft op weefselproductie door deze cellen. NP-cellen werden gekweekt in verschillende hydrogelen van verschillende stijfheden of in aanwezigheid van een focale adhesieremmer. NP-cellen reageerden met verhoogde weefselproductie op een verlaagde polymeerconcentratie in gelMA, dat bestaat uit een polymeer gemaakt van gelatine afkomstig van type I collageen en aanhechtingspunten voor cellen bevat. In alginaat en agarose hydrogelen, die beide gemaakt zijn van algen en bestaan uit lange ketens van suikerachtige moleculen, zijn deze aanhechtingspunten niet aanwezig en had verandering van polymeerconcentratie geen effect. Met confocale microscopie zagen we volledige, langgerekte focale adhesies in de NP-cellen gekweekt

in 2D op glas of in 3D in type II collageen hydrogelen. In cellen gekweekt in agarose waren onvolledige, ronde focale adhesies aanwezig. In gelMA hydrogelen werden zowel volledige als onvolledige focale adhesies gevonden. De toevoeging van een adhesieremmer had alleen effect op cellen die werden gekweekt in hydrogelen met aanhechtingspunten, namelijk gelMA en type II collageen.

Bij het kiezen van een hydrogel voor NP-regeneratie moet dus rekening worden gehouden met het type polymeer waar deze uit bestaat, of de NP-cellen hiermee kunnen binden en de stijfheid van de hydrogel. Agarose biedt NP-cellen de beste ondersteuning bij regeneratie, maar kan door het lichaam niet worden afgebroken. Type II collageen biedt ook redelijke ondersteuning voor regeneratie en kan wel worden afgebroken door het lichaam. Deze zou voor implantatie wellicht een betere keuze zijn.

Signalen in de omgeving

Groeifactoren zijn de boodschappers van het lichaam. Ze geven signalen door aan cellen die voor groei van het weefsel zorgen. Bekende groeifactoren voor de regeneratie van de tussenwervelschijf zijn BMPs. Hoewel BMP2 en BMP7 in dit onderzoeksveld het meest worden gebruikt, laten de resultaten in hoofdstuk 5 zien dat ze voor het regenereren van de tussenwervelschijf wellicht niet de beste keuze zijn. BMP4, de heterodimeer BMP4/7H en de combinatie BMP4+7, maar ook BMP6 en heterodimeer BMP2/6H stimuleerden meer weefselproductie door NP-cellen ten opzichte van andere BMPs. In een concentratie-afhankelijke responscurve werd bevestigd dat een lagere concentratie BMP4 nodig was dan voor dezelfde hoeveelheid weefselproductie met BMP7. In co-kweken van NP-cellen met stamcellen werd de hoogste hoeveelheid weefsel gemeten in aanwezigheid van BMP4.

Conclusie

In dit proefschrift hebben we geprobeerd om verschillende factoren te identificeren die invloed hebben op weefseregeneratie door cellen in de NP. Hierbij lag de focus op de osmolariteit, de interactie tussen de 3D-omgeving en de cellen, en groeifactoren. In dit proefschrift hebben we laten zien dat:

- Als NP-cellen worden gebruikt voor regeneratie het cruciaal is om de osmolariteit van de omgeving hoog te houden. Echter, enkel een fysiologische osmolariteit is niet voldoende om volledige regeneratie te bereiken.
- Voor het kweken van NP cellen in 3D hydrogelen, de samenstelling, stijfheid en bindingscapaciteit belangrijke factoren zijn.
- Groeifactoren, zoals BMPs, gebruikt kunnen worden om NP-cellen te stimuleren om weefsel aan te maken. BMP4 komt uit ons onderzoek als beste kandidaat naar voren om de NP te regenereren.

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Curriculum vitae

Anita Krouwels was born on July 14, 1983 in Haarlem, the Netherlands. She graduaded from high school in May, 2001 and moved to Enschede to study Biomedical Engineering at the University of Twente. Her bachelor thesis was performed at the Dutch Burn Center in Beverwijk and her master thesis at the Polymer Chemistry and Biomaterials research group at the University of Twente, both in the field of regenerative medicine combined with biomaterials. After graduation in 2008, she worked for Medisse, a start-up company in biomedical materials, Mallinckrodt Baker, a chemical company, and Qtis/e, a start-up company developing growing heart valves. In 2012, she started a PhD in the Orthopeadics department of the University Medical Center in Utrecht as part of the Regenerative Medicine program, under supervision of prof. dr. W.J.A. Dhert, prof. dr. F.C. Öner, prof. dr. M.A. Tryfonidou, and dr. L.B. Creemers. The focus of the research was the regeneration of the intervertebral disc, achieved by adjusting medium osmolality, application of biomaterials, and using growth factors. The main results are presented in this thesis. After that she was a postdoc with a focus on education at the Eindhoven University of Technology in the Orthopeadic Biomechanics group of prof. dr. K. Ito. Currently, she is working as a volunteer teaching Dutch.

Anita lives in Bilthoven with Stephan Janssen and their son Colin.