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In vitro studies for the development of biological repair strategies of intervertebral disc degeneration

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“If you can't explain it simply, you don't understand it well enough.”

Albert Einstein

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List of Abbreviations

AB	Alginate bead(s)
AF	Annulus fibrosus
Agg	Aggrecan
bFGF	Basic fibroblast growth factor
BMP	Bone morphogenetic protein(s)
BSA	Bovine serum albumin
CD	Cluster of differentiation
cDNA	Copy deoxyribonucleic acid
CEP	Cartilaginous endplate(s)
c-FOS	Cellular FBJ osteosarcoma oncogene
CFU-F	Colony forming unit-fibroblast(s)
CHO	Chondrogenic medium
CO_2	Carbon dioxide
Col	Collagen
DMEM	Dulbecco's Modified Eagle Medium
DMEM-HG	Dulbecco's Modified Eagle Medium high glucose
DMMB	1,9-Dimethylmethylene blue
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediamine tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FCS	Foetal calf serum
FOXF1	Forkhead box protein F1
GAG	Glycosaminoglycan(s)
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GFP	Green fluorescent protein
GLUT	Glucose transporter
HCl	Hydrochloric acid
HEPES	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
HIF	Hypoxia-inducible factor
HRP	Horseradish peroxidase
IVD	Intervertebral disc(s)
KCl	Potassium chloride
MEM	Minimum Essential Medium
MMP	Matrix metalloproteinase
mRNA	Messenger ribonucleic acid
MSC	Mesenchymal stem cell(s)
Na_2HPO_4	Disodium hydrogen phosphate
NaCl	Sodium chloride
NaH_2PO_4	Sodium dihydrogen phosphate
NaOH	Sodium hydroxide
NaN_3	Sodium azide
NEA	Non-essential amino acids
NP	Nucleus pulposus
O_2	Oxygen
P	Pellet
PAX-1	Paired box protein Pax-1
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGF-BB	Platelet-derived growth factor subunit B dimer
PRP	Platelet-rich plasma
PTN	Pleiotrophin

RLT	RNeasy™ Mini Kit lysis buffer
RNA	Ribonucleic acid
Rox	Reference dye for RT-PCR
RPL30	Ribosomal protein L30
RPS18	Ribosomal protein S18
RT-PCR	Reverse transcriptase polymerase chain reaction
STRO-1	Cell surface protein
Sox9	Sex determining region Y-box 9
TGF	Transforming growth factor
Tris	Tris(hydroxymethyl)aminomethane
TTBS	Tris-triton buffered saline
VEGF	Vascular endothelial growth factor

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1. Introduction

Reasons for the degeneration of the intervertebral disc (IVD) are as multifactorial as its consequences are variable. Virtually everybody will once in his lifetime suffer from low back pain – a symptom, which is associated with disc degeneration. But still not all mechanisms underlying this process of degeneration are clarified in detail. Accordingly, efforts to biologically regenerate this unique tissue are faced with challenging problems.

The present study aimed to develop biological repair strategies for cases of disc degeneration. As a first step, questions regarding general involvement of microenvironmental changes in glucose and oxygen concentration within the IVD during degeneration were addressed. In parallel, biological repair strategies using different cell types, culture systems and growth factor supplementations were developed, as well as an organ culture model system.

Below, structure and function of the intervertebral disc, processes during disc degeneration and biological strategies of disc regeneration are introduced.

1.1. Intervertebral Disc

1.1.1. Anatomy

At all times, the human IVD attracted the attention of scientists and with development and improvement of technical methods, the knowledge about disc morphology and functionality became more detailed and more complex.

The IVD lies between two vertebral bodies of the spinal column. It has a complex structure consisting of a gelatinous core called nucleus pulposus (NP), which is surround by a cartilaginous ring called annulus fibrosus (AF). Moreover, the NP is confined cranially and caudally by cartilaginous endplates (CEP) (Fig. 1) [214].

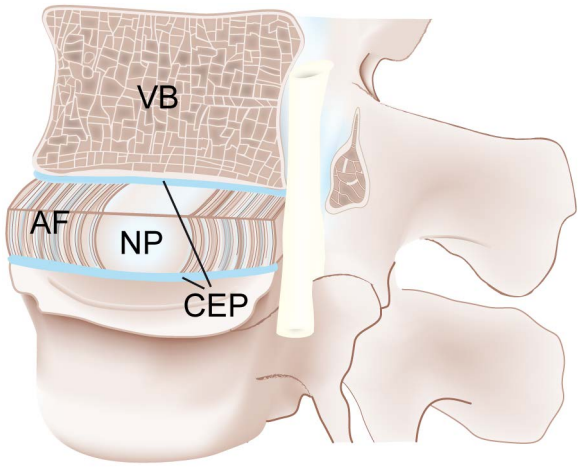

Fig. 1: Schematic view of a spinal segment and the intervertebral disc (IVD)
One spinal segment is composed of one IVD between two vertebral bodies (VB). The IVD itself consists of the centrally localised nucleus pulposus (NP) surrounded by the annulus fibrosus (AF) and the cartilaginous endplates (CEP) acting as boundaries of the IVD to the vertebral bodies.

The NP is the central component of the IVD and developed from the notochord [32, 187]. The cells within the NP are described to exhibit a chondrocyte-like phenotype, which is characterised by the expression of the matrix molecules aggrecan, the major proteoglycan of the NP, and collagen type II [219]. The small and round NP cells are distributed with a very low cell density (5,000 cells/mm^3) within the tissue [150]. The extracellular matrix of the NP is mainly composed of randomly organised collagen type II fibrils and proteoglycans such as aggrecan forming a highly hydrated gel at the ratio of 1:27 (articular cartilage 1:2) [166]. Aggrecan is highly hydrophilic as it is composed of monomers consisting of a core protein substituted with chains of the glycosaminoglycans (GAG) chondroitin and keratan sulphate bound to hyaluronic acid by link protein (Fig. 2) [118]. The tightly packed and highly charged sulphate groups of GAG generate a swelling pressure that provides resistance to compression [95, 257].

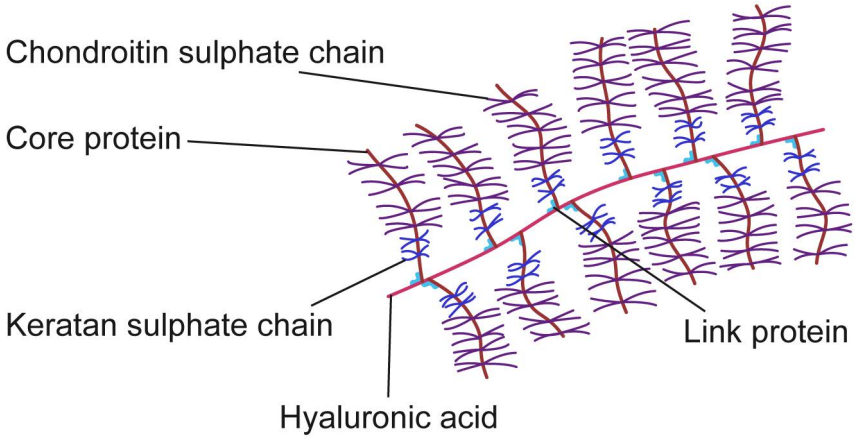

Fig. 2: Schematic structure of an aggrecan molecule
Within an aggrecan molecule the highly negatively charged chondroitin sulphate chains and keratan sulphate chains are bound to the core protein. This complex is fixed by the link protein to a hyaluronic acid macromolecule.

The surrounding AF confines the swelling pressure of the NP. In contrast to NP tissue, AF tissue is less hydrated and is mainly composed of collagen type I [2]. The AF consists of a series of 15-25 concentric lamellae [149]. In each one, parallel fibres of collagen type I run diagonally between the vertebrae at an angle of 120° to the adjacent lamella (Fig. 3). The tension of the collagen fibrils provides flexibility. The balance between the swelling pressure of the NP and the tension of the AF maintains disc height at rest [55]. Cells within the AF are adapted to their environment. They are aligned parallelly to the collagen fibres and the cells' morphology changes from oval to an elongated, thin shape from the inner to outer regions. Particularly in the outer region, the AF cells tend to be fibroblast-like [30].

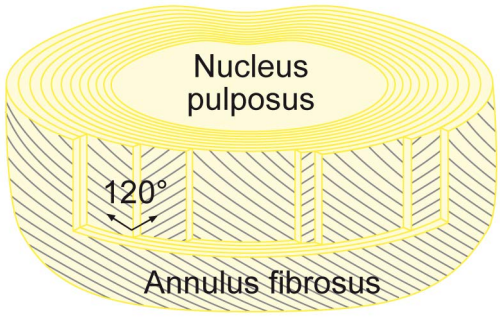

Fig. 3: Schematic design of annulus fibrosus (AF) lamellae
The AF consists of a series of 15-25 concentric lamellae. In each one, parallel fibres of collagen type I run diagonally between the vertebrae at an angle of 120° to the adjacent lamella.

The CEP separates the disc from the cortical bone of the adjacent vertebral bodies. In this thin horizontal layer of hyaline cartilage, chondrocytes are located in a highly oriented collagen fibril network parallel to the vertebral body [211].

Apart from the outermost lamellae of the AF, the healthy disc is completely avascular and aneural [220]. The extracellular matrix of all three tissues compartments is maintained by the respective cells, which regulate the balance between degradation and synthesis by continuous matrix turnover [258].
In the present study, analysis was focussed on the NP as cells within the NP are more affected by deprivation of oxygen and glucose than cells of the CEP or AF due to the greater distance to blood vessels. Thus, NP tissue is the main target in the development of biological repair strategies.

1.1.2. Biomechanics

The IVD together with the adjacent vertebral bodies forms a so called motion segment with six degrees of freedom [81]. All in all, 23 IVD form the major joints of the human vertebral column, providing flexibility and transmitting mechanical loads arising from body weight or muscle activity [52, 151]. The composition of the matrices of NP and AF displays thereby optimal adaption to the naturally occurring loads. While the NP with its swelling pressure is able to withstand compressive stress mainly in axial direction [84], the highly oriented collagen fibres of the AF tolerate tensile stress occurring during flexion, extension, disc bulging or rotation [149, 233]. The perfect interactions of the tissues allow complex movements of the whole spine in every degree of freedom of the three-dimensional Cartesian coordinate system (Fig. 4).

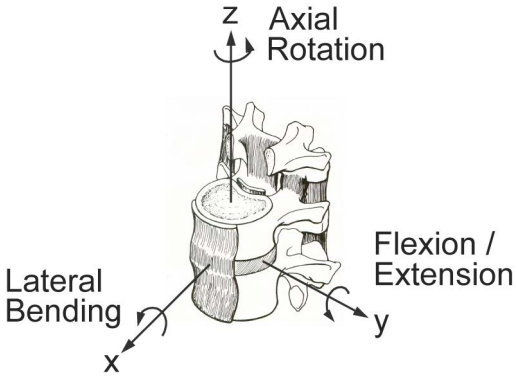

Fig. 4: Schema of three directions of spinal movement
The intervertebral disc between two vertebral bodies provides flexibility of the spinal segment in every degree of freedom of the Cartesian coordinate system.

1.2. Aetiology of Disc Degeneration

With a lifetime prevalence of more than 80%, back pain causes a social and economic burden of about 49 billion Euro per year corresponding to 2.2% of the German gross domestic product [277]. Although disc degeneration is in many cases asymptomatic [25, 50], it is often associated with back pain in terms of sciatica or disc herniation [142]. As a matter of fact, the IVD undergoes extensive structural alterations with ageing. Thereby it seems impossible to separate naturally occurring ageing effects from pathological - degenerative - changes. Furthermore, highly individual progression of disc degeneration and parameters, which are difficult to quantify, make general definitions difficult [258]. However, many grading systems exist to classify the changes of the disc macroscopically and microscopically [27, 117, 190, 247, 280]. Summarizing all changes of the IVD tissue as a whole, disc degeneration can be described as a progressive disorganisation of the whole tissue starting very early in human life. The border between NP and AF becomes indistinct and the whole tissue gets less hydrated [143, 258]. The NP becomes less gel-like and more fibrotic [31], cell cluster formation occurs [73, 232], as well as cell death due to apoptosis and necrosis [64, 253, 254]. Cleft formation and fissures can be seen [258, 263] and nerves and blood vessels penetrate the tissue more and more [160].

All these macroscopic alterations are based on changes in matrix composition and cell activity within the disc tissue [2, 125]. The most obvious and serious change is the degradation of proteoglycans [13, 143], while the changes in the collagen network are less dramatic [77, 258]. The delicate balance between matrix synthesis and matrix degradation within the healthy disc becomes deranged [255, 258]. An increase in matrix degrading enzymes like aggrecanases [185] and matrix metalloproteinases (MMP) [62, 124, 275, 286] during disc degeneration leads to increased fragmentation of collagens and proteoglycans [125, 213]. The subsequent loss of highly charged sulphate groups decreases the swelling pressure and thus leads to a loss of tissue hydration, mechanical functionality, and disc height [258].

The causes and mechanisms of disc degeneration are not clarified yet. Below several known influencing factors are described.

1.2.1. Nutritional Influences

In principle, viability of all cells is directly coupled to the availability of the most important nutrient glucose and oxygen. Since the IVD is the largest avascular tissue of the human body, cells within the IVD are adapted to the hypoxic environment and thus use anaerobic glycolysis to generate energy from glucose [78, 96]. Cells mainly depend on nutrient supply from the capillaries of the adjacent vertebral bodies [40] and - to a minor part - from the AF periphery [220] (Fig. 5).

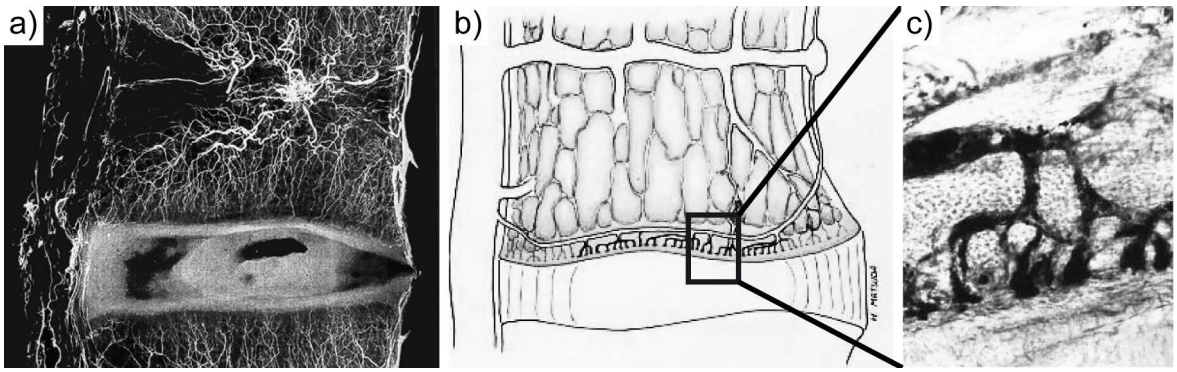

Fig. 5: Blood supply of the intervertebral disc (IVD)
Blood vessels of the vertebral bodies displayed a) radiographically after injection of a contrast agent and b) schematically showing the blood supply of the IVD by capillaries, which end at the cartilaginous endplate (CEP). The magnification in c) shows the capillaries stained in black at the border to the CEP. Adapted from [40, 258].

As the distance between the capillaries and a cell in the NP can be up to 8 mm [78] and diffusion is the main mechanism for nutrient transport [53, 113], steep gradients in the availability of nutrients exist, falling from the periphery to the centre of the disc and resulting from discrepancies between the duration of nutrient transport and the rate of consumption by the cells [259]. The CEP thereby acts as a semipermeable membrane allowing faster diffusion of small, uncharged molecules such as oxygen or water compared to anionic or large molecules such as growth factors [256]. With progressing disc degeneration and calcification of the CEP [212, 261], transport routes for glucose and oxygen are impaired. Also, metabolic products such as lactic acid accumulate, as they cannot be removed adequately leading to a decreasing pH value [46]. Such a deprivation of nutrients and alteration of the physicochemical environment is thought to lead to reduced cell metabolism, decreased production of matrix, increased matrix degradation or even cell death and thus to disc degeneration [21, 22, 79]. In contrast to most of the previous studies, which concentrated on short-term experiments, the present

study focussed on effects of glucose and oxygen reduction after long-term culture of NP cells.

With progressing disc degeneration, vascularisation of the disc tissue was shown [114, 160, 184] displaying a possible mechanism to overcome nutrient deprivation. Several studies were able to demonstrate the involvement of angiogenic factors during the vascularisation process. Johnson and colleagues demonstrated the presence of pleiotrophin (PTN) in pathologic human IVD, and suggested that it may play a role in neovascularisation of diseased or damaged disc tissue [102]. Vascular endothelial growth factor (VEGF) was also found in capillaries of the herniated IVD suggesting an active neovascularisation [70, 251]. VEGF can be activated by the transcription factor hypoxia-inducible factor 1α (HIF1α) [228]. Thus, HIF1α might play a role as regulator of the vascularisation process. Others reported on HIF1α as a contributor to cell survival [68] by stabilising the expression of glucose transporter 1 (GLUT-1) [196] for glucose uptake and galectin-3, which contributes to cell spreading [222]. Furthermore, the injection of basic fibroblast growth factor (bFGF) into rabbit discs led to higher vascularisation and faster resorption of herniated tissue [163]. However, up to now, no study has yet clarified the entire mechanism of vascularisation and the influence of oxygen environment. Thus, alterations in the expression of angiogenic factors after oxygen reduction were investigated in the present study.

1.2.2. Mechanical Influences

For many years, mechanical loading was thought to play a major role in disc degeneration. Permanent inappropriate biomechanical stress such as overloading or shear stress lead to injuries within the tissue causing structural damage [89, 229]. Animal models supported this fact. Both, injury to the disc [91, 121] and experimental overloading [88, 139] were shown to induce disc degeneration. Epidemiological studies demonstrated a correlation between disc degeneration and environmental factors such as heavy physical work [266], occupational driving [17] or obesity [45]. However, although various ergonomic interventions followed this knowledge, the incidence of disc degeneration continued to rise [258] demonstrating that mechanical loading is a risk factor but not necessarily the reason for disc degeneration. Twin studies demonstrated that regardless of

differences in physical occupation, twins had the same degree of disc degeneration [264] pointing out that genetic influences are stronger than physical influences.

1.2.3. Genetic Influences

In recent years, many epidemiological studies on twins or defined population groups have shown that genetic factors contribute considerably to disc degeneration [7, 18, 107, 268]. Further studies reported a strong familial predisposition [106, 155] for disc degeneration or herniation and showed a heritability of 60% [16, 221]. There are a number of genes that have been shown to be associated with disc degeneration including genes encoding structural proteins such as collagen type I [192, 250], collagen type IX [12, 105, 109, 236], aggrecan [115, 218, 274], and MMP3 [246]. In addition, interleukins [179, 235] and vitamin D receptor [37, 116, 265, 267] were found to be associated with disc degeneration. It is evident that mutations in these genes may cause structural changes of the matrix composition, biochemistry and function of the IVD [258], which may lead to disc degeneration.

1.3. Tissue Engineering

Current treatments of disc degeneration and the associated low back pain involve mainly non-surgical treatment modalities such as physical therapy, life style modifications or anti-inflammatory medication to reduce pain [3, 285]. If these treatments do not lead to pain relief, surgical treatments such as spinal fusion and discectomy are the methods of choice [51, 285]. These methods are known to result in a fast pain relief but also accelerate further disc degeneration as the mechanics of the adjacent motion segments are altered [51, 132, 181]. In recent years, new surgical treatments are being investigated using disc prostheses, which aim to provide the necessary flexibility to the spine and maintain spinal movement [24, 43, 69]. However, all these strategies currently only treat the symptoms of disc degeneration. None of them aims to restore the natural disc function rebuilds healthy disc tissue or at least stops further tissue degradation.

At this point, regenerative medicine and tissue engineering approaches of the IVD appear particularly promising. Different strategies such as the injection of growth factors into the IVD [10, 94, 248], gene therapy approaches [176, 272, 290] and cell-based therapies [158, 167, 175] are an increasing research field. The present work focuses on the strategy of cell-based therapies for NP tissue.
As autologous chondrocyte transplantation for articular cartilage [29, 189] is successful in clinical application, similar approaches were adopted for cell-based IVD tissue engineering [108, 154, 180]. However, there are many concerns and challenging questions about biological repair strategies of the IVD. The main issues including cell types and their availability, the choice of an appropriate culture system and medium composition, benefits of mechanical loading, and the advantages and disadvantages of animal models will be pointed out subsequently.

1.3.1. Cell Types

The identification of a suitable source of cells is one of the major issues of IVD tissue engineering. For the regeneration of NP tissue mainly two cell types are of interest: disc cells themselves and mesenchymal stem cells (MSC) as potential precursors.

Disc cells

The usage of autologous disc cells provides the advantage of having the correct phenotype and possibly being able to produce an adequate extracellular matrix. The major problem is the availability of non-degenerated healthy human disc cells. Unlike the autologous chondrocyte transplantation where biopsies are taken from non-load-bearing areas [29], such areas do not exist in discs. Therefore, cells have to be obtained from the degenerated disc level where cells might be senescent [67, 215], already dead or dying [27] by apoptosis [64] or necrosis [259]. Furthermore, the cells' phenotype might already be altered by degeneration or the cells might have the genetic predisposition to degenerate [51]. On the other hand, taking a biopsy of a healthy disc by e.g. needle puncture leads to damages in the structure and, together with the removal of NP tissue, would alter the mechanical function of this segment and induce further degeneration [121, 153, 289]. Additionally, parts of the AF tissue might be removed simultaneously leading

to an isolation of a heterogeneous cell population of fibroblastic AF cells with NP cells which differ in morphology and metabolism [80]. Another important point is the low cell number of the NP tissue. Thus, either a great volume of tissue has to be removed or cells have to be expanded *in vitro* over a long time to obtain sufficient cell numbers for tissue engineering.

Another possibility is the use of allogeneic NP cells as the CEP and the surrounding AF separate the NP from blood supply and hence prevent immune cell exposure [285]. Nomura *et al.* found no immune response after injection of allogeneic NP cells into rabbit IVD [178]. However, the availability of allogeneic human NP tissue leads to the same problems as autogenous cell retrieval from the patient. The removal of disc tissue will induce disc degeneration in the respective IVD.

Mesenchymal stem cells

Autologous MSC represent a healthy alternative to disc cells and can be obtained in large numbers from bone marrow [144], adipose tissue [293] or other tissue types [23]. The study of MSC was initiated by Friedenstein *et al.* [56] and in 1999, Pittenger *et al.* [191] were the first to demonstrate the capacity of MSC to differentiate into cells of the connective tissue lineage [33, 202] (Fig. 6).

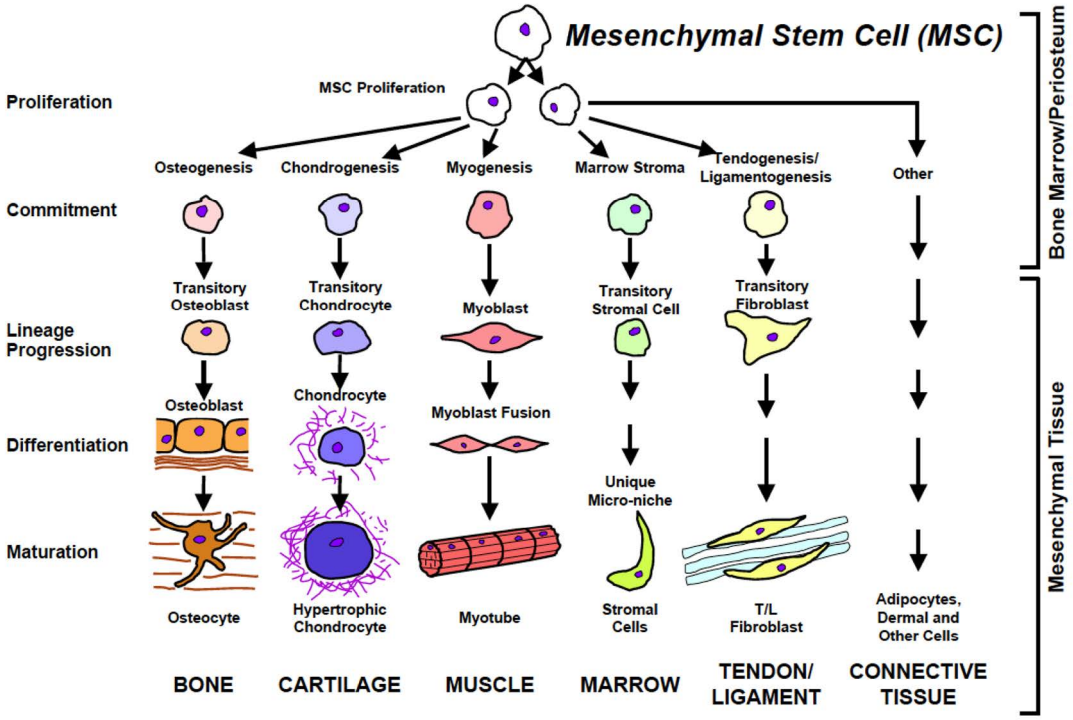

Fig. 6: Differentiation potential of mesenchymal stem cells (MSC)
The MSC differentiation potential comprises a multitude of mesenchymal tissues such as bone, cartilage and muscle [33].

Subsequent studies confirmed the multipotency of these cells by differentiation into cells of bone [14, 177], fat [15], hyaline cartilage [144], and many more. MSC can be isolated via density-gradient centrifugation and plastic adherence. However, a set of surface markers can also be used to isolate more homogeneous cell populations. These markers include immunopositivity for the cell surface protein STRO-1, cluster of differentiation (CD) 73, CD105, CD106, CD145, and CD166, combined with negative immunoreactivity for CD11b, CD31, CD34, CD45, and CD117 [47, 202]. The capability of MSC to differentiate to NP cells was suggested by several groups [128, 199, 260]. But the lack of definitive markers, phenotype and origin of NP cells may hamper MSC-based tissue engineering approaches, as a NP-like phenotype cannot be defined. Although Steck *et al.* suggested that chondrogenic differentiation of MSC leads to a phenotype, which resembles the molecular profile of IVD cells more than articular cartilage [239], a very general set of chondrogenic markers (aggrecan, collagen type II, and the transcription factor Sex determining region Y-box 9 (Sox9)) is always used to characterise chondrogenic differentiation. These markers are expressed by both, NP cells and chondrocytes, which make a clear proof of the discogenic phenotype impossible. Even within the IVD, three different cartilaginous tissues exist with AF, NP and CEP. For the matrix of NP and CEP, the major components of the matrix are the same, but the ratio of proteoglycan to collagen is about 2:1 in the CEP and 27:1 in the matrix of the NP [166] resulting in a large difference in biochemical and functional properties. Other markers such as HIF1α and GLUT-1 [195], CD44 [205], cytokeratin 19 [130, 165], as well as the transcription factors Paired box protein Pax-1 and Forkhead box protein F1 (FOXF1) [164] have been proposed as NP cell markers, but it is not clear if they are suitable for all species and the proof of these factors being markers to validate MSC differentiation to NP cells still needs to be established.

In the present study, both, MSC and NP cells were used for experiments addressing the development of cell-based therapies for NP tissue to compare effects of the different parameters on NP cell and MSC behaviour.

1.3.2. Culture Systems and Cell Carriers

The first aspect after the isolation of cells for tissue engineering approaches is their expansion to increase the cell number, which is typically performed in monolayer culture. During expansion, NP cells dedifferentiate and alter their matrix production [80, 137] and a subsequent proper redifferentiation needs to be confirmed. Furthermore, the usage of foetal calf serum (FCS) during expansion causes the problem of cross-contamination and might induce an immune response [123, 231]. In clinical use, these problems might be solved by the usage of the patient's own serum or human platelet lysate [36, 224].

For adequate (re-)formation of the chondrogenic phenotype, three-dimensional culture is indispensable. Since Pittenger *et al.* established the chondrogenic differentiation of MSC in pellet culture using transforming growth factor β (TGF-β) [191], various methods have been described to culture NP cells or MSC during chondrogenic differentiation. Pellet culture mimics the mesenchymal condensation, which occurs during embryogenesis [198]. However, the high cell density is unphysiological compared to the native cell density in NP tissue. Thus, several carrier systems have been developed using e.g. alginate [38, 203, 205], chitosan [135, 200, 217], polylactid/polyglycolic acid systems [197, 230], collagens [8, 170] or hyaluronic acid [11, 225, 241] where the cell density can be varied. Most of these carrier systems were evaluated regarding their effects in supporting matrix formation. However, their clinical applicability regarding biocompatibility and immunogenicity has to be considered, as well as their mechanical properties after implantation as weight-bearing elements [92, 108].

In the present work, pellet culture was used to analyse effects on the differentiation of MSC. Investigations of environmental effects on NP cells behaviour were tested in alginate beads to simulate the *in vivo* situation without cell-cell contacts. For cell injection approaches an albumin-hyaluronan hydrogel was used as a carrier system.

Furthermore, chondrogenic differentiation of MSC requires the addition of growth factors, which are also known to positively influence disc cell behaviour [183, 237]. Previous studies have documented the beneficial effects of growth factors such as TGF-β [63, 156, 207], insulin-like growth factor 1 (IGF-1) [65, 194], members of the bone morphogenetic protein (BMP) family [133, 287, 291] and growth and differentiation factor 5 (GDF-5) [39, 59, 244]. However, as most growth factors do

not have any clinical approval, their applicability for disc degeneration is limited so far. Therefore, the present study examined the usage of platelet rich plasma (PRP) as it contains high concentrations of growth factors, such as platelet-derived growth factor subunit B dimer (PDGF-BB), IGF and TGF-β [35, 271]. PRP can easily be purified from the patient's own blood, thereby eliminating the possibility of transmitting diseases and simultaneously avoiding the need for animal supplements during the *in vitro* preparation of the tissue-engineered constructs. Studies by Chen *et al.* showed positive effects of PRP on MSC proliferation as well as regenerative effects of PRP in a large animal model of IVD degeneration proposing PRP as a potential therapeutic agent in IVD tissue engineering [36]. The present study focussed on the comparison in the usage of PRP (mixture of several growth factors) compared to TGF-β_1 alone. TGF-β was the first growth factor to be introduced for chondrogenesis of MSC [104, 191]. Beside its ability to promote chondrogenesis, TGF-β was also shown to inhibit osteogenic and adipogenic differentiation of MSC [98, 292]. Disc cells are able to respond to TGF-β by an increase in proteoglycan synthesis and decreased MMP2 secretion [63, 186]. Furthermore, IVD cells are also able to produce TGF-β preferentially on sites of tissue damage as shown in histological studies [173, 252].

1.3.3. Mechanical Loading

The IVD as a load-bearing structure is exposed to daily recurring loads [52]. The biological response to these stimuli varies according to the IVD cell type and the type, magnitude, frequency and duration of loading [127, 229]. Focussing on NP tissue, the major types of *in vivo* loading are compressive and shear stress, as well as hydrostatic pressure due to the swelling properties of NP tissue [174]. Indeed, all of these loading types will occur simultaneously leading to very complex loading combinations *in vivo*. As NP cells experience predominantly hydrostatic pressure *in vivo*, the present study focussed on influences of hydrostatic pressure on NP cells in tissue engineering approaches.

Experiments applying cyclic compression to IVD of mouse [139] or rat tails [90] demonstrated positive influences on extracellular matrix synthesis as long as loading protocols retained their frequency at approximately 1 Hz in brief daily sessions [87, 146, 147, 242]. Excessive loading with higher or lower frequencies

and longer duration led to up-regulation of genes involved in matrix turnover [145, 283] and apoptosis of cells [140]. Several investigators applied external load to NP cells in three-dimensional culture to analyse NP cell reactions to mechanical stimulation [110, 111, 171, 203] and reported positive influences on matrix synthesis. Others used mechanical loading to improve the functionality of tissue-engineered constructs [82, 161, 249].

In general, the response of the cells to mechanical signals and the subsequent transfer and translation into biochemical signals that trigger pathway activation, alterations in gene expression and protein synthesis are known as mechanotransduction [227]. For IVD cells, the mechanotransduction pathways have only been sparsely investigated. Mechanical factors appear to regulate responses of the IVD cells through mechanisms involving intracellular calcium ion flux and cytoskeletal remodelling that may regulate downstream effects such as gene expression and posttranslational biosynthesis [229]. In articular chondrocytes, which are similar to IVD cells, a number of transmembrane receptors, including CD44, anchorin II and integrins have been identified that are involved in mechanotransduction [138, 157, 162, 238]. Le Maitre *et al*. were the first to show that mechanosensing of human NP cells is triggered by $\alpha 5\beta 1$ integrin and is dependent on the degeneration grade [127]. The same group also showed an up-regulation of the mechanosensitive transcription factor cellular Finkel; Biskis; Jinkins (FBJ) osteosarcoma oncogene (c-FOS) after exposure to dynamic hydrostatic pressure in NP cells [126]. Therefore, in the present study, the up-regulation of c-FOS expression was used to verify effects induced by hydrostatic pressure.

1.3.4. Animal Models

For disc degeneration - as for many other diseases - model systems play an important role to analyse the development and progress of pathological alterations and to test novel therapeutic approaches. Compared to cell culture experiments where effects can be demonstrated with isolated cells, animal models allow to study effects *in vivo* within the complete tissue/organism over a certain time period [141]. However, in terms of disc degeneration many differences exist between animal species and humans. Independent of the reason for disc degeneration in animals (naturally occurring [66, 159, 262] or experimentally induced [1, 28, 88,

270]), degenerative changes may differ significantly from those seen in humans as there are differences in cell population, tissue composition, anatomy, development and mechanical properties [9]. The differences in cell population can be explained by differences in the disc development. In humans, the NP is formed by notochordal cells during development but the number of these cells decreases rapidly after birth [86]. By contrast, most other species such as mouse, rat, rabbit and pig retain notochordal cells throughout much of their lifetime [32]. Notochordal cells are completely different from mature human NP cells. They are highly vacuolated and produce a large amount of hyaluronan [85, 240]. Due to their ability to increase proteoglycan synthesis [5], notochordal cells are often seen as progenitor cells which may eventually be able to differentiate to mature NP cells [9]. Hence, experimental data of animals retaining notochordal cells have to be evaluated carefully as they do not display the situation in the adult human. However, recently, several studies were published reporting on the existence of notochordal cells in adult human IVD tissue [165, 209, 276]. This fact changes the long lasting point of view of the disappearance of notochordal cells in humans with age and relativises the arguments against animal models with notochordal cell populations in the NP. However, it has to be stated that there is no naturally occurring disc degeneration in rodents, which makes them an unsuitable model to study repair mechanisms for disc degeneration.

Another limitation of animal models is the difference in anatomy between the species. First of all, most animals are quadruped and mechanical loading occurs in a completely different way. While the upright position of human leads to compressive forces due to the body weight, loading in quadrupeds results from muscle contractions and tension of passive structures resulting possibly in higher forces in animals than humans [279]. Furthermore, disc diameter, height and shape also differ from species to species (Fig. 7). Thus, time should be spent on the selection of the appropriate animal model to study specific aspects of disc biology. Even though these models do not display the unique situation in humans, they can serve to develop new experimental techniques [9].

Fig. 7: Relative sizes of intervertebral discs (IVD) in different species
From left to right: human lumbar IVD, L4–L5; bovine caudal IVD, C1–C2; sheep thoracic IVD, T11-T12; rat lumbar and tail IVD (arrows show the IVD location) [9].

Alternatives to *in vivo* animal models are *in vitro* explant cultures of whole IVD. In recent years, various models were described in the literature using IVD of rat [136, 204], rabbit [71], sheep [58], bovine tails [120, 129] or even humans [60]. These models offer the opportunity to analyse the effect of growth factors, protease inhibitors or cellular therapies directly in the environment within disc tissue under controlled *in vitro* conditions, additionally reducing the number of animals experiments [9]. As IVD of the bovine tail are described to be very similar to human IVD [182], in the present study a bovine organ culture was developed to simulate cell therapies.

1.4. Aim of the Study

As disc degeneration is often associated with back pain, it has a high clinical relevance. The reasons leading to disc degeneration are multifactorial and are influenced by age, genetics, mechanical and nutritional aspects. However, clinical symptoms vary from patient to patient. In general, structural changes within the disc matrix result in a failure of the functional interaction within a very sensible and balanced system. Within a European framework (GENODISC) these complex relationships between the different factors were investigated transnationally.
The overall aims of the GENODISC project were experimental investigations for novel diagnostics, prevention and repair of disc degeneration-linked pathologies. The present work addressed two of the work packages of the project dealing with environmental stress on IVD cells (part A) and the development of repair strategies (part B). The understanding of the environmental stress is very important to rate the feasibility of repair strategies.

Part A of the present study focussed on influences of degeneration-associated alterations of the disc microenvironment on NP cells. As shown before, environmental factors such as glucose concentration, oxygen concentration, pH value and osmolarity affect disc cell metabolism [172, 203, 259, 281] by investigating the short-term effects. In the present study, for the first time, long-term experiments were carried out which allowed redifferentiation of NP cells in three-dimensional culture before environmental changes of glucose and oxygen concentration were applied. The main questions were:

- How does glucose deprivation influence bovine NP cells after long-term three-dimensional culture in alginate beads?
- Does this glucose deprivation also influence mechanosensitivity of bovine NP cells?
- Does long-term adaption to specific oxygen environment (to either 21% oxygen or 6% oxygen) influence NP cell differentiation in pellet culture?
- Does this long-term adaption alter NP cell responses to a subsequent short-term reduction of oxygen?

Part B focussed on the development of strategies for disc regeneration. One aim of the study was to evaluate PRP with regard to its suitability as autologous biomaterial during NP tissue engineering approaches using human MSC or NP cells. Studies by Chen *et al.* showed positive effects of PRP on MSC proliferation as well as regenerative effects of PRP in a large animal model of IVD degeneration making PRP a potential therapeutic agent in IVD tissue engineering [36]. Thus, the influence of medium supplementation with PRP and mechanical loading during NP cell tissue engineering was investigated compared to medium supplementation with TGF-β addressing the following questions:

- Is PRP able to replace TGF-β as supplement of chondrogenic medium and is it able to induce appropriate chondrogenic differentiation?
- Are the effects of chondrogenic medium comparable or differing between MSC and NP cells?
- Does additional mechanical loading with hydrostatic pressure influence chondrogenic differentiation of MSC and NP cells?

Additionally, an organ culture system to simulate cell therapies was introduced and characterised. First of all, the experimental procedure was established. Afterwards, a possible cell therapy was simulated by the injection of either cells alone or cells embedded within an albumin-hyaluronan hydrogel. The following issues were investigated:

- How does long-term cultivation and static loading alter structure and composition of bovine disc organ cultures?
- Does this system allow cell injections and which effects can be observed?

2. Material and Methods

2.1. Material

2.1.1. Reagents

Tab. 1: Reagents

Reagent	Company
0.5% Trypsin/0.2% EDTA (10x)	Biochrom AG
Acetic acid	Roth®
Acetone	AppliChem GmbH
AEC Single Solution	Zytomed Systems GmbH
Alcian Blue 8GX	Sigma-Aldrich®
Alginate	Sigma-Aldrich®
Aquatex®	Merck KGaA
Ascorbate-2-phosphate	Sigma-Aldrich®
Biocoll (1.077 g/ml)	Biochrom AG
Bisbenzimide H33342	AppliChem GmbH
Blasticidin S HCl	invitrogen™
Bovine serum albumin	Sigma-Aldrich®
Carbon dioxide	MIT IndustrieGase AG
Collagenase type I	Sigma-Aldrich®
Dako pen	Dako
Dexamethasone	Sigma-Aldrich®
Dimethyl sulfoxide	Merck KGaA
Disodium hydrogen phosphate	Merck KGaA
DMEM	Gibco®
DMEM-HG	Gibco®
Dulbecco's MEM	Biochrom AG
Dulbecco's MEM without glucose	Biochrom AG
Dulbecco's PBS	PAA Laboratories GmbH
Ethanol	VWR International
Ethylenediamine tetraacetic acid	VWR International
FBS South American Origin	CAMBREX
Foetal Bovine Serum Standard Quality, EU approved	PAA Laboratories GmbH
Foetal Bovine Serum, Origin: EU-approved (South America)	Biochrom AG
Formalin	Merck KGaA
Fungizone® Antimycotic, liquid	Gibco®
Hematoxylin	Merck KGaA
HEPES	Biochrom AG
Hyaluronidase	SERVA Electrophoresis GmbH

Reagent	Company
Hydrochloric acid	AppliChem GmbH
Hydrogen peroxide 30%	Merck KGaA
Insulin	Sigma-Aldrich®
Isopropanol	Fluka
KCl	AppliChem GmbH
L-glutamine	PAA Laboratories GmbH
MEM	invitrogen™
Methanol	Fluka
NEA	Biochrom AG
Nitrogen	MIT IndustrieGase AG
Nuclear fast red	Merck KGaA
Oligo-desoxythymidine primers	Eurofins MWG Synthesis GmbH
Paraffin Paraplast Plus	McCormick™ SCIENTIFIC
Penicillin-Streptomycin, liquid	invitrogen™
Phenol red, 0.5%	Biochrom AG
Platinum® SYBR® Green qPCR SuperMix-UDG	invitrogen™
Primer random $p(dN)_6$	Roche Diagnostics
Proline	Sigma-Aldrich®
Pronase	Roche Diagnostics
Pure water	Ampuwa®
RLT buffer	QIAGEN GmbH
Rnasin® RNase inhibitor	Promega GmbH
Rox reference dye	invitrogen™
Sodium azide	SERVA Electrophoresis
Sodium chloride	Merck KGaA
Sodium dihydrogen phosphate	Merck KGaA
Sodium hydroxide	AppliChem GmbH
Sodium pyruvate	Sigma-Aldrich®
Sodium selenite	Sigma-Aldrich®
Transferrin	Sigma-Aldrich®
Transforming growth factor-β_1, recombinant human	tebu-bio
Triton X-100	Sigma-Aldrich®
Trypan Blue solution	Sigma-Aldrich®
Türk's solution	Merck KGaA
Urea	Merck KGaA
Vitro-Clud®	Merck KGaA
Xylol	Riedel-de Haën
β-Mercaptoethanol	Sigma-Aldrich®

2.1.2. Consumable Supplies

Tab. 2: Consumable supplies

Consumable supplies	Company
20G needle	B. Braun Melsungen AG
Cell culture flasks	nunc™
Cell culture inserts (polycarbonate membrane, 8 µm pore size)	nunc™
Cell culture plates for inserts	nunc™
Cell culture plates	TPP Techno Plastic Products AG
Cell strainer (40 µm)	BD Falcon™
Celluloseacetate membrane 0.2 µm	RENNER GmbH
Combination cap	Fresenius SE & Co. KGaA
CryoTube™ Vials	nunc™
Dual-volume syringe	kindly provided by TETEC AG
Lab-TEK™ Chamber Slides (with detachable top)	nunc™
Luer-lock mixing adapter	kindly provided by TETEC AG
MicroAmp® Fast Optical 96-Well Reaction Plate (0.1 ml)	Applied Biosystems Inc.
Parafilm	Pechiney Plastic Packaging
PCR Sealers™ Microseal® 'B' Film	Bio-Rad Laboratories
SuperFrost Plus® glass slides	Thermo Fisher Scientific Inc.
Syringes	BD Syringes™

2.1.3. Solutions

Tab. 3: Solutions

Solution	Composition
Alginate Solution	1.2% (w/v) alginate in 0.9% NaCl
Aqueous ammonia solution	37 mM NH_4OH
Buffered 4% formalin solution	30 mM NaH_2PO_4 + 45 mM Na_2HPO_4 in 4% formalin, pH 7.0
Dissolving buffer for alginate beads	28 mM EDTA, 150 mM NaCl, pH 8.0
NaCl/KCl	5 M NaCl, 0.4 M KCl
TTBS	50 mM tris, 0.88% NaCl, 0.1% NaN_3, 0.1% Triton X-100, pH 6.7

2.1.4. Primers

Primers were synthesised by Thermo Fisher Scientific (Germany).

Tab. 4: Primers -human-

mRNA	Primer sequence	PCR product size (bp)
Agg forward	5' - TCT GTA ACC CAG GCT CCA AC - 3'	199
Agg reverse	5' - CTG GCA AAA TCC CCA CTA AA - 3'	
bFGF forward	5' - CCG TTA CCT GGC TAT GAA GG - 3'	158
bFGF reverse	5' - ACT GCC CAG TTC GTT TCA GT - 3'	
c-FOS forward	5' - CCG GGG ATA GCC TCT CTT AC - 3'	216
c-FOS reverse	5' - GGT GAG GGG CTC TGG TCT - 3'	
Col1 forward	5' - TGA CCT CAA GAT GTG CCA CT - 3'	197
Col1 reverse	5' - ACC AGA CAT CCC TCT TGT CC - 3'	
Col2 forward	5' - CGC ACC TGC AGA GAC CTG AA - 3'	162
Col2 reverse	5' - TCT TCT TGG GAA CGT TTG CTG G - 3'	
GAPDH forward	5' - GAA GGT GAA GGT CGG AGT C - 3'	224
GAPDH reverse	5' - GAA GAT GGT GAT GGG ATT TC - 3'	
HIF1α forward	5' - CAG CTA TTT GCG TGT GAG GA - 3'	236
HIF1α reverse	5' - CCT CAT GGT CAC ATG GAT GA - 3'	
MMP2 forward	5' - AAG AAC CAG ATC ACA TAC AGG ATC A - 3'	185
MMP2 reverse	5' - GTA TCC ATC GCC ATG CTC C - 3'	
MMP3 forward	5' - GGA GAT GCC CAC TTT GAT GAT - 3'	187
MMP3 reverse	5' - CAT CTT GAG ACA GGC GGA AC - 3'	
MMP13 forward	5' - TTG AGC TGG ACT CAT TGT CG - 3'	172
MMP13 reverse	5' - GGA GCC TCT CAG TCA TGG AG - 3'	
PTN forward	5' - GCA AAC CAT GAA GAC CCA GA - 3'	189
PTN reverse	5' - GGC TTG GAG ATG GTG ACA GT - 3'	
RPS18 forward	5' - TGT GGT GTT GAG GAA AGC AG - 3'	75
RPS18 reverse	5' - AC CGT TCC ACC TCA TCC TC - 3'	
Sox9 forward	5' - AAT CTC CTG GAC CCC TTC AT - 3'	198
Sox9 reverse	5' - GTC CTC CTC GCT CTC CTC CTT CT - 3'	
VEGF forward	5' - TGC AGA TTA TGC GGA TCA AA - 3'	220
VEGF reverse	5' - AAA TGC TTT CTC CGC TCT GA - 3'	

Tab. 5: Primers -bovine-

mRNA	Primer sequence	PCR product size (bp)
Agg forward	5' - ACA GCG CCT ACC AAG ACA AG - 3'	155
Agg reverse	5' - ACG ATG CCT TTT ACC ACG AC - 3'	
Aggrecanase forward	5' - CTC CCA TGA CGA TTC CAA GT - 3'	155
Aggrecanase reverse	5' - TAC CGT GAC CAT CAT CCA GA - 3'	
bFGF forward	5' - GTG CAA ACC GTT ACC TTG CT - 3'	165
bFGF reverse	3' - ACT GCC CAG TTC GTT TCA GT - 3'	
c-FOS forward	5' - CGG CTT TGC AGA CAG AGA TT - 3'	148
c-FOS reverse	5' - CCC CCA CTC AGA TCA AGA GA - 3'	
Col1 forward	5' - TGA GAG AGG GGT TGT TGG AC - 3'	197
Col1 reverse	5' - AGG TTC ACC CTT CAC ACC TG - 3'	
Col2 forward	5' - CCT GTA GGA CCT TTG GGT CA - 3'	145
Col2 reverse	5' - ATA GCG CCG TTG TGT AGG AC - 3'	
GAPDH forward	5' - ACC CAG AAG ACT GTG GAT GG - 3'	178
GAPDH reverse	5' - CAA CAG ACA CGT TGG GAG TG - 3'	
HIF1α forward	5' - AAC TAG CCG GGG GAG AAC TA - 3'	167
HIF1α reverse	5' - ATG GAT GAG GAA TGG GTT CA - 3'	
MMP2 forward	5' - ACC AGA GCA CCA TTG AGA CC - 3'	208
MMP2 reverse	5' - AAC CGT AGC GGA GTC ACA TC - 3'	
MMP3 forward	5' - AAT CAG TTC TGG GCC ATC AG - 3'	237
MMP3 reverse	5' - CTC TGA TTC AAC CCC TGG AA - 3'	
MMP13 forward	5' - CAT GAG TTT GGC CAT TCC TT - 3'	179
MMP13 reverse	5' - GGC GTT TTG GGA TGT TTA GA - 3'	
PTN forward	5' - GCA AAC CAT GAA GAC CCA GA - 3'	189
PTN reverse	5' - GGC TTG GAG ATG GTG ACA GT - 3'	
RPL30 forward	5' - AGG AAG GCT CAA CGA GAA CA - 3'	155
RPL30 reverse	5' - CGA GGA GCA GAA ACC TTC AC - 3'	
VEGF forward	5' - TTG CCT TGC TGC TCT ACC TT - 3'	196
VEGF reverse	5' - ACA CAG GAC GGC TTG AAA AT - 3'	

2.1.5. Antibodies

Tab. 6: Antibodies

Antibody	Produced in	Target species	Specificity	Company
Anti-human Aggrecan AG1-IGD-G2 Domains Antibody	Goat	Human	Agg	R&D Systems®
Anti-human Collagen II Antibody	Rabbit	Human	Col2	Rockland Immunochemicals, Inc.

Antibody	Produced in	Target species	Specificity	Company
Donkey anti-goat IgG	Donkey	Goat	IgG, $F(ab')_2$-B	santa cruz biotechnology®
Goat anti-rabbit IgG	Goat	Rabbit	IgG, $F(ab')_2$	invitrogen™
IgG from goat serum	Goat	-	IgG	Sigma-Aldrich®
IgG from rabbit serum	Rabbit	-	IgG	DIANOVA GmbH

2.1.6. Media

Tab. 7: Media

Medium		Composition
Chondrogenic medium		DMEM-HG (Gibco®) + 2 µl/ml phenol red + 0.1 mM dexamethasone, 0.2 mM ascorbate-2-phosphate, 1 mM sodium pyruvate, 5 mg/ml transferrin, 5 ng/ml sodium selenite, 0.35 mM proline, 1.25 mg/ml BSA, 15 mg/ml insulin, 10 ng/ml TGF-β_1
Glucose media	G0:	Dulbecco's MEM without glucose (Biochrom AG), 1% FCS (Biochrom AG), 2.5% HEPES, 1% L−glutamine, 1% NEA, 1% penicillin-streptomycin, 0.5% Fungizone®, 1% NaCl/KCl
	G1:	90% Dulbecco's MEM without glucose + 10% Dulbecco's MEM (Biochrom AG), 1% FCS (Biochrom AG), 2.5% HEPES, 1% L−glutamine, 1% NEA, 1% penicillin-streptomycin, 0.5% Fungizone®, 1% NaCl/KCl
	G2:	Dulbecco's MEM (Biochrom AG), 1% FCS (Biochrom AG), 2.5% HEPES, 1% L−glutamine, 1% NEA, 1% penicillin-streptomycin, 0.5% Fungizone®, 1% NaCl/KCl
IVD transport medium		DMEM (Gibco®), 1% L−glutamine, 1% NEA, 5% penicillin-streptomycin, 5% Fungizone®, 1.5% NaCl/KCl
MSC expansion medium		Dulbecco's MEM (Biochrom AG) + 10% FCS (CAMBREX), 1% L−glutamine, 1% penicillin-streptomycin, 0.5% Fungizone®
NP expansion medium		DMEM (Gibco®) + 5% FCS (Biochrom AG), 1% L−glutamine, 1% NEA, 1% penicillin-streptomycin, 0.5% Fungizone®, 1.5% NaCl/KCl
SCP1-GFP medium		MEM (invitrogen™) + 10% FCS (PAA), 1% penicillin-streptomycin, 0.5% Fungizone®, 10 µg/ml blasticidin

2.1.7. Kits

Tab. 8: Kits

Kit	Company
Blyscan™ Sulfated Glycosaminoglycan Assay	Biocolor, Ltd.
Omniscript™ RT Kit	QIAGEN GmbH
QIAshredder™	QIAGEN GmbH
Quantikine® Human TGF-β1 Immunoassay	R&D Systems Europe, Ltd.
RNase-Free DNase Set	QIAGEN GmbH
RNeasy™ Mini Kit	QIAGEN GmbH

2.1.8. Equipment

Tab. 9: Equipment

Equipment	Type	Company
Camera	Camedia C-5060 Wide Zoom	OLYMPUS
Camera	DFC420C	Leica
Centrifuge	UniCen FR	Herolab GmbH Laborgeräte
Centrifuge	Biofuge13	Heraeus Holding GmbH
Clean bench	Heraeus LaminAIR®	Heraeus Holding GmbH
Custom-made pressure chamber		Wissenschaftliche Werkstatt Feinwerktechnik, Ulm University
Counting chamber	Neubauer, double	Jordan Gamma GmbH
Embedding device	Histokinette RH/12E	Reichert-Jung
Image processing software	MetaMorph AF, version 1.4.0	Leica
Incubator	BBD 6220	Heraeus Holding GmbH
Incubator	HeraCell 240	Heraeus Holding GmbH
Microscope	DMI6000 B	Leica
Microscope	Olympus IX70	OLYMPUS
Microtome	DDM-0036	MEDIM
Mixing device	Vibrax VXR	IKA®-Werke GmbH & CO. KG
PCR device	Thermocycler	Biometra GmbH
Photometer	infinite M200	Tecan
RT-PCR device	StepOnePlus™ Real-Time PCR System	Applied Biosystems Inc.
Statistical analysis software	JMP™, version 5.0.1.2	SAS Institute Inc.
Water bath		Labortechnik Medingen

2.2. Methods

2.2.1. Isolation/Preparation of Cells

Bovine nucleus pulposus cells

Bovine NP cells, which were included in the current study as a model for healthy IVD cells, were isolated from five to six pooled segments of one cattle tail (n=15, age < 2 years) shortly after slaughtering. Cattle tails were dissected by removing all muscles and ligaments to expose IVD. Subsequently, the whole IVD was prepared under sterile conditions for cell isolation by separating the NP from the adjacent AF (Fig. 8).

NP tissue was digested in 0.6 mg/ml collagenase I in IVD transport medium for up to 5 h at 37 °C under stirring. The obtained cell suspension was passed through a 40 µm cell strainer to remove residual tissue debris, centrifuged at 250 g for 10 min, washed once with 50 ml phosphate buffered saline (PBS) and seeded at 20,000 cells/cm^2 with NP expansion medium in cell culture flasks. Cells were grown to subconfluence, trypsinised and further expanded or cryo-preserved in liquid nitrogen in NP expansion medium containing 20% FCS and 10% dimethyl sulfoxide (DMSO).

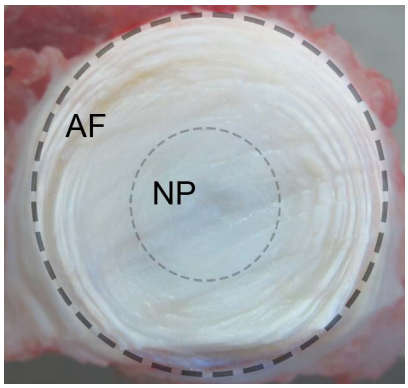

Fig. 8: Bovine intervertebral disc
For cell isolation the nucleus pulposus (NP) was separated from the adjacent annulus fibrosus (AF).

Human nucleus pulposus cells

Human NP cells (n=27, 27-61 years) were isolated from human disc samples obtained from patients undergoing disc surgery after informed consent and approval from the ethics committee of Ulm University, Germany (59/08). NP tissue and AF tissue were separated during surgery and stored in IVD transport medium while being transported to the institute. NP tissue was digested in 0.6 mg/ml

collagenase I in IVD transport medium at 37 °C under stirring for 2-4 h depending on the amount of tissue. The obtained cell suspension was treated as described above for bovine cells.

Human mesenchymal stem cells

Human MSC (n=14, 18-31 years) were obtained from bone marrow aspirates taken either from the proximal tibia during anterior cruciate ligament replacement or from iliac crest during hip replacement (German Armed Forces Hospital, Ulm, Germany) after informed consent in accordance with the terms of the ethics committee of Ulm University, Germany (01/08). Isolation of the cells was performed by density gradient centrifugation and adhesion to tissue culture plastic [191].

Accordingly, 2-3 ml bone marrow aspirate was layered onto 5 ml Biocoll and centrifuged (400 g, 30 min, room temperature). Mononuclear cells were taken from the interphase of the gradient and were washed with 2-3 volumes MSC expansion medium (250 g, 10 min, room temperature). Cells were counted in a counting chamber with Türk's solution and seeded at 80,000-160,000 cells/cm^2 in cell culture flasks with MSC expansion medium. Medium was changed after 24 h and subsequently twice a week. After 7-14 days, several colony-forming unit-fibroblasts (CFU-F) were visible and were cultivated up to one more week. These adherent cell populations forming CFU-F are thought to contain stem cells of non-hematopoietic tissues [56]. To distribute cells, CFU-F were washed twice with PBS and 1 ml trypsin/ethylenediamine tetraacetic acid (EDTA) was added for 1 min at 37 °C. After that, medium was added and changed after 24 h. Cells were grown to subconfluence, trypsinised and further expanded or cryo-preserved in liquid nitrogen in MSC expansion medium containing 20% FCS and 10% DMSO.

2.2.2. Cultivation of Cells

Nucleus pulposus cells

Human and bovine NP cells were treated identically. NP cells stored in liquid nitrogen were thawed in a water bath (37 °C) and added to 9 ml NP expansion medium to dilute DMSO. Cells were centrifuged (250 g, 10 min, room temperature), resuspended in NP expansion medium, counted in a counting

chamber with trypan blue and used for cell expansion. Cells were seeded in cell culture flasks at 3,500 cells/cm^2 and cultured in NP expansion medium at 6% O_2, 37 °C, 8.5% CO_2 and saturated humidity. Medium was changed twice a week. At subconfluence, NP were trypsinised and reseeded for further expansion up to passage 4 or used for experiments.

Mesenchymal stem cells

MSC stored in liquid nitrogen were thawed in a water bath (37 °C) and added to 9 ml MSC expansion medium to dilute DMSO. Cells were centrifuged (250 g, 10 min, room temperature), resuspended in MSC expansion medium, counted in a counting chamber with trypan blue and used for cell expansion. Cells were seeded in cell culture flasks at 2,000-3,500 cells/cm^2 and cultured in MSC expansion medium at 21% O_2, 37 °C, 8.5% CO_2 and saturated humidity. Medium was changed twice a week. At subconfluence, MSC were trypsinised and reseeded for further expansion up to passage 4 or used for experiments.

SCP1-GFP cells

The SCP1-GFP cells were kindly provided by Dr. Denitsa Docheva (Experimental Surgery and Regenerative Medicine, Department of Surgery, Director: Prof. Schieker, Ludwig-Maximilians-University (LMU), Munich, Germany) and described previously [26]. This stable cell line is transfected with the green fluorescent protein (GFP) and is therefore a useful tool for injection experiments in the organ culture system (see 2.2.5.). Due to the fluorescence SCP1-GFP cells can be detected within the non-fluorescent IVD tissue and thus give information about cell distribution and survival of injected cells within the organ culture system.

SCP1-GFP cells stored in liquid nitrogen were thawed in a water bath (37 °C) and added to 9 ml SCP1-GFP medium to dilute DMSO. Cells were centrifuged (250 g, 10 min, room temperature), resuspended in SCP1-GFP medium, counted in a counting chamber with trypan blue and used for cell expansion. Cells were initially seeded in cell culture flasks at 10,000 cells/cm^2 and cultured in SCP1-GFP medium at 21% O_2, 37 °C, 8.5% CO_2 and saturated humidity. Medium was changed twice a week. At subconfluence, SCP1-GFP were trypsinised and reseeded at 1,000 cells/cm^2 for further expansion. Cells between passage 86 and 95 were used for experiments.

2.2.3. Differentiation of Cells

Pellet culture

To induce chondrogenic differentiation of MSC as well as differentiation of NP cells, cells were transferred to three-dimensional pellet cultures for three to four weeks. For this purpose, cells were trypsinised counted and 400,000 cells were transferred into one Eppendorf cup and centrifuged at 250 g for 10 min to form a single pellet. The supernatant was replaced by 500 µl chondrogenic medium and cells were centrifuged at 250 g for further 10 min. Afterwards, the lid of the Eppendorf cup was punctured to allow gas exchange and the cups were cultivated at 37 °C, 8.5% CO_2, saturated humidity and either 6% or 21% O_2 depending on the study design (see 2.3.). The medium was changed twice a week.

Alginate beads culture

Alginate beads were used as further three-dimensional culture system for chondrogenic differentiation. For this purpose, cells were trypsinised, counted and 4,000,000 cells/ml were resuspended in alginate solution. For polymerisation, the cell-alginate solution was transferred into a syringe and dropped through a 22 G needle in a stirred 102 mM $CaCl_2$-solution. Hence, cells were completely encapsulated by alginate. After 10 min of polymerisation, alginate beads were washed three times with PBS and 40 alginate beads each were transferred into one well of a 6-well plate and covered with 5 ml of chondrogenic medium. The alginate beads were incubated at 37 °C, 8.5% CO_2, saturated humidity and either 6% or 21% O_2 depending on the study design (see 2.3.). The medium was changed twice a week.

2.2.4. Mechanical Loading

Hydrostatic pressure was applied to simulate physiological load of the NP within the spine. After chondrogenic differentiation, depending on the study design (see 2.3.) a part of the samples was exposed once to mechanical stimulation by hydrostatic pressure. Seven pellets or 40 alginate beads were transferred into syringes containing 3 or 5 ml of study specific medium, respectively. The cap of

the syringes were sealed with parafilm and the syringes were put into a custom-made pressure chamber filled with 37 °C tempered fluid (Fig. 9) as described recently by our group [203]. Cyclic hydrostatic pressure was applied for 30 min with 2.5 MPa and 0.1 Hz. This corresponds to a quite high physiological load, occurring for example when lifting a load with a round back [278]. Control cultures were maintained under equal, but mechanically unloaded conditions in an incubator.

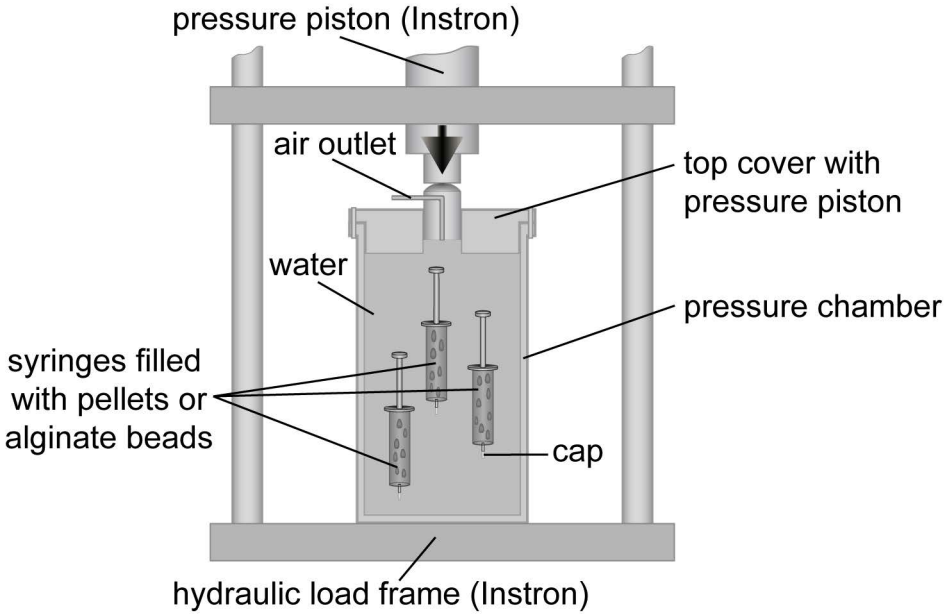

Fig. 9: Schema of the hydrostatic pressure device
Syringes filled with medium and pellets or alginate beads were put in a water-filled pressure chamber. Cyclic hydrostatic pressure was applied via a pressure piston.

2.2.5. Organ Culture

Albumin-hyaluronan hydrogel

The albumin-hyaluronan hydrogel was kindly provided by Prof. Jürgen Mollenhauer (Natural and Medical Sciences Institute at the University of Tübingen, Germany) and tested as a carrier system to inject cells into the organ culture system. The defined composition and the production process of the gel was described by Scholz *et al.* [225]. In the present study, hydrogels were prepared in the following steps. First, high molecular weight hyaluronic acid was mixed at a ratio of 1:3 with the respective suitable cell culture medium for target cells, which will be seeded in the hydrogel afterwards, vortexed and centrifuged at 250 g for 5 min to prepare the hyaluronic acid stock solution. Afterwards, the final

albumin-hyaluronan hydrogel was prepared by mixing 75% hyaluronic acid stock solution with 8.75% human maleimido-albumin and 16.25% cell suspension or medium for cell-free albumin-hyaluronan hydrogels. The cell suspension was prepared in the respective suitable cell culture medium and seeded accordingly resulting in a final cell concentration of 500,000 NP cells per ml hydrogel or 800,000 MSC or SCP1-GFP cells per ml hydrogel. The final albumin-hyaluronan hydrogel and the polymerisation agent were mixed at a ratio of 4:1 in a dual-chamber syringe with a Luer-lock mixing device and a 20 G needle (Fig. 10a). Through this system approximately 50 µl of the hydrogel were injected into IVD punches in the organ culture system described below (Fig. 10b-d).

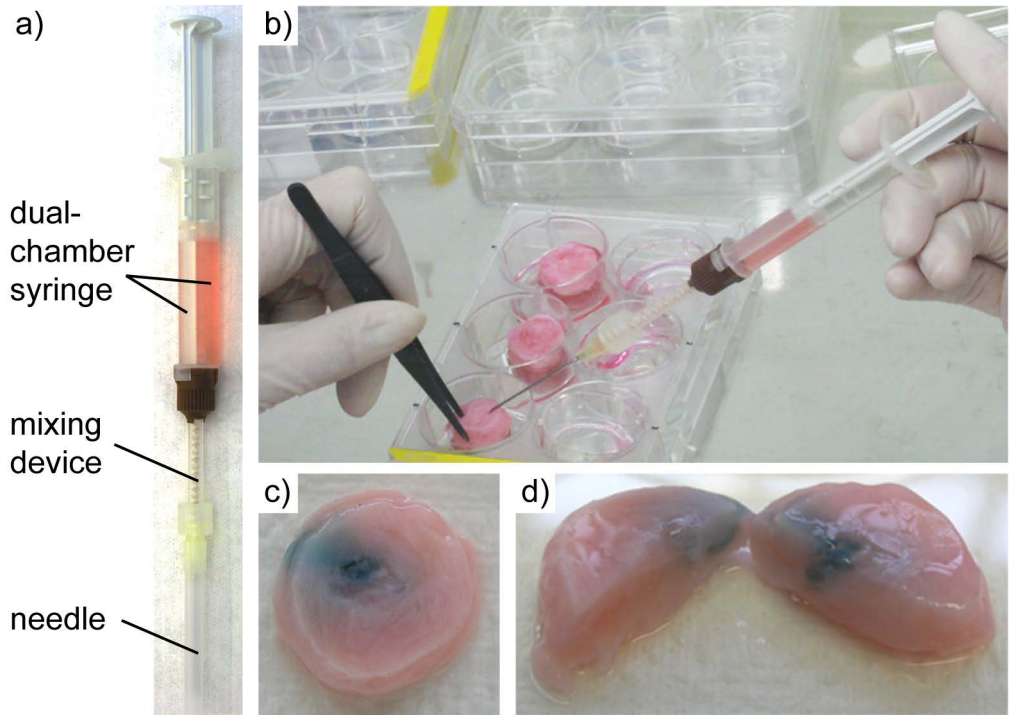

Fig. 10: Injection of albumin-hyaluronan hydrogel into the intervertebral disc (IVD) punches
The albumin-hyaluronan hydrogel was injected into the centre of the IVD punches with a dual-chamber syringe with a mixing adaptor and fixing the punches with a sterile forceps (a). Injection with a coloured albumin-hyaluronan hydrogel show disposition of the gel within the IVD punch (b).

Organ culture system

An organ culture system was established to provide a tool for *in vitro* testing of cell therapies addressing disc regeneration. In separate approaches the experimental set-up was established and first experiments for its suitability were performed. Cattle tails (n=14, age < 2 years) were dissected by removing all muscles and ligaments to expose caudal IVD. Subsequently, the whole IVD was prepared

under sterile conditions separating it from the adjacent vertebral bodies by dissection of the disc tissue as close as possible to the CEP. In preliminary experiments several approaches were tested. The cultivation of whole discs was compared to standardised punched discs (diameter 13 mm, see Fig. 11b) with an intact NP and few surrounding AF lamellae. Due to their standardised size, IVD punches were used in all subsequent experiments. IVD punches were cultured for up to five weeks in NP expansion medium at 6% O_2, 37 °C, 8.5% CO_2 and saturated humidity in differing experimental set-ups. First, static loading (Fig. 11c, left) using 6-well plates with cell culture inserts was compared to unloaded conditions (Fig. 11c, right). Secondly, IVD were needle-punctured under x-ray control prior to preparation to induce degenerative processes as described before [121] and facilitate subsequent injection tests (Fig. 11a). Thirdly, cell therapy was simulated by injecting either a carrier-free cell suspension of SCP1-GFP cells in SCP1-GFP medium, cells seeded in an albumin-hyaluronan hydrogel or the albumin-hyaluronan hydrogel without additional cells prepared as described above.

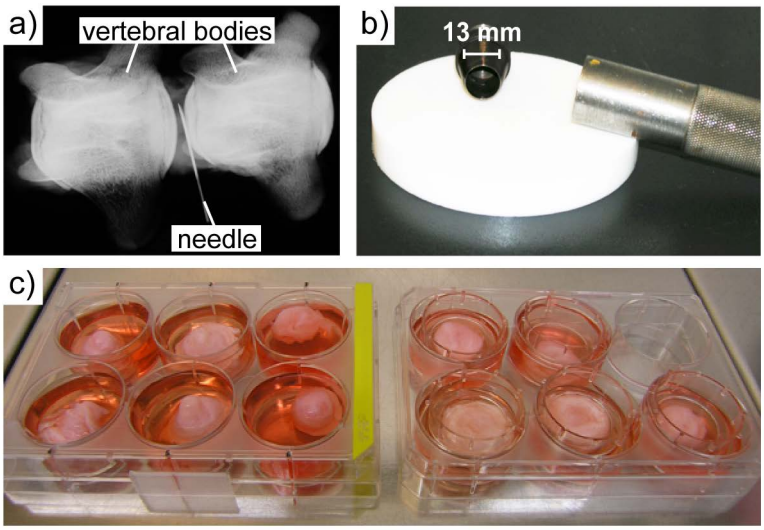

Fig. 11: Experimental set-ups for the organ culture system
Different experimental set-ups were tested to establish the organ culture system. In a first step, effects of needle puncture of the disc within one motion segment under x-ray control (a) were tested. Furthermore, the comparison of 13 mm punches (b) against whole discs as well as unloaded (c, left) *versus* loaded (c, right) culture were tested for their influences on organ culture.

2.2.6. Gene Expression

The analysis of mRNA expression in cells was performed to obtain information about chondrogenic differentiation of MSC, NP cell differentiation as well as changes in mRNA expression due to influences of the cells' environment.

RNA isolation

Cell lysates were collected at day 0 (500,000 cells) and at the end of the respective cell experiment. Cells in suspension were centrifuged at 250 g for 5 min and the pellet obtained was lysed in 350 µl RLT buffer containing 10 µl/ml β-mercaptoethanol. For RNA isolation from pellet cultures, five pellets per group were pooled and treated mechanically with a pestle prior to lysis. Alginate beads were dissolved in 3 volumes of dissolving buffer for 10 min. Afterwards, the solution was centrifuged at 250 g for 5 min and the cell pellet obtained was lysed in 350 µl RLT buffer containing 10 µl/ml β-mercaptoethanol. Lysates were either processed directly or stored at -80 °C. For RNA isolation, lysates were homogenised with QIAshredder™ (except for lysates of pellet cultures) and total RNA was isolated using the RNeasy™ Mini Kit and RNase-Free DNase Set, all according to the manufacturer instructions. RNA concentration was measured photometrically.

cDNA synthesis

1 µg RNA was transcribed into cDNA using the Omniscript™ RT Kit completed with oligo-deoxythymidine primers (5 µM), random hexamer primers (50 µM) and RNase inhibitor (10 units) in a total volume of 20 µl according to the manufacturer instructions. The obtained cDNA was diluted at a ratio of 1:10 in water and used for polymerase chain reaction (PCR).

Real-time reverse transcriptase polymerase chain reaction

Real-time reverse transcriptase polymerase chain reaction (RT-PCR) was performed in a total volume of 25 µl in 96-well plates sealed with tape. Samples contained 12.5 µl Platinum® SYBR® Green qPCR SuperMix-UDG, 0.5 µl Rox reference dye, 0.4 µM of each primer and 2 µl cDNA. Specific primer pairs (Tab. 4 and 5) were designed using published gene sequences (PubMed, NCBI Entrez

Nucleotide Database). For the analysis of the mRNA expression, cloned amplification products were provided and used as standards for real-time RT-PCR (Cycling: 50 °C for 2 min; 95 °C for 2 min; 40 cycles with 95 °C for 15 sec, 60 °C for 1 min and 95 °C for 15 sec; 60 °C for 1 min; melting curve: heating in 0.3 °C steps from 60 °C to 95 °C; 95 °C for 15 sec). The amount of each respective amplification product was determined relative to the house-keeping genes glyceraldehyde-3-phosphate dehydrogenase (GAPDH, bovine or human), ribosomal protein L30 (RPL30, bovine) or ribosomal protein S18 (RPS18, human). Normalised values of samples collected at the end of the experiments were compared either between the different experimental groups, to the control group, or between mechanically stimulated and non-stimulated samples.

2.2.7. Histology

Stainings were documented via bright field microscopy.

Alcian blue staining

Chondrogenic differentiation of MSC as well as differentiation of NP cells in pellet cultures and organ culture samples were analysed in paraffin sections for proteoglycans. Incubation with the cationic dye alcian blue stains polyanionic GAG chains of proteoglycans of the extracellular matrix [226]. Proteoglycans, in addition to collagens, represent a major component of the cartilage matrix [61] and were therefore investigated here.

For the preparation of the paraffin sections, pellets or IVD punches were washed with PBS and fixed with buffered 4% formalin solution for 2 h or two days, respectively, washed with PBS and dehydrated in a graded series of ethanol (70%, 96% and 100% for 2 h each). IVD punch embedding continued directly with the washing step with acetone, while pellets were incubated over night in 100% ethanol, before continuing with a washing step with acetone and incubation in acetone for 2 h. Subsequently, samples were incubated with liquid paraffin for 2 h, embedded in paraffin blocks and air-dried. Paraffin sections with a thickness of approximately 7 µm were prepared and mounted on glass slides.

Prior to staining, sections were deparaffinised at 60 °C for 15 min, then washed twice in xylol for 5 min each and hydrated in a graded series of ethanol (100%, 96%, 70% and 50% for 5 min each). After washing with pure water and acidifying

with 3% acetic acid for 5 min, sections were stained with 1% alcian blue in 3% acetic acid for 30 min and washed with pure water. Counterstaining was carried out with nuclear fast red for 3 min. Sections were washed with pure water, 96% ethanol and twice with 100% ethanol and were incubated with xylol for 5 min before being mounted with Vitro-Clud® and a cover glass.

Immunocytochemical detection of aggrecan and collagen type II

Aggrecan, the main proteoglycan and collagen type II, the main collagen of NP tissue [255], were detected immunocytochemically by visualising the binding of the primary antibody via the horseradish peroxidase (HRP) reaction [193]. Sections of pellet cultures were prepared and deparaffinised as described above.

After washing with pure water and PBS, sections were encircled with a Dako pen and washed with pure water. For collagen type II staining, two digestion steps were inserted: sections were digested with 2 mg/ml hyaluronidase in PBS (pH 5.5) for 15 min at 37 °C, washed three times with PBS and further digested in 1 mg/ml pronase in PBS (pH 7.4) for 30 min at 37 °C. Thereafter, endogenous peroxidases were blocked with 3% hydrogen peroxide in methanol for 15 min. Sections were washed twice with pure water and once with tris-triton buffered saline (TTBS) before blocking with 2% bovine serum albumin (BSA) in TTBS for 60 min. The primary antibody (goat anti-human Aggrecan, 5 µg/ml in PBS with 1% BSA or rabbit anti-human collagen II, 20 µg/ml in PBS with 1% BSA) or control antibody (goat IgG or rabbit IgG in the same concentration) was added over night at 4 °C. After washing three times with TTBS, the secondary antibody (donkey anti-goat IgG/goat anti-rabbit IgG) was added for 30 min. After washing again three times with TTBS, Streptavidin-HRP-Conjugate (from ZytoChem HRP Kit) was added. Sections were washed three times with TTBS and 3-amino-9-ethylcarbazole (AEC) Single Solution was added up to 15 min until red staining was observed under the microscope. Washing twice with pure water stopped the reaction and counterstaining was carried out with haematoxylin for 1 min. After washing again twice with pure water, aqueous ammonia solution was added shortly. Pellet sections were washed with pure water and mounted with Aquatex® and a cover glass.

2.2.8. Biochemical Assays

Detection of glycosaminoglycans in cell culture supernatants (DMMB assay)

The amount of GAG released by the IVD punches into the cell culture medium during organ culture was analysed using Blyscan™ Sulfated Glycosaminoglycan Assay following the manufacturer instructions based on the binding of 1,9-dimethylmethylene blue (DMMB) to GAG [243]. Briefly, a dilution series of chondroitin 4-sulfate standards was prepared. Cell culture supernatants were centrifuged at maximum speed for 10 min to remove debris. Subsequently, 100 µl of each sample were transferred to an Eppendorf cup. Depending on the sample, 1:50 and 1:100 dilutions of the sample in cell culture medium were measured, too. Each sample was measured in duplicate. Subsequently, 1 ml Blyscan dye reagent was added to each Eppendorf cup and cups were mixed gently for 30 min. During this time period, an insoluble sulphated GAG-dye complex formed and precipitated from the unbound soluble dye. After centrifugation at maximum speed for 10 min, the supernatant was removed carefully and 1 ml of dissociation reagent was added to each cup to solubilise the precipitated pellet. After the complex had dissolved, standards and samples were transferred to a 96-well microtitre plate and absorbance was measured at 656 nm. GAG content was calculated referring to the standard curve.

Proliferation assay

In the course of the analysis of PRP, the potential of the culture media to promote proliferation of MSC was tested by a growth curve assay. 3,500 MSC/cm^2 were seeded in 6-well plates and cultured with 2 ml of chondrogenic medium supplemented with either 10 ng/ml TGF-β_1 or 10% pooled human PRP. Medium was changed twice a week. Cell morphology was evaluated microscopically and the cell number was counted by haemocytometer in duplicate at various time points after cell seeding.

Determination of TGF-β_1 concentration in platelet-rich plasma

TGF-β_1 concentration in PRP was analysed quantitatively using a Quantikine enzyme-linked immunosorbent assay (ELISA) kit following the manufacturer instructions. Briefly, a dilution series of TGF-β_1 standards was prepared. 100 µl of

PRP samples were activated with 100 µl 2.5 N acetic acid/10 M urea, incubated at room temperature for 10 min and neutralised by the addition of 100 µl 2.7 N NaOH/1 M 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES). Standards and samples were added to a 96-well microtitre plate coated with TGF-β_1 receptor II. By adding a polyclonal antibody against TGF-β_1 conjugated to horseradish peroxidase, binding was visualised via a chromogen reaction. After addition of the substrate solution, colour intensity correlated with the amount of TGF-β_1 that was initially bound.

2.2.9. Statistical Analysis

A paired non-parametric Wilcoxon signed rank test was performed to determine differences in data of gene expression normalised to house-keeping genes. The test was used to point out differences between data of day 0 compared to the end of the experiment, between different groups analysed within one experiment, as well as to show differences between mechanically stimulated and non-stimulated samples. Statistical differences of $p < 0.05$ were considered significant. Multiple testing was not considered. The number of samples is indicated at each individual analysis. Gene expression analyses are expressed as individual values with the median marked in red if not indicated otherwise.

2.3. Specific Study Design

The present studies were dealing with aspects of disc degeneration (part A) and regeneration (part B). For these two parts specific experimental designs were developed.

2.3.1. Influence of Environmental Factors During Disc Degeneration

Part A focussed on environmental changes during disc degeneration. The effects of glucose concentration and oxygen on NP cells were investigated.

Effect of glucose concentration on nucleus pulposus cells

This study focussed on effects of glucose concentration as reduced glucose supply is considered to be a key contributor to disc degeneration due to calcification of the CEP blocking the main route of transport of nutrients and metabolites [21]. The purpose of this study was to investigate the influence of glucose deprivation in healthy bovine NP cells (Fig. 12). After long-term alginate beads culture in high-glucose chondrogenic medium for 23 days, part of the alginate beads were transferred to NP expansion medium with less glucose and without TGF-β_1 for three days. Subsequently, glucose concentration was further reduced for 24 h followed by a single mechanical stimulation with hydrostatic pressure. Gene expression of the mechanosensitive transcription factor c-FOS as well as matrix molecules and MMPs was analysed to further elucidate the complex role of the glucose environment in the pathogenesis of disc degeneration.

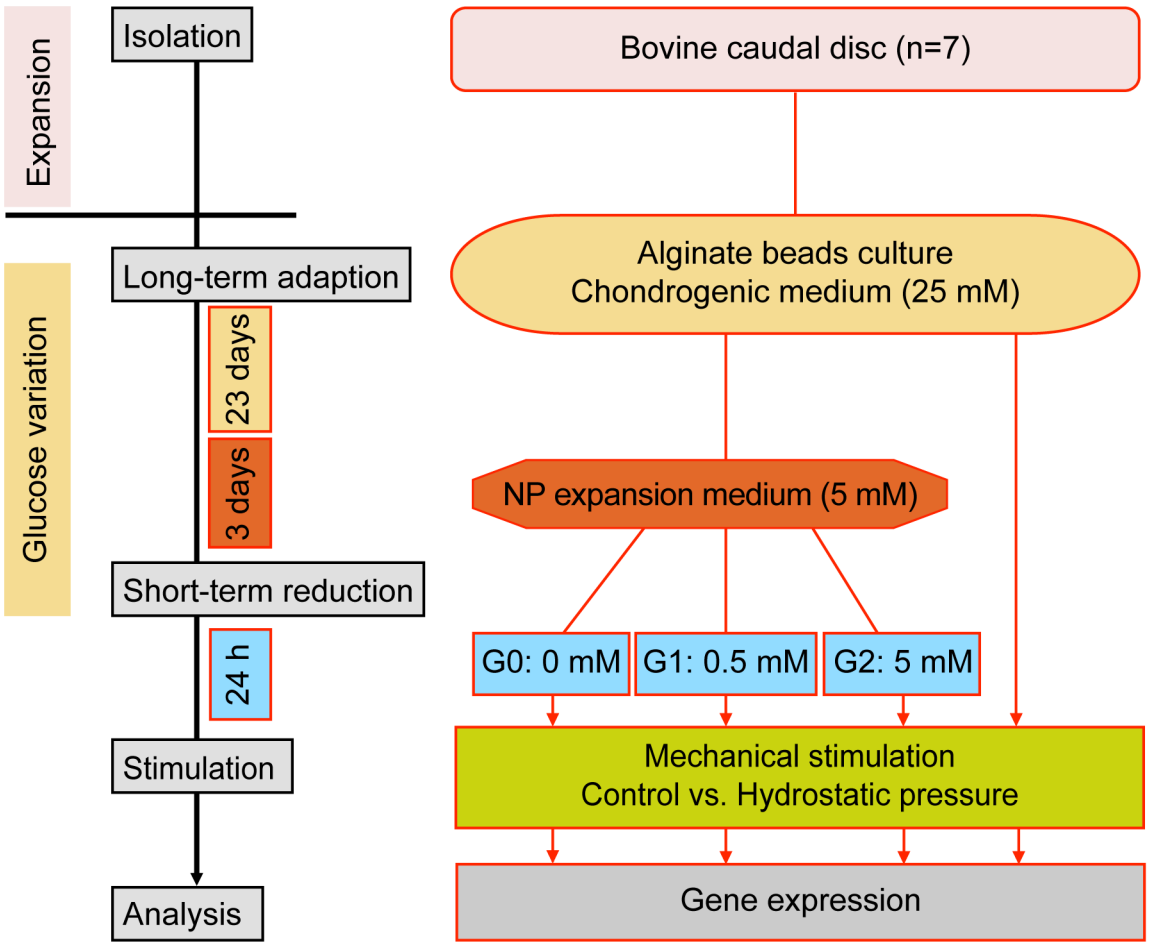

Fig. 12: Study design to analyse effects of glucose environment
Analysis of glucose environment included investigations of different glucose concentrations and aspects of mechanical stimulation.

Effect of oxygen concentration on nucleus pulposus cells

This study focussed on oxygen supply as one critical factor for NP cell metabolism, which is reduced due to disc degeneration. Although it is known that cells of the NP are adapted to low oxygen concentrations *in situ* because of the avascular environment of the disc, several *in vitro* studies have been carried out at disc-unphysiological 21% oxygen [5, 75, 119, 284]. Therefore, the effect of oxygen concentration over a long-term adaption in pellet culture was investigated comparing disc-unphysiological 21% oxygen (group A) with disc-normoxic 6% oxygen (group B) in chondrogenic medium (Fig. 13). Human NP cells from degenerated tissue were compared to healthy bovine caudal NP cells. In addition, the effects on gene expression levels after a further short-term reduction to 1% oxygen were investigated, thereby simulating decreased oxygen supply as it may occur during degeneration. Gene expression of matrix molecules, as well as angiogenic factors and MMPs was analysed to further elucidate the complex role of the oxygen environment in the pathogenesis of disc degeneration.

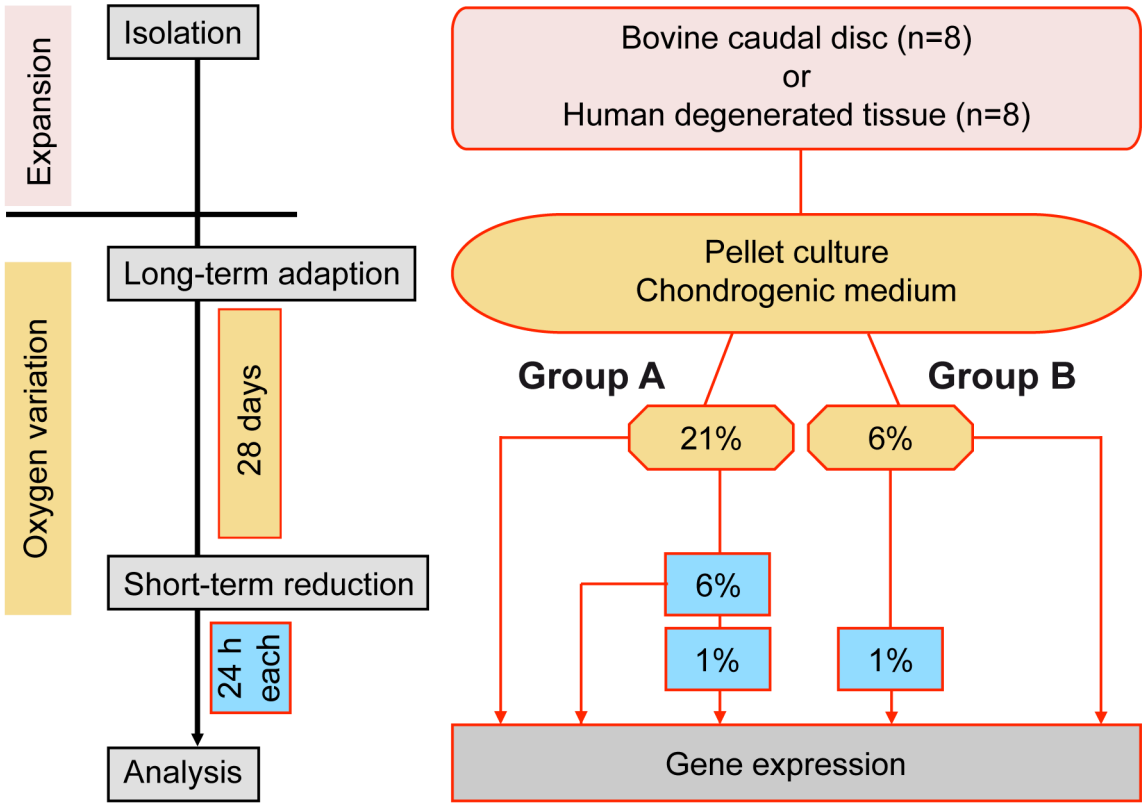

Fig. 13: Study design to analyse effects of oxygen environment
Analysis of oxygen environment included comparison of gene expression after cultivation at different oxygen concentrations.

2.3.2. Strategies of Disc Regeneration

Beside the establishment of an organ culture system to simulate cell therapies, the influence of medium composition and mechanical loading during NP cell tissue engineering was investigated in part B to reveal new insights for disc regeneration.

Effect of platelet-rich plasma and hydrostatic pressure

The aim of the study was to evaluate PRP with regard to its suitability as autologous biomaterial during NP tissue engineering approaches. For PRP preparation, whole blood-derived pooled platelet concentrates from 20 patients were processed to obtain 1 litre of pooled PRP, which was used throughout the complete study. The PRP was kindly provided by Prof. Hubert Schrezenmeier, Director of the Institute of Transfusion Medicine in Ulm, Germany.

The influence of chondrogenic medium composition on the differentiation of human MSC to a NP-like phenotype was compared by using supplementation with either 10% pooled human PRP or 10 ng/ml recombinant TGF-β_1. Furthermore, the efficiency of the differentiation in two three-dimensional culture systems (pellet culture *versus* alginate beads) and the influence of additional hydrostatic pressure was evaluated (Fig. 14). Due to the lack of an adequate NP cell marker to confirm differentiation of MSC and the alternative possibility to use NP cells for tissue engineering approaches, all experiments with MSC were carried out simultaneously with NP cells to compare effects of the different parameters on NP cell and MSC behaviour.

The proliferative capacity of PRP was investigated by growth curve analysis for MSC and human NP cells.

Results were analysed microscopically, by gene expression analysis and by (immuno-)histology.

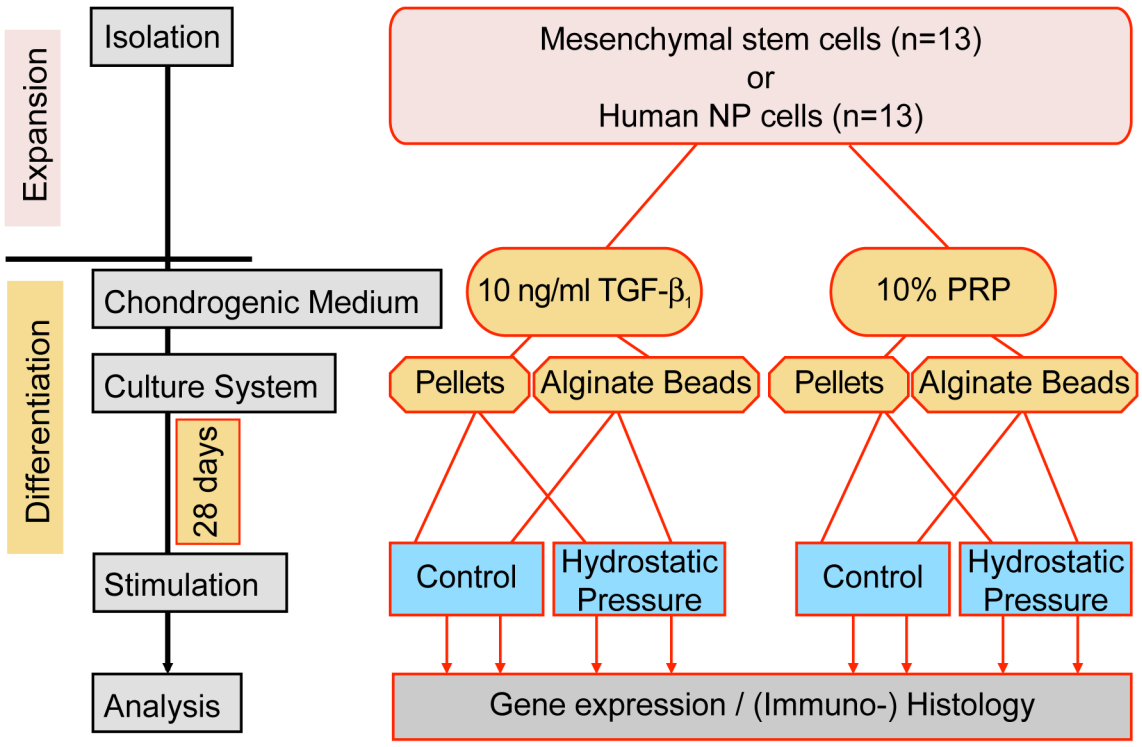

Fig. 14: Study design to analyse effects of platelet-rich plasma (PRP)
Analysis of PRP included investigations of different compositions of culture media, different three-dimensional cultures and aspects of mechanical stimulation.

3. Results

Part A - Influence of Environmental Factors During Disc Degeneration

Part A of this work focussed on environmental changes during disc degeneration. The effects of the glucose concentration and oxygen on NP cells as environmental factors and their role during disc degeneration were investigated.

3.1. Effect of Glucose Concentration on Nucleus Pulposus Cells

With progressing disc degeneration and calcification of the CEP the main route of transport of nutrients and metabolites is impaired. The consequential reduced glucose supply is supposed to be a key contributor to disc degeneration. Thus, the influence of glucose deprivation on NP cells with and without additional application of mechanical loading was investigated by mRNA expression analysis.

After three weeks of differentiation in high-glucose chondrogenic medium (25 mM glucose), NP cell gene expression of aggrecan, collagen type II and collagen type I was up-regulated by trend compared to day 0 (Fig. 15a). MMP2 expression by contrast increased significantly and MMP3, MMP13, and aggrecanase expression was decreased significantly (Fig. 15b).
Subsequent reduction of glucose concentration from high-glucose medium to NP expansion medium (5 mM glucose) for three days decreased expression of collagen type II and collagen type I significantly, even below levels of day 0. Further reduction of glucose concentration from NP expansion medium (5 mM glucose, 5% FCS) to one of the glucose media (1% FCS + G0: 0 mM, G1: 0.5 mM, G2: 5 mM glucose) for 24 h revealed a trend towards a positive correlation between glucose concentration and gene expression of matrix molecules (Fig. 15a). The same changes were shown for all matrix degrading enzymes that were investigated (Fig. 15b).

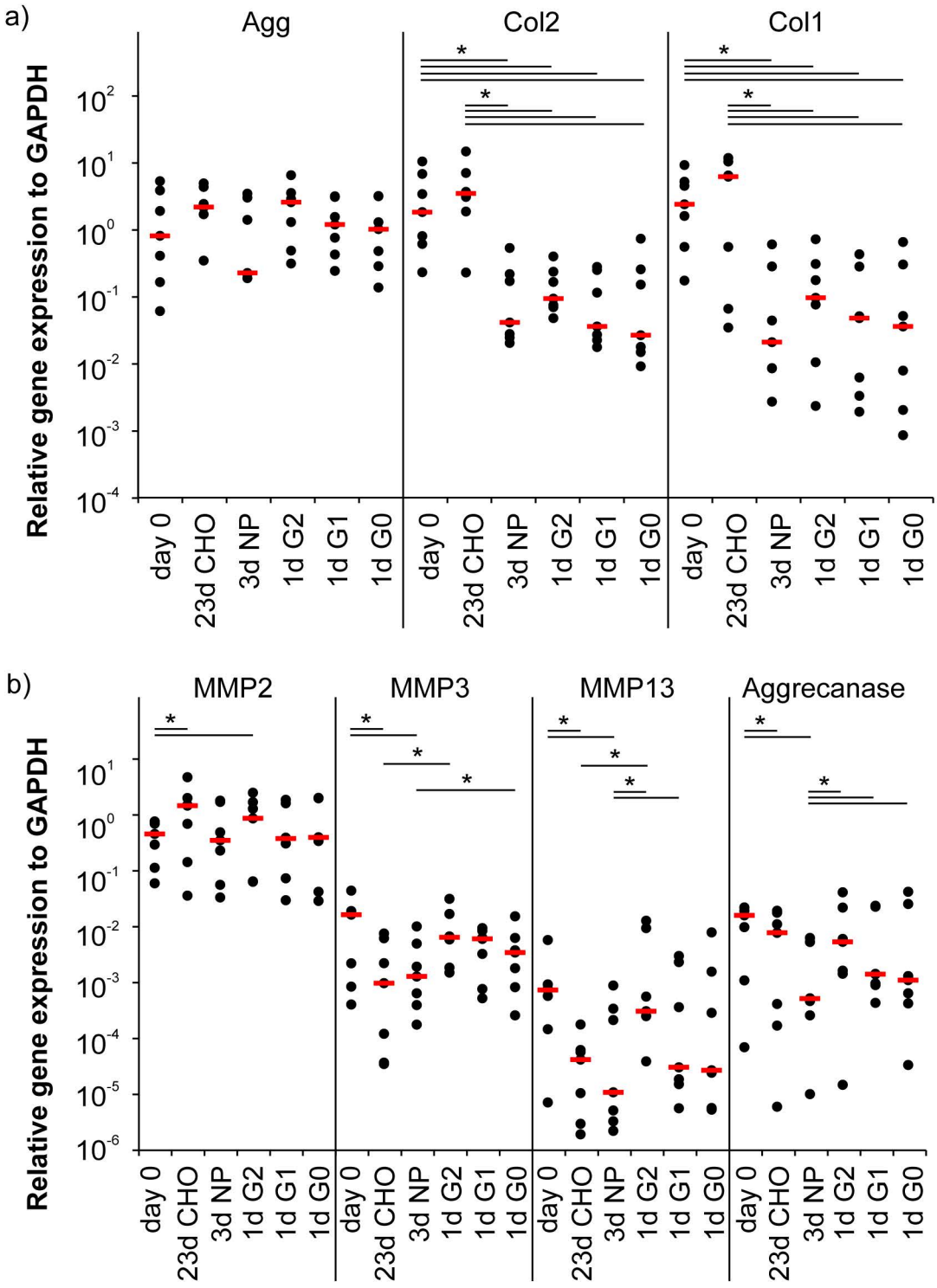

Fig. 15: Quantitative analysis of gene expression after glucose reduction
mRNA expression of the matrix molecules aggrecan (Agg), collagen type II (Col2) and collagen type I (Col1) (a) and the matrix degrading enzymes matrix metalloproteinase 2 (MMP2), matrix metalloproteinase 3 (MMP3), matrix metalloproteinase 13 (MMP13) and aggrecanase (b) after short-term reduction (three days) of glucose concentration from chondrogenic medium (CHO, 25 mM) to NP expansion medium (NP, 5 mM) followed by either glucose media G2 (5 mM), G1 (0.5 mM) or G0 (0 mM). Black dots represent gene expression values of every single donor, red bars represent the median of all gene expression values, n=7, * significant difference between different media and points in time, $p < 0.05$.

Mechanical stimulation by hydrostatic pressure demonstrated great differences between the four media used. The greatest mechanically induced effects were shown in NP cells, which were not exposed to glucose reduction and were maintained in high-glucose medium (25 mM) supplemented with TGF-β_1 (CHO) (Fig. 16a). Within this group, mechanical loading increased c-FOS and collagen type I significantly, whereas aggrecan and collagen type II expression increased more moderately. In contrast, gene expression of MMP2, MMP3, MMP13 and aggrecanase tended to decrease within this group after mechanical stimulation (Fig. 16b). In cells exposed to glucose reduction, the mechanically induced changes in gene expression were minimal and no distinct correlation with glucose concentration could be pointed out.

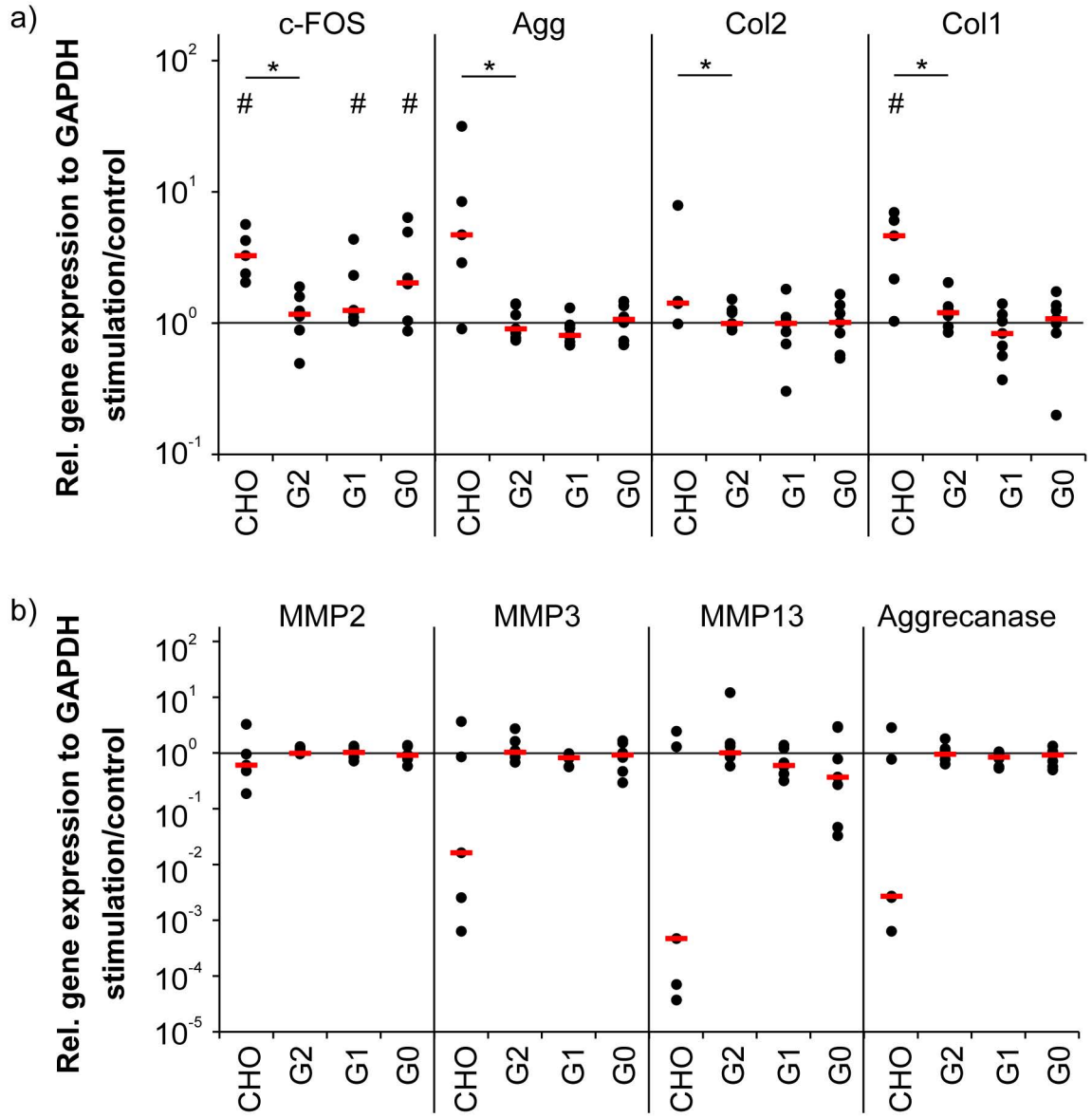

Fig. 16: Quantitative analysis of chondrogenic differentiation after mechanical stimulation
mRNA expression after mechanical stimulation with hydrostatic pressure. The mechanosensitive transcription factor c-FOS as well as the matrix molecules aggrecan (Agg), collagen type II (Col2), collagen type I (Col1) (a) and the matrix degrading enzymes matrix metalloproteinase 2 (MMP2), matrix metalloproteinase 3 (MMP3), matrix metalloproteinase 13 (MMP13), and aggrecanase (b) from individual donors were analysed after short-term reduction of glucose concentration followed by a single mechanical stimulation with hydrostatic pressure in chondrogenic medium (CHO, 25 mM) or glucose media G2 (5 mM), G1 (0.5 mM) or G0 (0 mM). The ratio of stimulation to control indicates the multiplication of induction after mechanical stimulation. Black dots represent gene expression values of every single donor, red bars represent the median of all gene expression values, n=5-7, # significant differences between stimulation and control, * significant difference between different media, $p < 0.05$.

3.2. Effect of Oxygen Concentration on Nucleus Pulposus Cells

In the avascular IVD, and particularly in the NP, oxygen concentration is very low. During disc degeneration oxygen supply might further decrease. On the other hand, during cell culture unphysiologically high oxygen concentration are often applied. Thus, the influence of oxygen environment on NP cells was investigated by mRNA expression analysis.

Long-term adaption

The impact of oxygen concentration on long-term differentiation of NP cells was compared by exposing them to either disc-hyperoxic 21% oxygen (group A) or disc-normoxic 6% oxygen (group B). Relative mRNA expression of matrix molecules, matrix degrading enzymes and angiogenic factors was analysed in relation to values at day 0 (Fig. 17). Due to limited cell numbers, not all of the parameters could be investigated for each donor resulting in varying sample sizes.

The mRNA expression of matrix molecules of bovine and human NP cells was altered by differentiation based on chondrogenic medium (Fig. 17a+d). In bovine NP cells, expression of aggrecan and collagen type II was significantly up-regulated at 21% and 6% oxygen compared to day 0. Collagen type I expression only increased significantly at 21% oxygen. The alterations of aggrecan and collagen type I were at 6% oxygen significantly lower than at 21% oxygen (Fig. 17a). In human NP cells, collagen type II and the transcription factor Sox9 were significantly up-regulated at 21% and 6% oxygen compared to day 0 while aggrecan expression only at 6% oxygen increased significantly. Collagen type I expression at 6% oxygen was significantly lower than at 21% (Fig. 17d).

The mRNA expression of matrix degrading enzymes after differentiation showed high individual variations and effects differed between bovine (Fig. 17b) and human NP cells (Fig. 17e). In bovine NP cells, expression of MMP3, MMP13 and aggrecanase remained unaltered or decreased. Expression of MMP13 and aggrecanase was significantly lower in cells exposed to 6% oxygen (Fig. 17b). In human NP cells, expression of MMP3 increased due to differentiation. The increase of MMP13 was weaker at 6% oxygen (Fig. 17e).

The changes in mRNA expression of angiogenic factors after differentiation were greatest for PTN that was significantly down-regulated in NP cells of both species

(Fig. 17c+f). In both species, the expression of HIF1α and angiogenic factors was lower at 6% than at 21% oxygen. Only the expression of VEGF in human NP cells was higher at 6% oxygen (Fig. 17f).

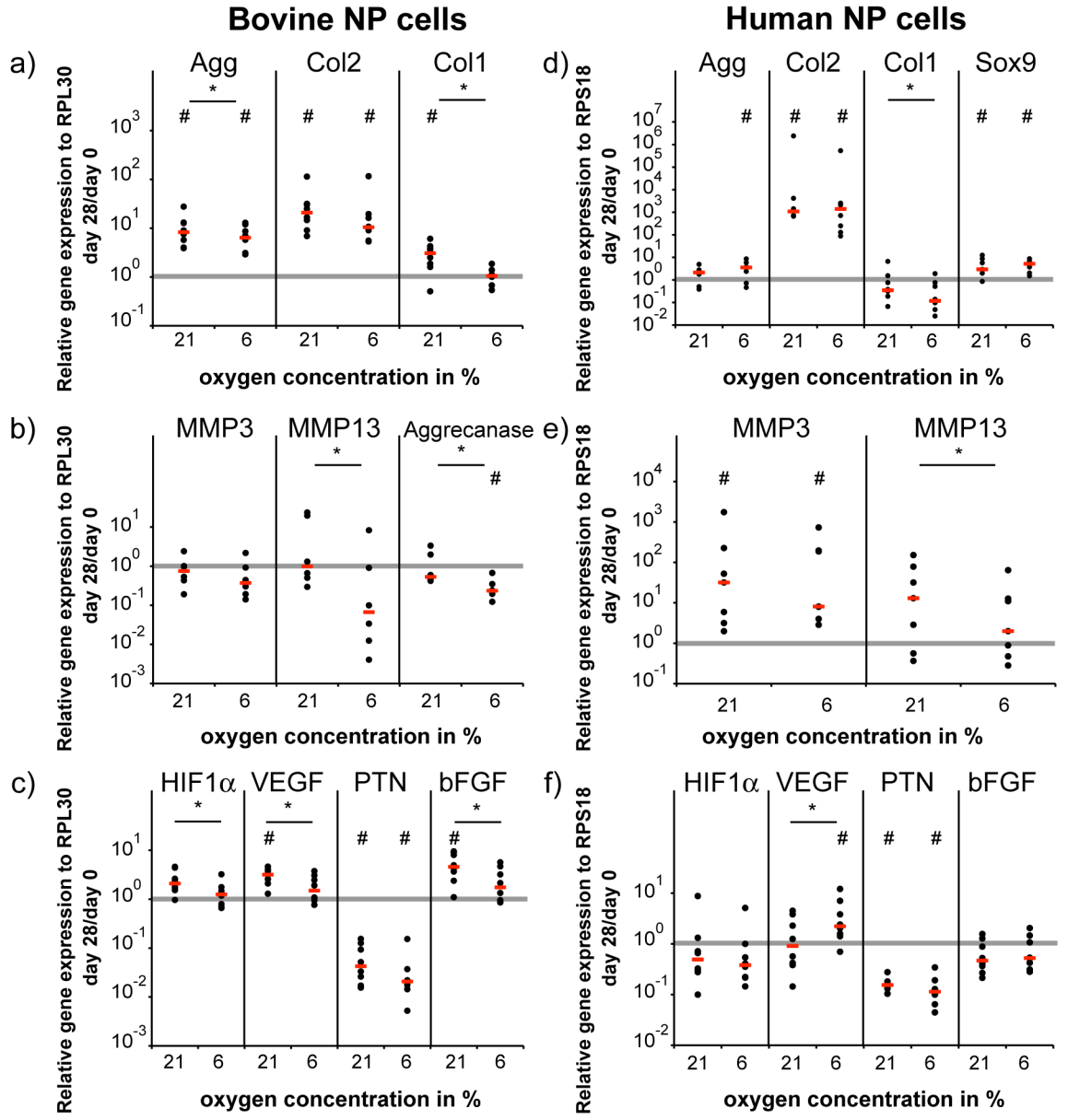

Fig. 17: Quantitative analysis of gene expression after differentiation at different oxygen concentrations

mRNA expression of bovine nucleus pulposus (NP) cells normalised to ribosomal protein L30 (RPL30) (n=8, a, b, c) and human NP cells normalised to ribosomal protein S18 (RPS18) (n=6-9, d, e, f) after four weeks of differentiation and long-term adaption to 21% or 6% oxygen relative to the mRNA expression at day 0. Expression of matrix molecules aggrecan (Agg), collagen type II (Col2), collagen type I (Col1) and the transcription factor sex determining region Y-box 9 (Sox9) (a, d), matrix degrading enzymes matrix metalloproteinase 3 (MMP3), matrix metalloproteinase 13 (MMP13), aggrecanase (b, e), as well as hypoxia-inducible factor 1α (HIF1α) and angiogenic factors vascular endothelial growth factor (VEGF), pleiotrophin (PTN), basic fibroblast growth factor (bFGF) (c, f) of every single donor are represented as black dots, red bars represent the median of all gene expression values and the grey line represents gene expression at day 0. # significant difference between day 0 and day 28, * significant difference between 21% and 6% oxygen, $p < 0.05$.

In general, gene expression of most genes was higher after cultivation at 21% compared to 6% oxygen. The effects were more pronounced in bovine compared to human NP cells.

Short-term reduction

A subsequent decrease in oxygen concentration after four weeks of adaption to either 21% (group A) or 6% oxygen (group B) to a final oxygen concentration of 1% altered gene expression of most target genes depending on the species and the long-term adaption to either 21% or 6% oxygen (Fig. 18-20).

In bovine NP cells, expression of matrix molecules collagen type II and type I was significantly down-regulated in cells of group A, which were adapted to 21% (Fig. 18a). Cells of group B, which were adapted to 6% oxygen, showed no significant alterations after oxygen reduction (Fig. 18b). Human NP cells showed nearly no alterations in gene expression independent from their long-term oxygen adaption (Fig. 18c+d).

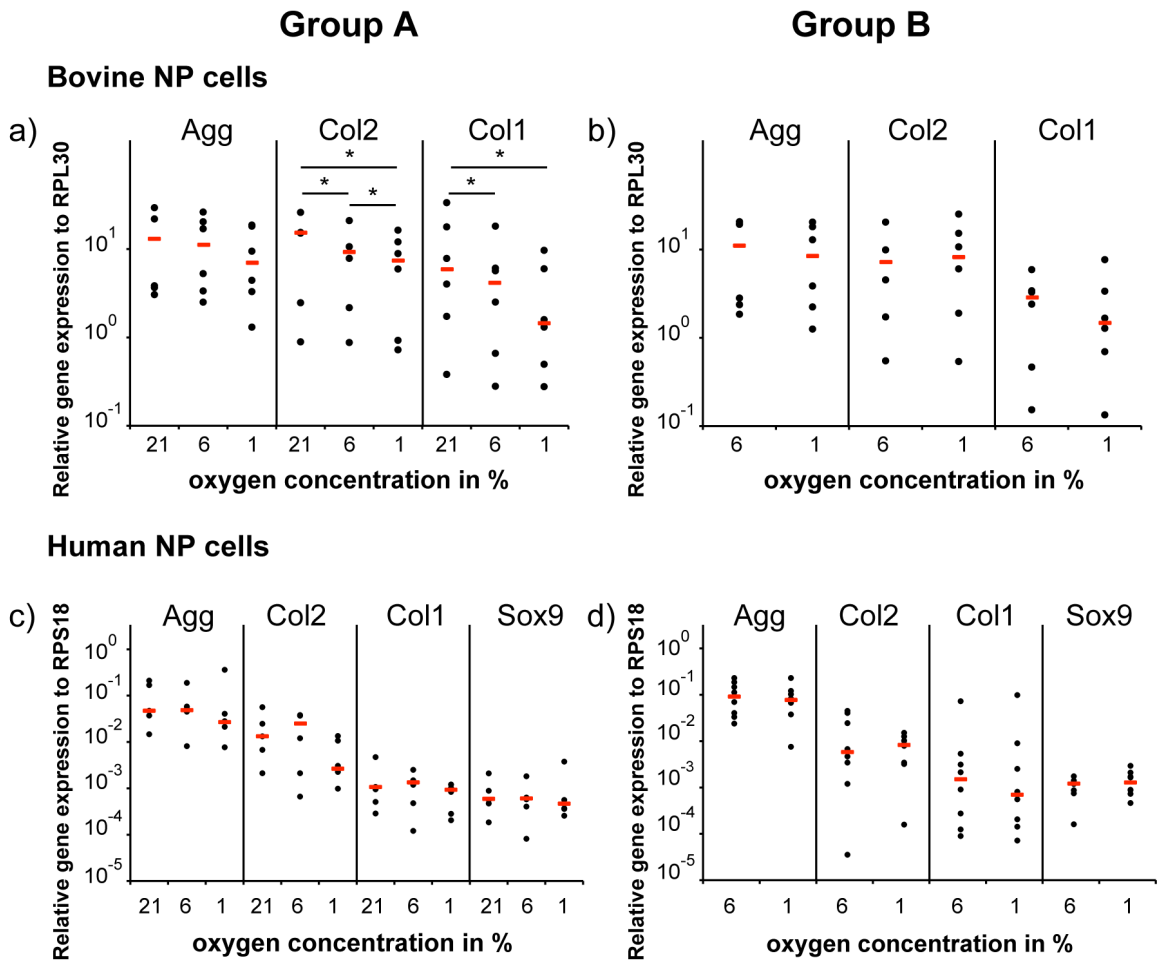

Fig. 18: Quantitative analysis of gene expression of matrix molecules after oxygen reduction
mRNA expression of matrix molecules aggrecan (Agg), collagen type II (Col2), collagen type I (Col1) and the transcription factor sex determining region Y-box 9 (Sox9) of bovine nucleus pulposus (NP) cells (n=6) normalised to ribosomal protein L30 (RPL30) (a, b) and human NP cells (n=5-8) normalised to ribosomal protein S18 (RPS18) (c, d) after short-term reduction of oxygen. Left column shows data of NP cells of group A, which were adapted to 21%, and oxygen was reduced stepwise to 6% and further to 1% oxygen for 24 h each. Right column shows data of NP cells of group B, which were adapted to 6% oxygen, and oxygen was reduced in one step to 1% for 24 h. Values of every single donor are represented as black dots and red bars represent the median of all gene expression values. * significant difference between the different oxygen concentrations, $p < 0.05$.

Gene expression of matrix degrading enzymes was significantly decreased in bovine NP cells of group A (Fig. 19a). Within group B, only expression of aggrecanase was down-regulated (Fig. 19b). Human NP cells, again, remained nearly unaltered, with the exception of MMP13, which was significantly down-regulated in group B after oxygen reduction (Fig. 19c+d).

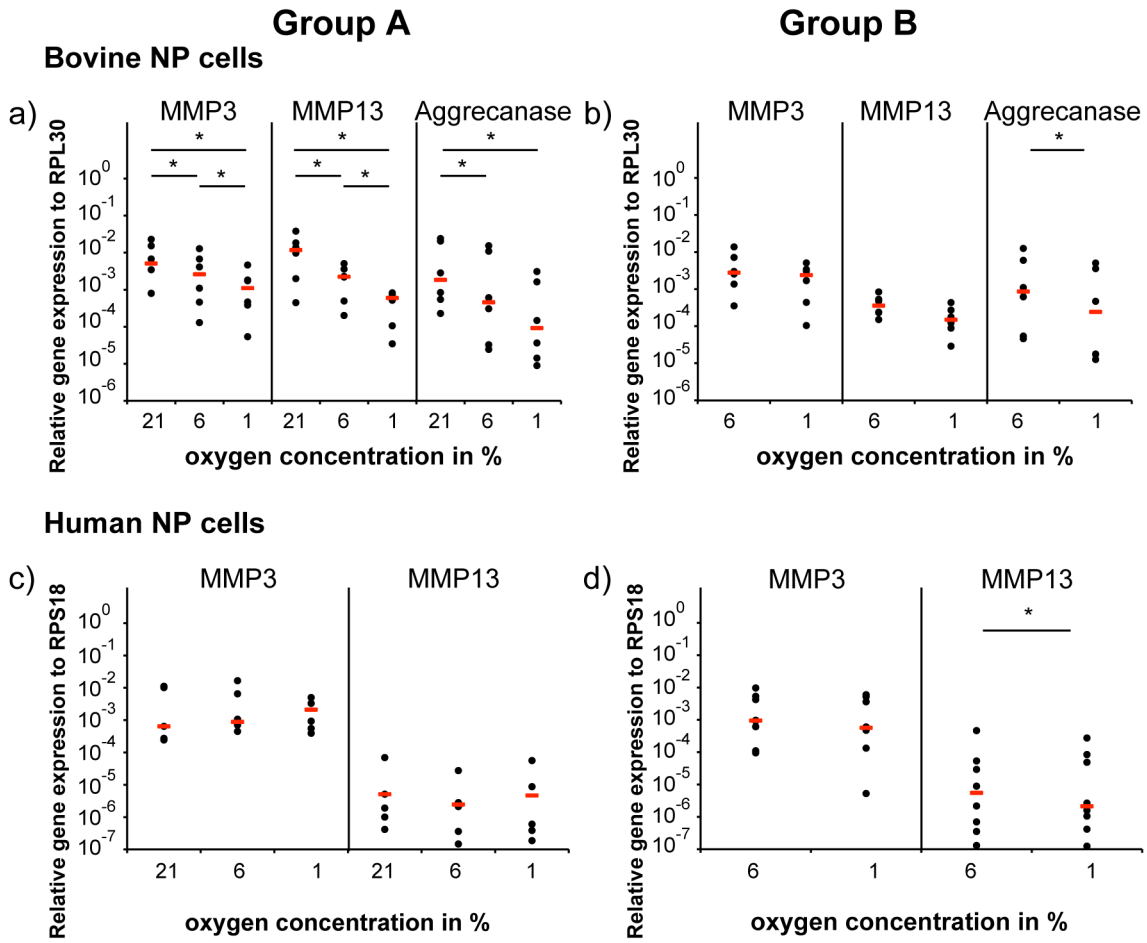

Fig. 19: Quantitative analysis of gene expression of matrix degrading enzymes after oxygen reduction
mRNA expression of matrix degrading enzymes matrix metalloproteinase 3 (MMP3), matrix metalloproteinase 13 (MMP13) and aggrecanase of bovine nucleus pulposus (NP) cells (n=6) normalised to ribosomal protein L30 (RPL30) (a, b) and human NP cells (n=5-8) normalised to ribosomal protein S18 (RPS18) (c, d) after short-term reduction of oxygen. Left column shows data of NP cells of group A, which were adapted to 21%, and oxygen was reduced stepwise to 6% and further to 1% oxygen for 24 h each. Right column shows data of NP cells of group B, which were adapted to 6% oxygen, and oxygen was reduced in one step to 1% for 24 h. Values of every single donor are represented as black dots and red bars represent the median of all gene expression values. * significant difference between the different oxygen concentrations, $p < 0.05$.

For NP cells of both species, the expression of HIF1α and angiogenic factors in cells adapted to 21% oxygen (group A) was significantly down-regulated by the reduction of oxygen with exception for VEGF, which was up-regulated (Fig. 20a+c). Cells adapted to 6% oxygen (group B) showed only little changes in gene expression, but VEGF was also up-regulated in this group (Fig. 20b+d).

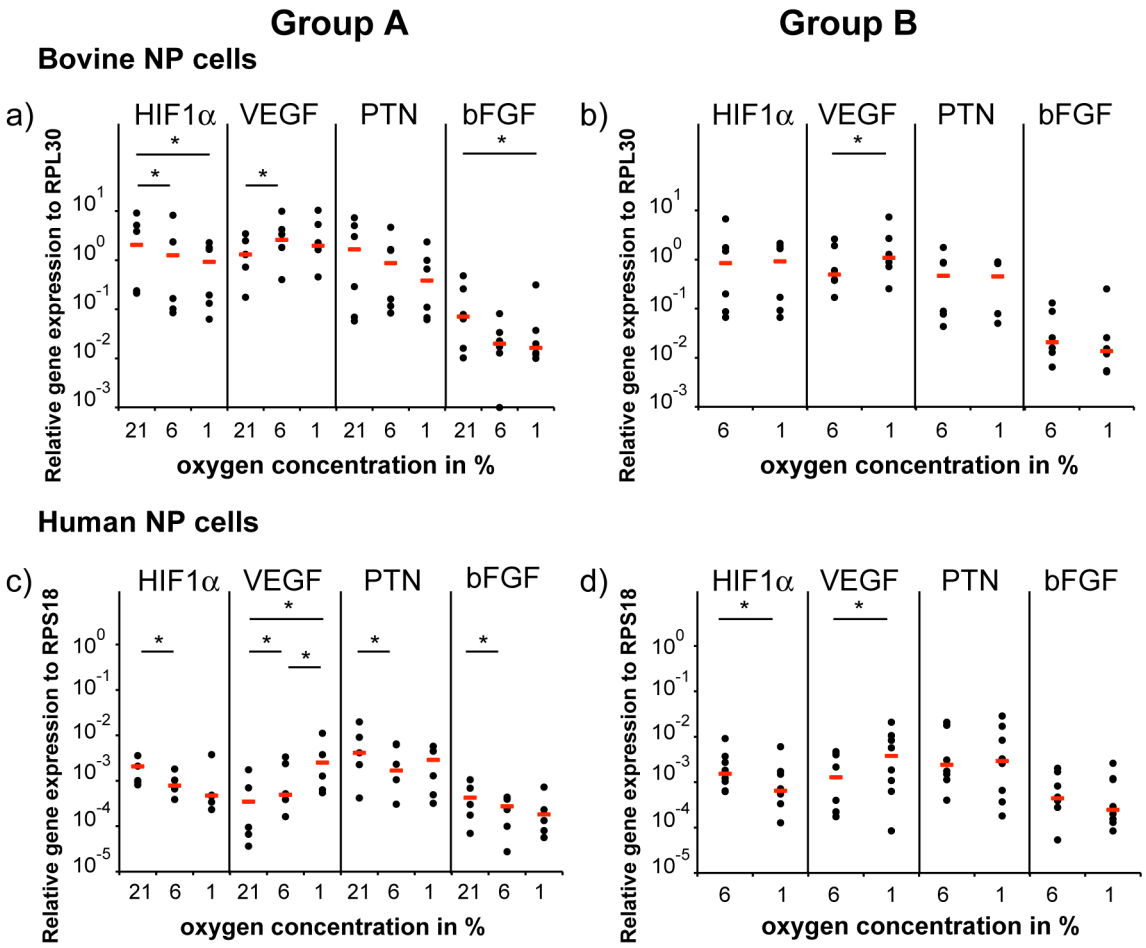

Fig. 20: Quantitative analysis of gene expression of angiogenic factors after oxygen reduction
mRNA expression of hypoxia-inducible factor 1α (HIF1α) and angiogenic factors vascular endothelial growth factor (VEGF), pleiotrophin (PTN), basic fibroblast growth factor (bFGF) of bovine nucleus pulposus (NP) cells (n=6) normalised to ribosomal protein L30 (RPL30) (a, b) and human NP cells (n=5-8) normalised to ribosomal protein S18 (RPS18) (c, d) after short-term reduction of oxygen. Left column shows data of NP cells of group A, which were adapted to 21%, and oxygen was reduced stepwise to 6% and further to 1% oxygen for 24 h each. Right column shows data of NP cells of group B, which were adapted to 6% oxygen, and oxygen was reduced in one step to 1% for 24 h. Values of every single donor are represented as black dots and red bars represent the median of all gene expression values. * significant difference between the different oxygen concentrations, $p < 0.05$.

In general, human NP cells were less sensitive to oxygen reduction than bovine NP cells and in both species pre-adaption of NP cells to 6% oxygen reduced sensitivity to further oxygen reduction.

Part B - Strategies of Disc Regeneration

Part B of this work focussed on strategies of disc regeneration. The influence of medium supplementation with PRP and mechanical loading during NP cell tissue engineering was investigated. Additionally, an organ culture system to simulate cell therapies was introduced and characterised.

3.3. Effect of Platelet-Rich Plasma and Hydrostatic Pressure

PRP is known to contain high concentrations of growth factors and thus appears to be a suitable autologous substitute avoiding recombinant growth factors in the clinical practice and cell culture substitutes of animal origin such as FCS since the patients own blood can be used for preparation. The capacity of PRP as chondrogenic inductor was compared to that of recombinant TGF-β_1 by analysing gene expression and histology with and without additional application of mechanical loading.

First, TGF-β_1 concentration in the pooled PRP, which was used for all experiments, was analysed and determined to be 146 ± 18 ng/ml. Thus, the addition of 10% PRP to the chondrogenic medium resulted in a reasonably comparable concentration to the 10 ng/ml TGF-β_1 which was used in the standard chondrogenic medium.

3.3.1. Gene Expression

The chondrogenic phenotype of human MSC and NP cells after four weeks in different culture systems and different media was analysed by comparing relative mRNA expression of aggrecan, collagen type II, collagen type I and Sox9 by quantitative RT-PCR with GAPDH as the house-keeping gene. Twelve independent experiments were carried out for each cell type. Due to limited cell numbers not all of the parameters could be investigated for each donor resulting in different sample sizes.

After four weeks of differentiation, higher expression of chondrogenic markers in MSC (Fig. 21a) as well as NP cells (Fig. 21b) was observed in alginate beads cultivated with TGF-β_1 compared to those cultured with PRP. Cells cultivated in pellet culture displayed similar gene expression patterns for MSC (Fig. 21c) and NP cells (Fig. 21d). TGF-β_1 medium induced up to 8,000-times greater effects in gene expression levels of matrix molecules compared to PRP for both cell types in pellet culture (Fig. 21c+d), with statistically significant results for aggrecan and collagen type II.

In addition to this influence of medium composition, a statistically significant differential gene expression pattern was observed between MSC and NP cells under the same experimental parameters. While NP cells (Fig. 21b+d) showed an order of magnitude greater expression of aggrecan, collagen type I expression was an order of magnitude lower than it was observed in MSC (Fig. 21a+c) for both culture systems and medium compositions. Expression levels of collagen type II and Sox9 were comparable. Moreover, comparing data of every single donor, MSC and NP cells showed a considerably greater expression (up to 23-fold) of aggrecan and collagen type II in pellet culture (Fig. 21c+d) compared to alginate beads (Fig. 21a+b).

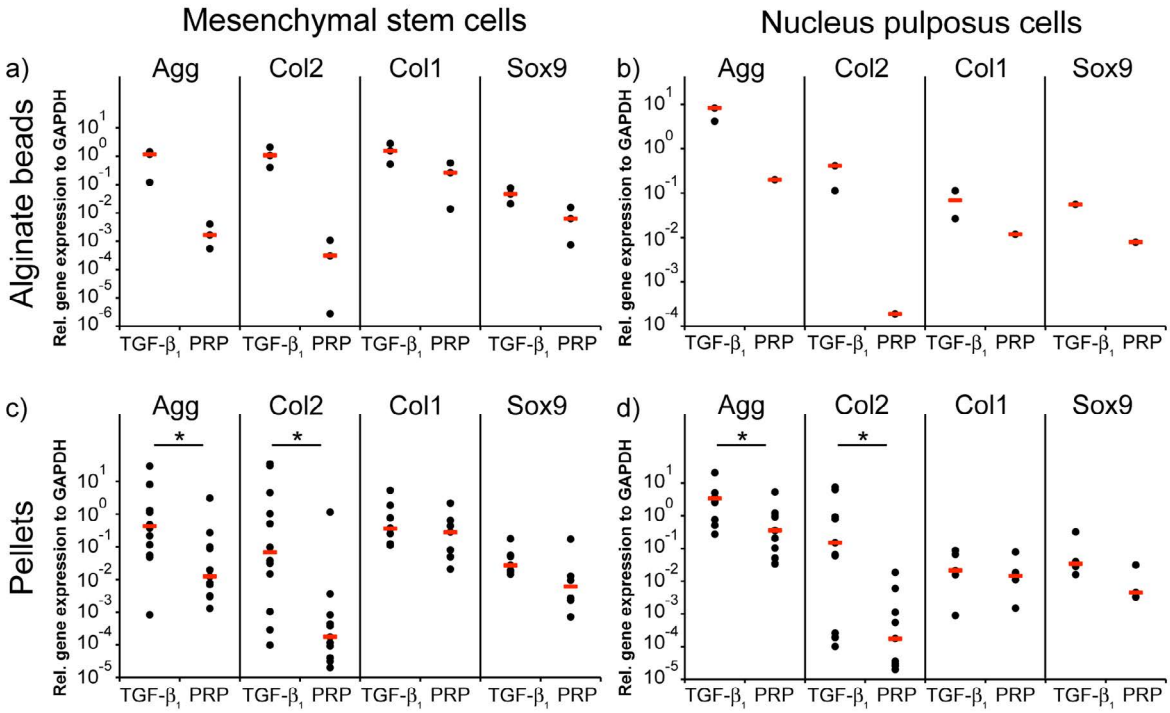

Fig. 21: Quantitative analysis of chondrogenic differentiation
mRNA expression of the chondrogenic differentiation markers aggrecan (Agg), collagen type II (Col2), collagen type I (Col1) and the transcription factor sex determining region Y-box 9 (Sox9) of mesenchymal stem cells (a, c) and nucleus pulposus cells (b, d) from individual donors after four weeks of chondrogenic differentiation in alginate beads (a, b) or pellet culture (c, d) with medium containing either TGF-β_1 or platelet-rich plasma (PRP). Levels of mRNA were normalised to glyceraldehyde-3-phosphate dehydrogenase (GAPDH), black dots represent gene expression values of every single donor, red bars represent the median of all gene expression values, n=1-12, *significant differences between different medium supplementations, $p < 0.05$.

The ratio of collagen type II to collagen type I (Col2/Col1) gene expression is a marker for the chondrogenic differentiation state of MSC. In NP tissue, collagen type II is the major collagen type. Thus, this ratio was used to evaluate an adequate induction of chondrogenesis of MSC and NP cells as the higher the ratio the better the NP-like phenotype of the cells. The Col2/Col1 ratio was higher for NP cells than for MSC (Fig. 22). As expected, a difference between PRP medium and TGF-β_1 medium was found. Individual donors reached a higher Col2/Col1 ratio for cells in pellet culture than in alginate beads. However, due to different donor numbers statistical evaluation was not possible. Additionally, when comparing MSC or NP cells of donors, which were exposed to both culture systems, collagen type II expression was always greater in pellet culture. Thus, for further studies, the pellet culture system was chosen.

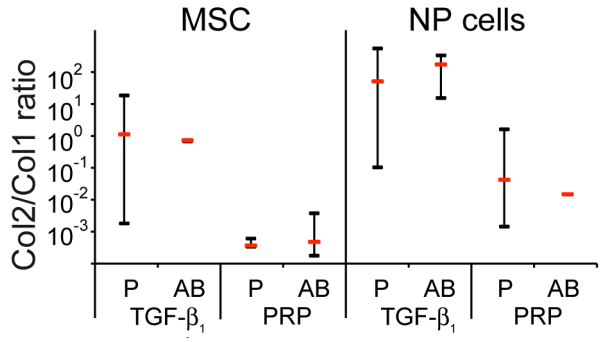

Fig. 22: Quantitative analysis of chondrogenic differentiation using collagen ratio
mRNA expression ratio of collagen type II normalised to collagen type I (Col2/Col1) of mesenchymal stem cells (MSC) and nucleus pulposus (NP) cells after four weeks of chondrogenic differentiation in pellet culture (P) or alginate beads (AB) in medium containing either TGF-β_1 or platelet-rich plasma (PRP). Col2/Col1 ratio is shown as a range of minimum and maximum values with the median marked in red, n=1-8.

However, MSC and NP cell differentiation based on mRNA expression levels displayed high variability between individual donors. Chondrogenic differentiation was only possible for approximately 75% of MSC and 83% of NP donors that were included in the analysis.

To investigate the influence of mechanical stimulation on MSC and NP differentiation, hydrostatic pressure was applied once for 30 min, at 0.1 Hz and 2.5 MPa. Up-regulation of the mechanosensitive transcription factor c-FOS after stimulation demonstrated a response of cells to this stimulus. Effects on matrix molecule expression showed high variations (Fig. 23). In pellets of certain MSC donors, a mechanically induced up-regulation of collagen type II and Sox9 was observed (Fig. 23a). However, in contrast to expected positive effects of mechanical stimulation, up-regulations were not significant. In general, less influence of mechanical stimulation on the expression of matrix molecules was observed in NP cells (Fig. 23b).

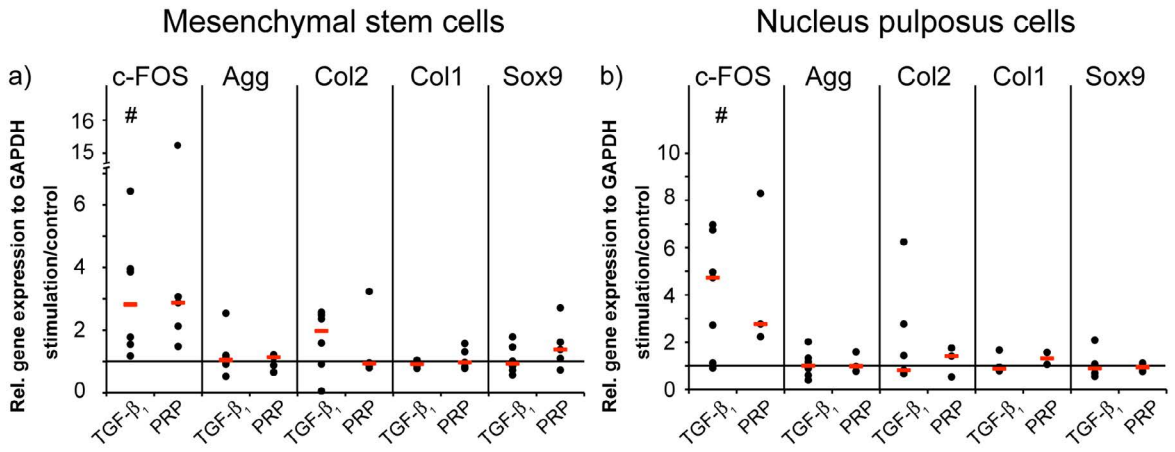

Fig. 23: Quantitative analysis of chondrogenic differentiation after mechanical stimulation
mRNA expression analysis after mechanical stimulation with hydrostatic pressure. The mechanosensitive transcription factor c-FOS as well as the chondrogenic differentiation markers aggrecan (Agg), collagen type II (Col2), collagen type I (Col1) and the transcription factor sex determining region Y-box 9 (Sox9) of mesenchymal stem cells (a) and nucleus pulposus cells (b) from individual donors were analysed after four weeks of chondrogenic differentiation in pellet culture in medium containing either TGF-β_1 or platelet-rich plasma (PRP) followed by mechanical stimulation with hydrostatic pressure. Levels of mRNA were normalised to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The ratio of stimulation to control indicates the multiplication of induction after mechanical stimulation, black dots represent gene expression values of every single donor, red bars represent the median of all gene expression values, n=2-6, # significant differences between stimulation and control, $p < 0.05$.

3.3.2. Histology

Results from gene expression analysis showed a strong influence of medium composition on mRNA level for both cell types. Thus, proteoglycans, aggrecan and collagen type II were also investigated at the protein level. Alcian blue staining as well as by immunohistology of collagen type II and aggrecan confirmed that matrix deposition was clearly greater in TGF-β_1-containing medium than in medium supplemented with PRP for both, MSC and NP cells, after four weeks of chondrogenic differentiation in pellet culture (Fig. 24).

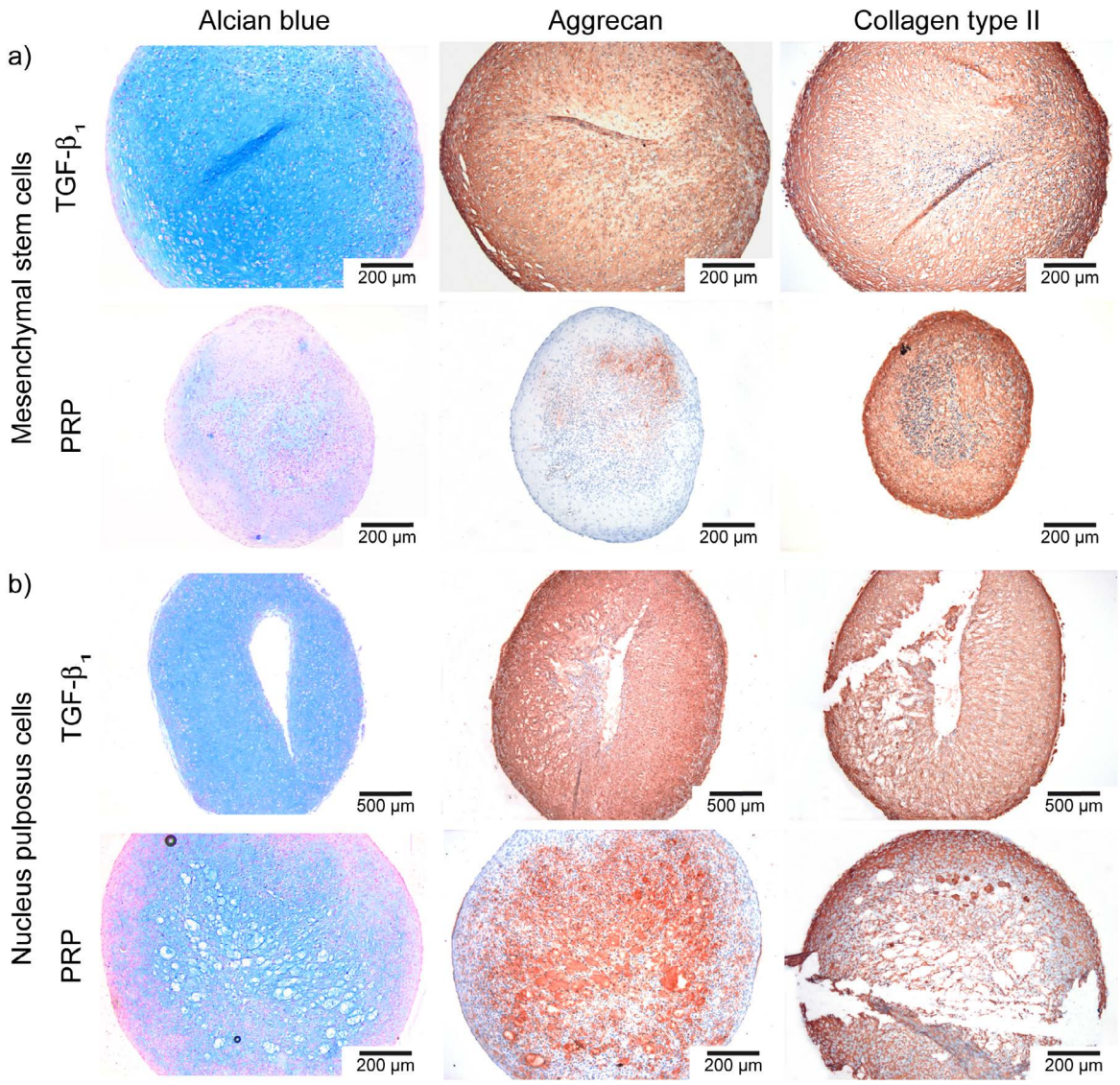

Fig. 24: Qualitative analysis of chondrogenic differentiation
Chondrogenic differentiation of mesenchymal stem cells (a) and nucleus pulposus cells (b) in pellet culture using either medium containing TGF-β_1 or platelet-rich plasma (PRP) was analysed histologically by alcian blue staining (proteoglycans represented by blue colour in first column) as well as by immunohistological detection aggrecan (brown colour in second column) and collagen type II (brown colour in third column) with specific antibodies after four weeks.

3.3.3. Proliferation assay

As PRP is known to contain a mixture of growth factors, the proliferative potential of the media was investigated comparing medium supplemented with PRP to medium containing TGF-β_1 for two exemplary MSC donors in monolayer culture (Fig. 25). MSC cells cultured with medium containing TGF-β_1 did not proliferate (Fig. 25a). Microscopic images taken at day 7 showed, that these MSC cells formed stellate colonies with only few cells attached to plastic having many filopodia (Fig. 25b). In contrast, using medium supplemented with PRP led to a

strong proliferation (Fig. 25a). MSC cells had a very elongated spindle-shaped morphology (Fig. 25c) and grew into a multilayer that detached from plastic after one week.

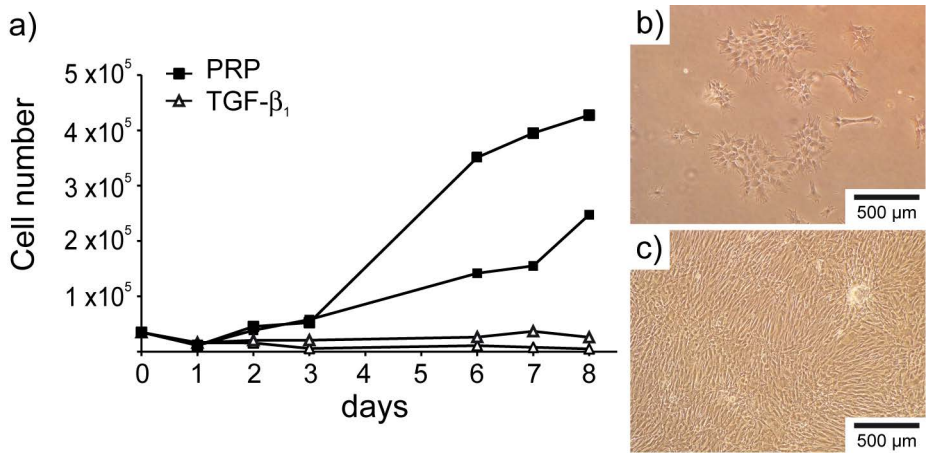

Fig. 25: Cell growth and morphology of mesenchymal stem cells (MSC)
Growth curve showing the absolute cell number of MSC from two donors (a) and representative microscopy images at day 7 of cells cultured in monolayer and medium containing either 10 ng/ml TGF-β_1 (b) or 10% platelet-rich plasma (PRP) (c). Initially 35,000 cells were seeded. Medium supplemented with TGF-β_1 (a; white triangles) did not induce proliferation and cells formed stellate colonies (b) whereas medium with PRP (a; black rectangles) led to strong proliferation and elongated, spindle-shaped morphology of the cells (c).

3.4. Characterisation of an Organ Culture System for Cell Therapy Simulation

As animal models implicate some disadvantages, the establishment of an *in vitro* organ culture system simulating disc degeneration would offer a tool for basic research of cell therapy approaches. Biological treatment of disc degeneration by cell-based approaches is a challenge because of the harsh environment in degenerated discs that influences the survival of injected cells. *In vitro* evaluation of cell therapy treatments requires standardised model systems that simulate the degenerative disc environment. The here presented model was characterised and tested in first cell injection experiments.

3.4.1. Macroscopic Characterisation

After 14 days of cultivation at loaded or unloaded conditions, no macroscopic differences were seen between whole and punched IVD (Fig. 26a). As caudal

bovine disc differ in diameter, punched IVD allowed standardised starting conditions for every single IVD and were used for all subsequent experiments.

A comparison of macroscopical parameters such as height, diameter and weight of punched IVD after 14 days at loaded or unloaded conditions revealed only little differences for diameter (Fig. 26c) and weight (Fig. 26d). However, regarding height, there were great differences between loaded and unloaded conditions (Fig. 26b). Due to the differences in swelling behaviour between NP and AF, the NP was extruded in direction of lowest resistance under unloaded conditions and hence the height of the IVD increased (Fig. 26a, arrow).

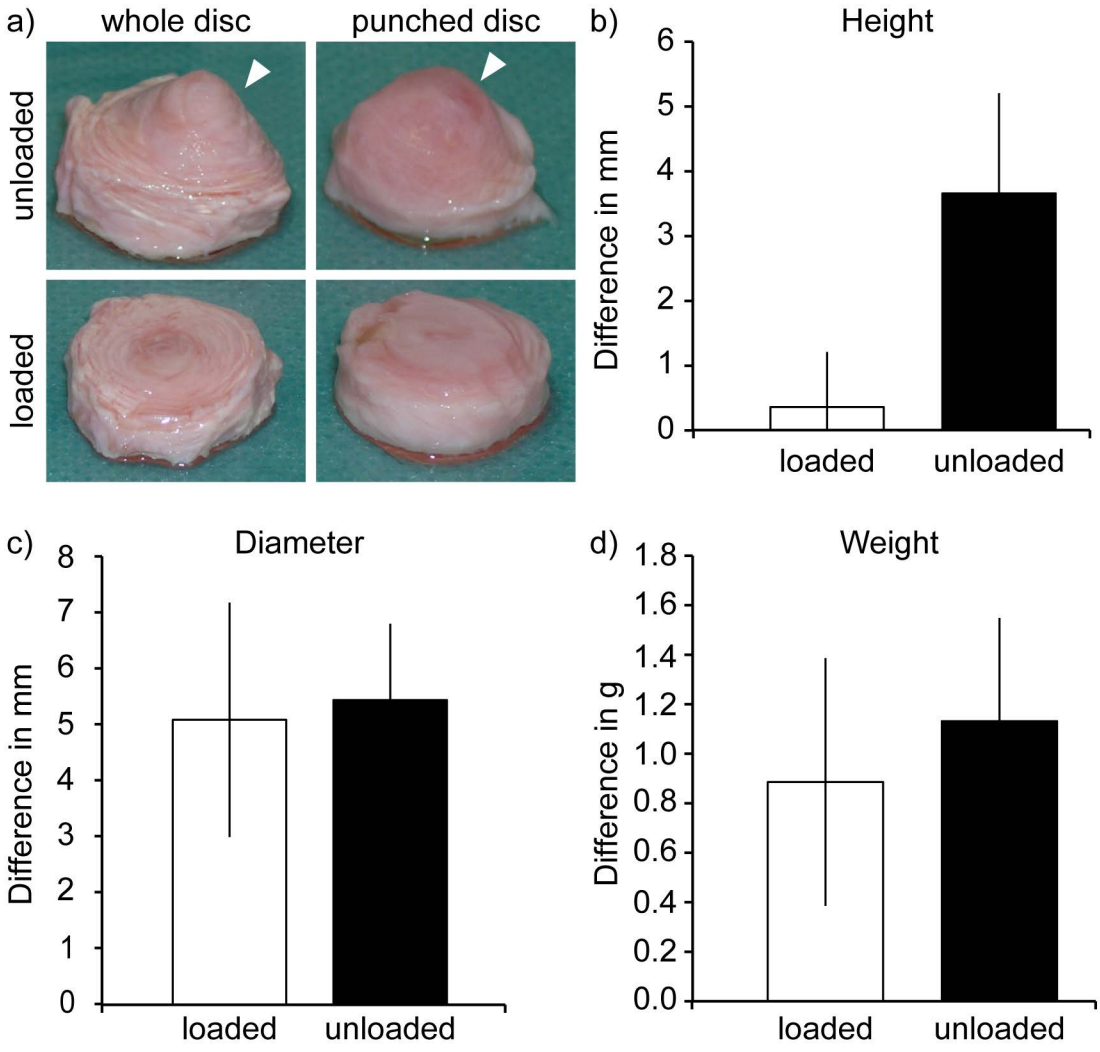

Fig. 26: Macroscopical parameters of intervertebral discs (IVD)
Whole IVD were compared macroscopically to punched IVD under unloaded (black columns) and loaded (white columns) culture conditions (a). Seven IVD punches of one donor were used for analysis of the physical parameters height (b), diameter (c) and weight (d). Parameters of the IVD were compared before and after cultivation and differences are shown as mean ± standard deviation.

3.4.2. Biochemical Characterisation

Investigations of the GAG release over a culture period of five weeks revealed diffusion of GAG from the disc tissue into the cell culture medium. Alcian blue staining of IVD punches at different points in time showed a decrease in staining intensity, which was directly correlated to the GAG amount decreasing within the tissue (Fig. 27a). Parallel analysis of the cell culture supernatant showed an exponential increase of GAG amount in the medium (Fig. 27b). No differences were observed between loaded and unloaded culture conditions.

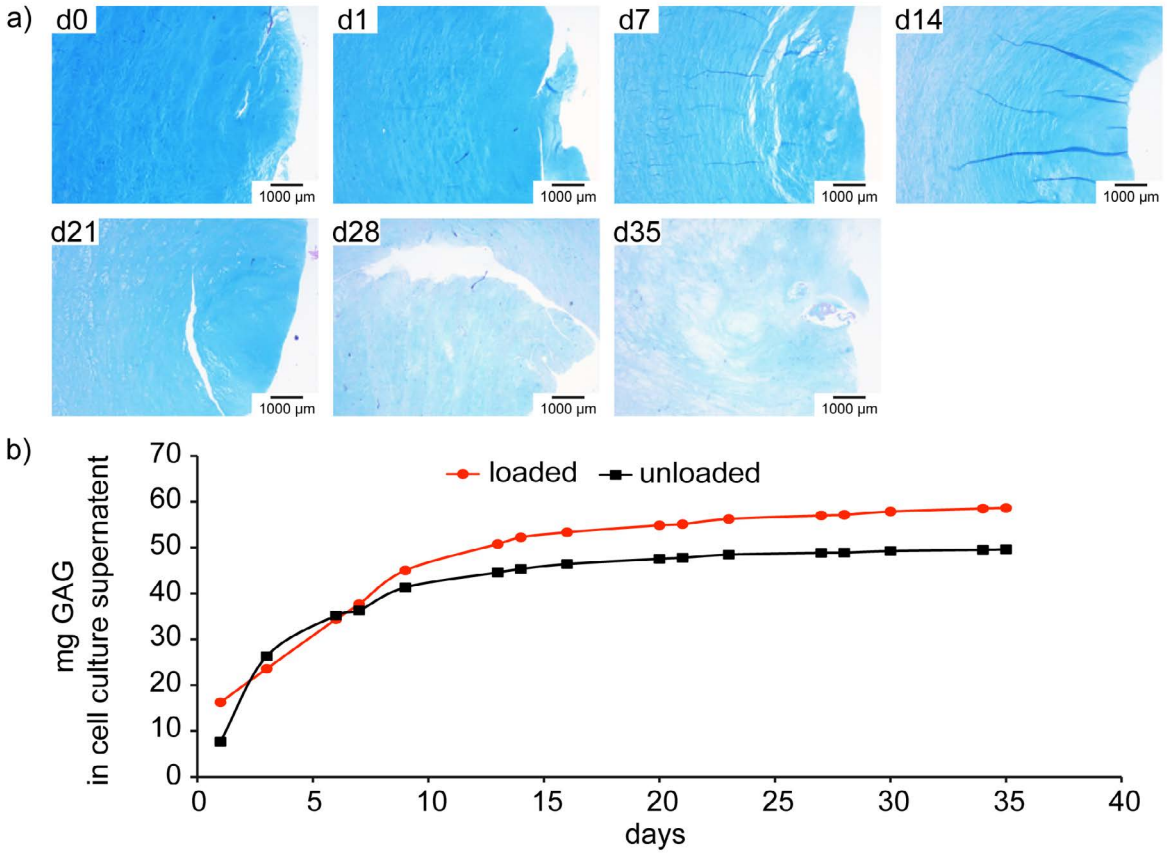

Fig. 27: Release of glycosaminoglycans (GAG) over the culture period
Exemplary alcian blue staining of intervertebral disc (IVD) punches of one donor at different points in time over the culture period of five weeks under loaded conditions (a). The release of GAG into the medium was analysed by DMMB assay for the same donor and results of one disc each cultured under loaded (red line) and unloaded (black line) conditions were plotted cumulatively against the culture period (b).

3.4.3. Cell Injection Experiments

The established organ culture system was used to test possible cell therapy approaches by injection of either (I) a carrier-free cell suspension of SCP1-GFP cells, (II) the albumin-hyaluronan hydrogel without cells or (III) cells seeded in

albumin-hyaluronan hydrogel. Thereby only very small volumes (approximately 50 µl) could be injected due to the high intradiscal pressure.

(I) After the injection of a carrier-free cell suspension of SCP1-GFP cells, the presence of several green-fluorescent SCP1-GFP cells was confirmed within the IVD punches after four days of culture (Fig. 28).

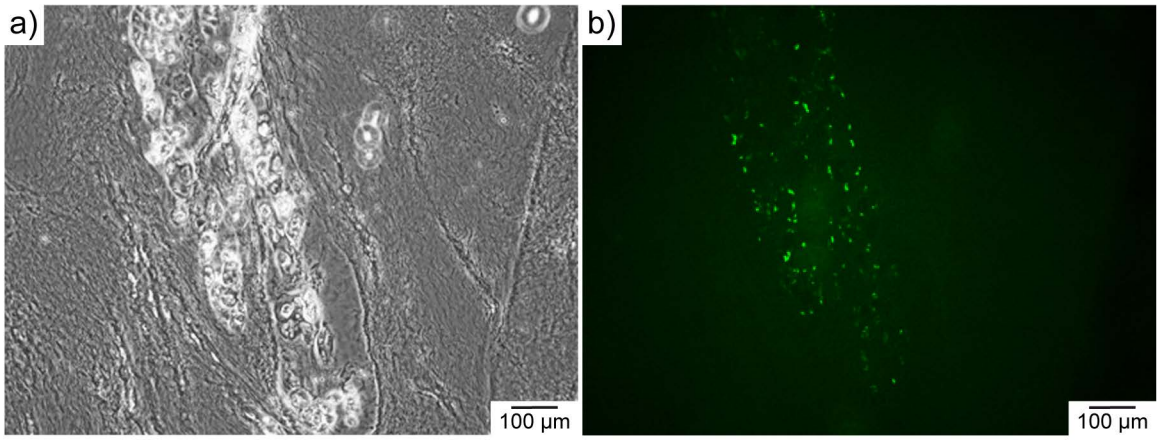

Fig. 28: Injection of SCP1-GFP cell suspension into intervertebral disc (IVD) punches
Phase contrast (a) and fluorescence microscopy (b) of SCP1-GFP cells within the IVD punches after four days of culture.

(II) An alternative way to bring cells into the disc is the usage of hydrogels as carrier system for a better embedding of the cells within the tissue. As a first step, the injection of a hydrogel without cells was tested for its feasibility. Here, an albumin-hyaluronan hydrogel was used with the ability to polymerise *in situ*. For comparison, two groups were formed. All discs within these two groups were needle-punctured to induce degenerative alterations. One day after needle-puncture, hydrogel was injected into the discs of one group while the other group was left without further treatment. After 14 days of cultivation, IVD punches which were cultivated without further treatment (Fig. 29a+b) were compared histologically to IVD punches treated with albumin-hyaluronan hydrogel (Fig. 29c+d). In these preliminary experiments, intense cell cluster formation was observed in the centre of punctured IVD punches without further treatment (Fig. 29b). IVD punches with albumin-hyaluronan hydrogel treatment showed less cell cluster formation (Fig. 29d).

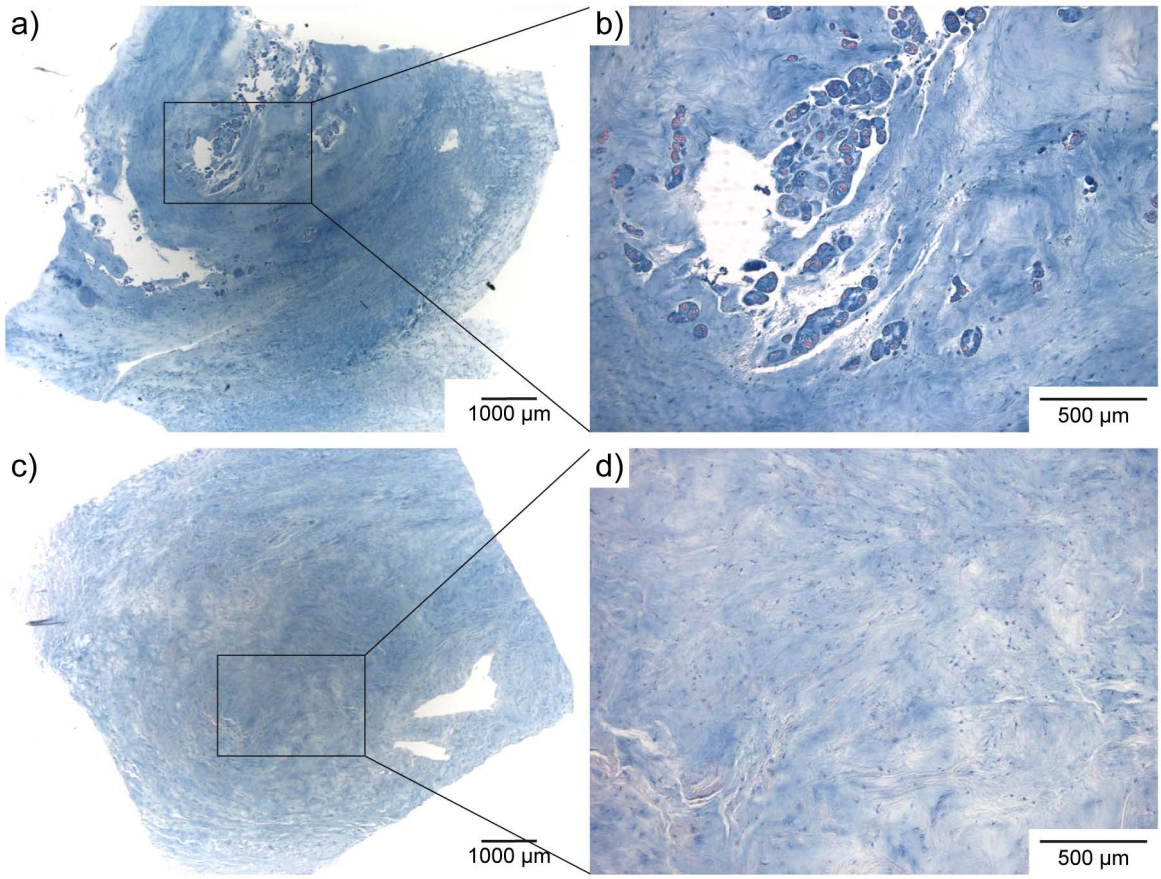

Fig. 29: Organ culture of punched intervertebral disc (IVD) with and without albumin-hyaluronan hydrogel injection
Exemplary alcian blue staining of IVD punches after a cultivation period of 14 days. A punctured IVD punch without further treatment (a, b: higher magnification) was compared to a punctured IVD punch after injection of albumin-hyaluronan hydrogel (c, d: higher magnification).

(III) In a next step, the effects of the hydrogel injection seeded with cells were compared to hydrogel without cells. The treatment of punctured IVD punches with SCP1-GFP cells seeded in the albumin-hyaluronan hydrogel was investigated two and 14 days after injection (Fig. 30). After two days, the cell-seeded hydrogel was clearly visible (Fig. 30a). At higher magnification, cells close to the injection side displayed a more intense alcian blue staining in the cells' periphery compared to the rest of the tissue (Fig. 30b, black arrow). After 14 days, still some deposits of the hydrogel were visible appearing in pink (Fig. 30d). Again a more intense staining surrounding some of the cells was observed (Fig. 30d, black arrow). Higher magnification of cells of the outer (Fig. 30e, *or*) and inner region (Fig. 30f, *ir*) of the IVD punch clearly demonstrated these differences in staining intensity.

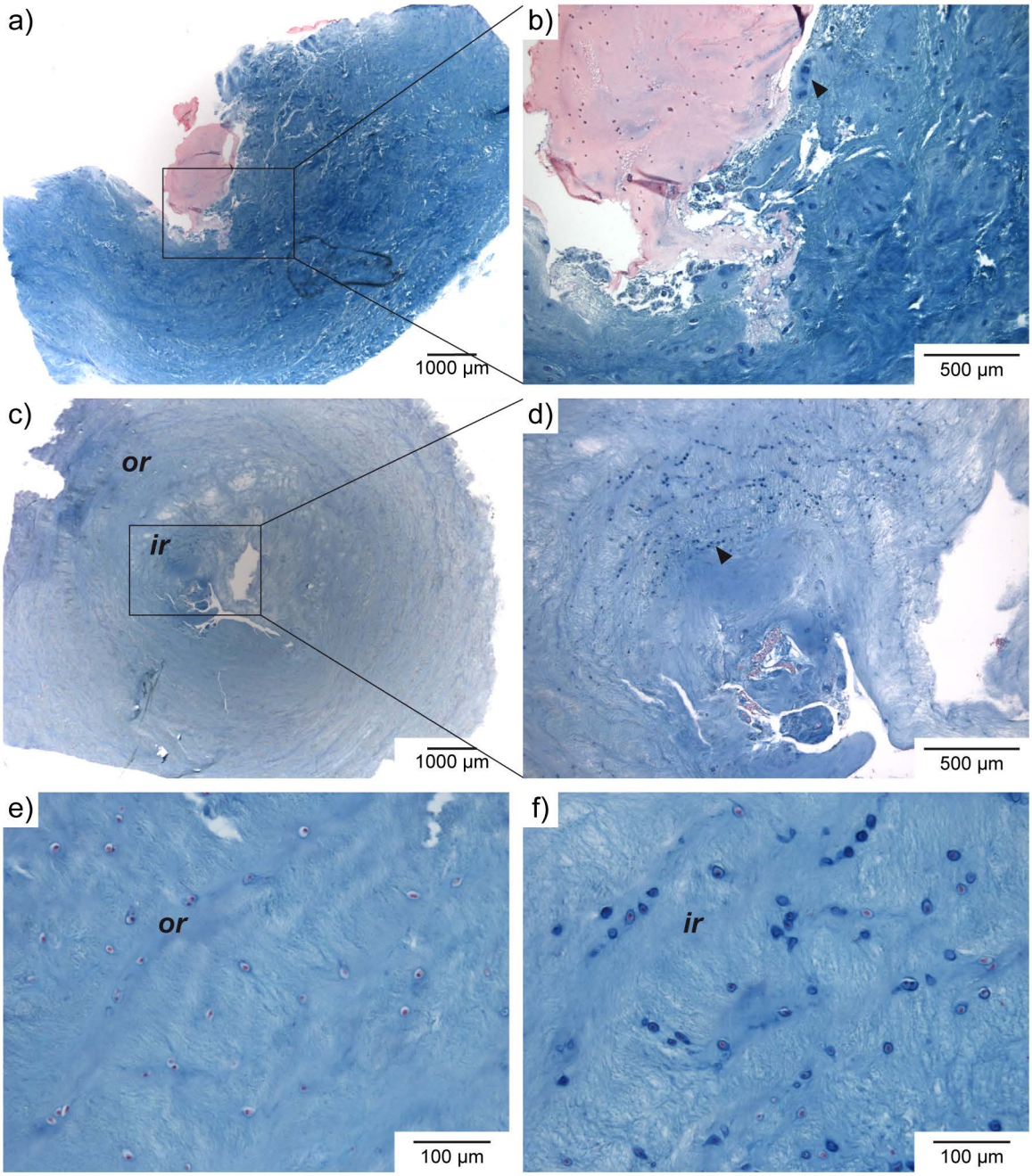

Fig. 30: Organ culture of punched intervertebral disc (IVD) after injection of SCP1-GFP cells in albumin-hyaluronan hydrogel
Exemplary alcian blue staining of IVD punches after injection with SCP1-GFP cells in albumin-hyaluronan hydrogel (pink) after a cultivation period of two days (a, b: higher magnification) and 14 days (c, d: higher magnifications). The outer (*or*) and inner region (*ir*) of c) are shown in e) and f), respectively.

The parallel analysis of the GAG release into the cell culture medium showed that the injection of the albumin-hyaluronan hydrogel with or without cells was not able to influence the amount of GAG released to the medium (Fig. 31).

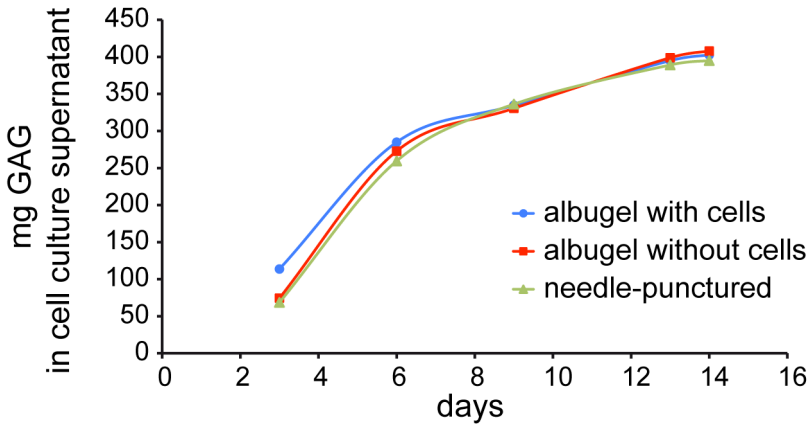

Fig. 31: Release of glycosaminoglycans (GAG) after albumin-hyaluronan hydrogel injection
Exemplary release of GAG of intervertebral disc punches after no treatment (green line) and albumin-hyaluronan hydrogel (albugel) injection with (blue line) and without (red line) SCP1-GFP cells into the medium was measured by DMMB assay for one donor and results were plotted cumulatively against the culture period.

4. Discussion

Part A - Influence of Environmental Factors During Disc Degeneration

The understanding of the influence of environmental stress during disc degeneration is very important. This study contributes to the disclosure of basic mechanisms and helps to evaluate the suitability of repair strategies.
In the present work, effects of variations of the glucose concentration and oxygen on NP cells were analysed as both environmental factors play a crucial role in disc degeneration.

4.1. Effect of Glucose Concentration on Nucleus Pulposus Cells

In this study, effects of glucose concentration were investigated as glucose deprivation is considered to be a key contributor to disc degeneration resulting from the calcification of the CEP, thus blocking the main route of transport of nutrients and metabolites [21]. The purpose of this study was to investigate the influence of glucose deprivation in combination with mechanical loading in healthy bovine NP cells.
The analysis revealed a moderate increase in gene expression of matrix-forming proteins after three weeks in high-glucose chondrogenic medium. Gene expression of matrix degrading enzymes by contrast was decreased significantly. Subsequent reduction of glucose concentration from high-glucose medium to physiological 5 mM glucose for three days decreased expression of collagen type II and collagen type I significantly. Further reduction of glucose concentration for 24 h revealed only little effect. The same trend was shown for all matrix degrading enzymes that were investigated. These findings indicate that the reduction of glucose concentration within the media immediately affects NP cell metabolism after long-term differentiation. Only few studies exist dealing with the effect of glucose deprivation on the expression of matrix molecules. In contrast to the present data, Johnson and colleagues showed an inhibited synthesis of collagen type I and type II after glucose deprivation in monolayer and alginate beads cultures of bovine NP cells [103]. However, a former study of our own group supported the results of this study. In short-term monolayer experiments using

bovine and human IVD cells a significant down-regulation of the gene expression of aggrecan, collagen type I and type II was shown [203]. Other studies demonstrated that glucose deprivation also influences IVD cell viability and metabolism. Bibby and colleagues reported on a significant reduction of cell viability after glucose reduction independent of the oxygen concentration in the environment [21]. In a follow-up study of the same authors, a reduction in glucose consumption was pointed out when cells were exposed to reduced glucose concentration depending on the pH value in the environment. Thus, a strong interaction of oxygen, glucose, lactate concentration and pH value was concluded [22]. Würtz and co-workers investigated the influence of glucose concentration on the behaviour of rat MSC in monolayer culture in terms of using MSC for IVD tissue engineering approaches [282]. In this study, cell proliferation and gene expression were analysed comparing a high-glucose medium (25 mM glucose) with an IVD-like glucose medium containing 5 mM glucose. Under these conditions, cell proliferation and gene expression of aggrecan and collagen type I was increased while collagen type II was not detectable. However, other influences such as osmolarity and pH value had stronger effects. This demonstrates that also MSC are influenced by the surrounding microenvironment. Another study by Li *et al.* using human MSC pointed out that a high concentration of glucose in the medium supported osteogenic differentiation [134]. All these studies together with the present results demonstrate the strong influence of glucose concentration on MSC and IVD cells. Deprivation of this essential nutrient leads to decreased metabolic activity. However, besides a high glucose concentration the chondrogenic differentiation medium also contained TGF-β_1. Therefore the observed effects could also be influenced by the supplementation with TGF-β_1. Clarification of this question is part of ongoing studies.

Furthermore, the influence of mechanical loading at different culture conditions was investigated with regard to medium composition. Great differences were pointed out between the four different media used after mechanical stimulation of NP cells by hydrostatic pressure.

First, the greatest mechanically induced effects were shown in NP cells, which were maintained in high-glucose chondrogenic medium (25 mM) supplemented with TGF-β_1. Gene expression of c-FOS and collagen type I increased

significantly, whereas aggrecan and collagen type II expression increased more moderately. In contrast, gene expression of MMP2, MMP3, MMP13 and aggrecanase tended to decrease within this group after mechanical stimulation. In conclusion of these results, the chondrogenic medium reveals positive effects on mechanotransduction, as c-FOS expression was up-regulated. At these medium conditions the effects of mechanical loading on matrix production seemed to be enhanced.

In recent literature, the influence of high glucose during mechanical loading was not taken into consideration. However, regarding studies without mechanical loading, there are findings that TGF-β stimulates matrix production via stimulation of Sox9 expression [131]. Another study of Hayes and Ralphes demonstrated the role of TGF-β during the fibrocartilaginous metaplasia of the maturing AF by pushing the gene expression profile of AF cells to the fibrocartilage phenotype [74]. These findings support results of the present study that collagen type I was up-regulated without mechanical stimulation.

Furthermore, the influence of TGF-β on other tissues and cell types was investigated. Tendons have a fibrocartilaginous structure. In a study of Maeda and co-workers the involvement of TGF-β in the transduction of physical forces to tenocytes was pointed out [148]. Another study by Huang *et al*. demonstrated a positive influence of TGF-β in combination with dynamic compression on chondrogenic matrix production of bovine MSC [82]. Thus, the positive influence of TGF-β during mechanical loading of NP cells reported by other authors supported the present findings.

Secondly, present results revealed only minor influences of mechanical loading at glucose-reduced conditions. Comparable results were published by a former study of our group after short-term experiments with bovine NP cells in alginate beads [203]. In this study only minor influences of hydrostatic pressure were shown independent of the glucose concentration used. It might be possible that the mechanical loading protocol was insufficient to alter cell reactions. Indeed, a recent study by Fernando and colleagues demonstrated an influence of mechanical loading conditions on the cells' reactions. The authors showed differences between the application of 1 Hz and 0.1 Hz [54]. Mechanical loading only influenced energy metabolism of NP cells after application of 1 Hz. Illien-Jünger and co-workers reported minor influences of mechanical loading on

matrix production [93]. In an ovine IVD organ culture model an insufficient nutrient supply by comparing influences of two media with differing glucose concentrations (11 mM *versus* 25 mM glucose) was simulated. Additionally, dynamic compression was applied. Although a decrease in cell viability after insufficient nutrition and unphysiological loading was demonstrated, no influence on metabolism and matrix production could be pointed out. These studies together with the recent results lead to the conclusion that mechanical loading cannot reverse effects of glucose-reduced conditions. Another recent study of our group dealing with influences of glucose concentration and mechanical loading in short-term experiments supports this conclusion [172]. Consequently, therapeutic interventions should focus on the restoration of adequate nutrient supply within the IVD.

4.2. Effect of Oxygen Concentration on Nucleus Pulposus Cells

This study aimed to provide new insights into two important aspects. First, the question which impact oxygen concentration has on long-term differentiation of NP cells was addressed comparing disc-hyperoxic 21% oxygen (group A) to disc-normoxic 6% oxygen (group B). The question if it is important to culture NP cells at reduced oxygen conditions should thereby be clarified. Secondly, the effect of oxygen reduction to 1% which might occur during disc degeneration was investigated starting from the two different pre-culture conditions: 21% (group A) or 6% oxygen (group B).

Results of the present study revealed that after differentiation and long-term adaptation to either 21% or 6% oxygen, gene expression of virtually all genes was higher at 21% oxygen. The effects were more pronounced in healthy bovine NP cells compared to human NP cells from degenerated tissue. However, although some significant differences were pointed out, these effects were quite low. From these results it could be suggested that it might not be relevant whether disc cells are cultured at atmospheric 21% oxygen or disc-normoxic 6% oxygen at least during long-term culture. The reduction of oxygen to 1% caused a decrease in gene expression under all conditions, with exception for the gene expression of VEGF, which was up-regulated in all cases under hypoxia. In general, human NP cells were less sensitive to oxygen reduction than bovine NP cells. In both

species, pre-adaption of NP cells to 6% oxygen reduced sensitivity to further oxygen reduction. From these findings it can be concluded that a steep gradient in oxygen variation is necessary to alter gene expression.

Regarding the consequences of oxygen concentration on matrix turnover during long-term culture, there are controversies in literature. While some studies pointed out that a fall in oxygen concentration reduced synthesis of extracellular matrix [79, 96], others reported an increased GAG accumulation at hypoxia [20, 168]. In agreement with previous studies [20, 34, 76, 284], a strong induction of chondrogenic markers was shown in bovine and human NP cells at both 21% and 6% oxygen, demonstrating that the chondrogenic induction of the matrix molecules aggrecan and collagen type II might be independent of oxygen concentration. However, the subsequent oxygen reduction to 1% oxygen decreased gene expression of NP cells although chondrogenic medium was still present. Summing up the present results of all genes involved in matrix turnover, several differences between bovine and human NP cells were observed. After differentiation, expression of aggrecan and collagen type II was up-regulated in both species. However, in bovine NP cells collagen type I was slightly up-regulated while it was diminished in human NP cells. MMPs showed differing gene expression patterns, too. While in bovine NP cells gene expression of MMP3 and MMP13 remained unaltered, differentiation caused an up-regulation of MMP3 in human NP cells. As the discs of both species are phenotypically very similar [80] and are described to contain no notochordal cells [9, 32], differences might be explained by differences in the age, degeneration state and different expansion periods.

Subsequent reduction to hypoxic 1% oxygen caused alterations in gene expression of matrix molecules as well as matrix degrading enzymes with differences between both species (human, bovine) and their preadaptation conditions (21%, 6% oxygen). The steeper the gradient in oxygen reduction was the stronger were the influences seen on gene expression. Consequently, the balance between matrix synthesis and matrix degradation might be deranged. Within disc tissue, the degradation of major proteoglycans changes the properties of the disc and hence influences the swelling properties of the NP [97, 245]. The major disc proteoglycan aggrecan was shown to inhibit nerve growth and endothelial cell adhesion and thus vascularisation of the disc [101]. Consequently, degeneration associated degradation of aggrecan reduces the water binding

capacity and thus the swelling ability that is necessary to maintain disc height and flexibility and might indirectly facilitate neovascularisation triggered by angiogenic factors.

Focussing on effects of oxygen concentration on angiogenic factors, literature again reveals controversial theories. On the one hand, the angiogenic factors HIF1α and VEGF were seen as possible survival factors in healthy discs [57, 206]. On the other hand, both factors were also shown to promote neovascularisation during disc degeneration [42, 70, 201]. In the present study, gene expression of angiogenic factors after long-term adaption in chondrogenic medium showed only minor alterations. Only PTN was significantly down-regulated. PTN occurrence in human disc tissue is associated with increased vascularisation [102] but PTN has many other functions such as acting as mitogenic factor triggering proliferation [44]. Thus, a down-regulation of PTN expression after differentiation might be explained by the shift of the cells from proliferation during expansion to differentiation processes where proliferation is stopped.

In bovine cells, gene expression of angiogenic factors was higher in cells cultivated for four weeks at 21% oxygen whereas in human cells the only difference between 21% and 6% oxygen was seen for VEGF, which was expressed at a higher level at 6% oxygen. Interestingly, the subsequent reduction of oxygen concentration caused a reduction of HIF1α, PTN and bFGF but an up-regulation in VEGF expression in all cases under hypoxia, which underlines its role in disc angiogenesis. In a recent review, the authors hypothesised that VEGF and HIF1α also act as survival factors in disc tissue, thereby influencing cell metabolism and apoptosis [210]. Others reported similar findings in human articular chondrocytes [41]. Regarding other cell types, HIF1α is only stable under hypoxic conditions and undergoes rapid degradation at normoxia (21% oxygen) [273]. In contrast, NP cells exhibit stable expression of HIF1α at normoxia [195, 208]. In rat NP cells, HIF1α was shown to stabilise the expression of glucose transporter GLUT-1 and GAPDH [4], which are required for anaerobic glycolysis, the main way of energy production in discs [78]. This role of HIF1α as regulator of metabolic activity might explain the high gene expression of HIF1α at 21% as found in the present study indicating higher metabolic activity of the cells. VEGF was also thought to regulate cell metabolism, but specific blocking of VEGF signalling induced apoptosis in NP cells [57]. Thus, VEGF was found to support

cell survival rather than cell metabolism. However, other studies examined human disc samples and demonstrated a correlation between VEGF and vascularisation of disc tissue [70, 251]. This dual capacity of VEGF as angiogenic factor and survival factor was also described for chondrocytes during bone development [288]. Consequently, the decrease in HIF1α expression and the increase in VEGF expression with decreasing oxygen concentration in the present study might be an overall shift of the cells from high metabolic activity to cell survival. This strategy might help the cells to survive short periods of oxygen deprivation.

Another interesting point of the present study was observed in cells of group B, which were already adapted to low oxygen concentration (6%) and therefore underwent a relatively small change in the oxygen gradient by reduction to 1% compared to group A. Within group B changes in gene expression due to oxygen reduction were relatively low. In contrast, in cells of group A where the change in the oxygen gradient was high (from 21% to 1% oxygen), effects on gene expression patterns were greater. This points out that the physiological adaption of NP cells to hypoxia occurring within disc tissue might be protective, avoiding severe effects on gene expression when cells are exposed to oxygen deprivation.

However, results and conclusions of the present study were drawn from mRNA expression levels and verification on protein level needs to be performed in order to support the findings. Posttranscriptional regulation might modify final outcomes. Furthermore, the isolated role of oxygen as one of many factors in the pathology of disc degeneration was investigated. *In vivo*, deprivation of glucose, accumulation of lactic acid and a shift to acidic pH as well as other alterations occur simultaneously in addition to possibly reduced oxygen concentrations and act multifactorial on gene expression of disc cells [259].

Summarising all findings of this study, results revealed that after long-term cultivation at different oxygen levels, although some differences exist in the expression of several genes between 6 and 21% oxygen, bovine and human NP cells are able to induce chondrogenic differentiation at both conditions, indicating that hyperoxia does not inhibit the differentiation process. The present data suggest that the different oxygen concentrations during long-term cell culture procedures might have only minor influence on the results. However, the final goal should be to culture cells in an environment as similar as possible to the *in vivo*

conditions as the adaption to other conditions might alter their reactions to further stimuli. This effect was demonstrated by the recent data of bovine cells, which were transferred from 21% to 6% and subsequently to 1% oxygen resulting in a reduction in the expression of several genes involved in NP homeostasis. The changes observed after transfer to 1% oxygen are less intense in cells adapted for a long period to 6% oxygen. In contrast, human NP cells are rather insensitive to alterations of oxygen level. These data indicate that alterations of oxygen level during IVD degeneration in humans might have minor consequences. Finally, a constant change observed after a reduction of oxygen level in all cases was the overexpression of the angiogenic factor VEGF, probably representing a tissue response to counteract oxygen deprivation during disc degeneration.

Part B - Strategies of Disc Regeneration

The development of strategies for cell-based therapies for disc regeneration is a growing research field. The present work focussed on influences of medium supplementation with PRP and mechanical loading during NP cell tissue engineering to clarify if it is possible to induce a disc-like phenotype with these parameters. Additionally, the question what might happen to cells during cell therapy in degenerative environment was addressed. Thus, an organ culture system to simulate cell therapies was established and characterised.

4.3. Effect of Platelet-Rich Plasma and Hydrostatic Pressure

Generation of a biological NP replacement by tissue engineering is considered to be a promising approach for the therapy of early stages of IVD degeneration. The most important parameters to create disc-like cell constructs by tissue engineering are medium composition, culture system, cell type and a possible functionalisation by mechanical stimulation [108]. Thus, effects of chondrogenic medium supplemented with either PRP or TGF-β_1 on gene expression of MSC and NP cells were compared in pellet culture and alginate beads. Furthermore, the influences of mechanical loading by hydrostatic pressure were evaluated.

Regarding the important aspect of medium composition, results of the present study revealed that in comparison to TGF-β_1-supplemented medium, human PRP did not induce adequate chondrogenic differentiation independent of culture system or cell type used. Gene expression levels of all four chondrogenic marker genes as well as matrix protein deposition were lower using PRP. In recent years, the use of PRP was intensively investigated, mainly as treatment for small bone defects in maxillofacial surgery [152]. However, the clinical relevance for using a mixture of growth factors as in autologous PRP [271] to accelerate healing also has a great potential for cartilaginous defects, particularly as the application of recombinant TGF-β_1 alone has not received any clinical approval yet. Several *in vivo* and *in vitro* studies working with animal cells showed promising results. PRP stimulated extracellular matrix metabolism of porcine IVD cells cultured in alginate beads [6]. Direct injection of gelatine hydrogel microspheres filled with PRP into degenerated discs in rabbits led histologically to a suppression of disc degeneration [169], enhanced mRNA expression of proteoglycan core protein and collagen type II and improved disc height [223]. Application of PRP in an *ex vivo* porcine organ culture system resulted in an up-regulation of chondrogenic markers, which was confirmed by parallel *in vivo* studies [36]. Interestingly, in the same study, the combined application of MSC and PRP resulted in an inclination towards osteogenesis. Others also showed this osteogenic capacity of PRP, which might counteract chondrogenesis [112, 269]. This aspect has to be considered regarding clinical application. However, for evaluation of the above described studies in animal models, it has to be kept in mind that, in contrast to humans, rabbits and pigs have notochordal cells throughout their lifetime, making it difficult to compare these results with the human system [9]. Application of PRP on MSC and chondrocytes of sheep, animals which also lack notochordal cells, failed to induce chondrogenesis and only promoted proliferation [48]. In the present study, the mixture of growth factors in PRP also appeared to promote proliferation rather than chondrogenic differentiation, even though the final TGF-β_1 concentration was similar in both media used. Proliferation and differentiation naturally do not occur simultaneously, thus, an increase in proliferation negatively influences the differentiation status.

Regarding the culture system, the present experiments revealed differences in gene expression levels of matrix molecules in MSC and NP cells. Pellet culture appeared to be better suitable than alginate beads as crucial matrix factors were expressed more reliably and efficiently in pellet culture and thus, chondrogenic differentiation was better supported. This result confirmed former studies comparing alginate beads with pellet culture of chondrocytes [19, 49].

Mechanical stimulation of tissue-engineered cell constructs appears to be a promising tool for the development of more functional biological implants which are able to withstand *in vivo* loads. In the present experiments, however, a general positive influence of mechanical stimulation by hydrostatic pressure on MSC or NP cell pellet cultures could not be shown. Even though expression of the mechanosensitive transcription factor c-FOS was up-regulated (positive control), gene expression of matrix molecules was almost unaltered. In contrast, other studies applying loads to MSC or NP cells reported positive effects. This might be explained by two parameters: the culture conditions and the loading protocol.

The first aspect concerning the culture conditions has to be regarded since most studies were performed by using animal cells embedded in alginate [83, 122] or agarose [82, 249] and applying dynamic compression. Compared to the present study using pellet culture, a completely different situation regarding cell-cell and cell-matrix interactions was applied that might influence mechanical signal transduction. In contrast to cells embedded in hydrogels, the highly condensed pellet culture in the present study might provide insufficient conditions due to an inadequate extracellular matrix composition. Taking clinical application and feasibility into account, recent research has focussed on injectable hydrogels with or without cells, as hydrogels provide a more physiological cell density than pellet culture, support a better anchorage within the tissue and might additionally restore disc height by a minimally invasive surgery [200, 217, 225].

Regarding the loading protocols, other studies reported positive effects of the application of hydrostatic pressure on pellet cultures of human chondrocytes [49]. However, in this study hydrostatic pressure was applied daily. Studies on rat tail models showed a great dependency of stimulatory effects on loading protocols [147, 283]. In conclusion, the application of multiple loading cycles might have a stronger influence on matrix protein gene expression than the single, short

stimulation cycle of 30 minutes that was applied in the present study. Application of other mechanical stimulation protocols will be part of further studies.

Experiments of the present study demonstrated that MSC and NP cell reactions in response to medium composition and mechanical loading differ in only few aspects, making both cell types equal candidates for tissue engineering approaches. In literature, pros and cons exist for the use of both cell types [23, 108, 198, 234]. Although the donor collective of the present study was quite young and homogenous with regard to average age, most concerns and problems were implicated due to the variability of the results among individual donors. This indicates limitations for clinical application. Not all patients might be suitable for NP tissue engineering as their genetic background possibly influences the effectiveness of this form of therapy. Additionally, the initial set-up for the cell culture phase of tissue engineering might influence the results. Quantity and quality of the cells taken from patients always differ as well as expansion time to reach sufficient cell numbers for tissue engineering approaches.
Summarising and bringing back to mind the initially introduced important aspects of tissue engineering, several questions were clarified. It was shown that although MSC and NP cells reacted with minor differences, in principle, both cell types reacted in a similar manner to changed parameters in the present tissue engineering approach. Furthermore, favourable results for the pellet culture compared to alginate beads were found. The promising expectations for the application of autologous PRP for NP tissue engineering could not be confirmed. Further studies focussing on protocols of tissue/cell sampling, cell culture and improvement of tissue engineering approaches by mechanical stimulation are required to find optimal therapies for disc disorders.

4.4. Organ Culture System for Cell Therapy Simulation

The biological treatment of disc degeneration by cell-based approaches is a challenge because of the harsh environment in degenerated discs. As no adequate animal model for disc degeneration exists and as the availability of complete human IVD for research purposes is very limited, standardised model

systems that simulate the degenerative disc environment are needed to test effects and survival of injected cells.

In the present study, such an organ culture system was established by using standardised punched bovine coccygeal IVD cultured under static loading. With proceeding culture time, GAG of the IVD were released into the cell culture medium and intense cell cluster formation was seen. Both are features mimicking situations, which can be similarly observed during disc degeneration. In literature, several IVD organ culture systems exist using IVD of various animal species. The group of Risbud and colleagues used whole rat IVD motion segments and was able to demonstrate IVD cell viability for one week and revealed positive effects of TGF-β on the maintenance of IVD cells during culture of the whole segment [204, 207]. Lim *et al.* used a similar system and demonstrated that IVD cells can survive for two weeks in this *in vitro* organ culture [136]. Haschtmann and colleagues developed an organ culture system of whole rabbit IVD and reported on the maintenance of IVD cell viability for up to four weeks accompanied by a decrease in cell metabolism and matrix molecule synthesis [71]. In this system, daily hyperosmotic stimulation did not affect cell viability or matrix synthesis [72]. However, the usage of rodent IVD organ culture systems remains debatable due to the occurrence of notochordal cells compared to human IVD. Gantenbein and co-workers established an ovine IVD organ culture system and cultured whole IVD with the CEP and uniaxial daily loading. After seven days in culture, unaltered cell viability and GAG synthesis rate was shown. However, gene expression of catabolic genes was up-regulated while anabolic genes were down-regulated [58]. Lee and colleagues established an organ culture system using whole bovine coccygeal IVD and compared culture of IVD with and without vertebral endplates under static loading in a self-made culture chamber. While tissue integrity was better with endplates, cells died due to limited nutrition. Without vertebral endplates, cell viability was maintained for up to seven days. Thus, it was concluded that culture without vertebral endplates is more suitable [129]. Korecki *et al.* compared static loading *versus* daily axial loading within the same system and demonstrated better suitability of static loading for IVD cell viability [120]. In a follow-up study, the same authors were able to show that needle puncture affects disc mechanics and cell viability unrecoverably [121]. Cell cluster formation was demonstrated close to the insertion site of the needle. Hence, it was concluded

that this cell cluster formation was induced by needle puncture. Cell clusters form via cell proliferation possibly in an effort to repair extracellular matrix [100]. These cell clusters are often found in the regions of the IVD showing the greatest macroscopic evidence of degeneration and are directly linked to increased immunolocalisation of stress-activated heat shock factors [232]. Thus, the cell cluster formation seen in the present model might - at least in part - be explained by the initial needle puncture, which was applied.

In a recent study of Jim and colleagues, the culture of bovine IVD with vertebral endplates, IVD with CEP and IVD without any endplate was compared and additional enzymatical digestion with trypsin was performed to establish a model for disc degeneration [99]. In IVD with vertebral endplates nutrition of the cells were insufficient and cell viability strongly decreased. For IVD with CEP and IVD without endplates no difference in maintenance for cell viability was observed. However, due to the deformation of the IVD without endplates, the authors concluded the best suitability for IVD with CEP. Additionally, treatment with trypsin reduced aggrecan synthesis, which could be overcome by the injection of TGF-β. A comparable study by Gawri *et al.* using human IVD with vertebral endplates, IVD with CEP and IVD without any endplate in addition to high and low nutrient levels also demonstrated the best suitability for IVD with CEP [60]. As the availability of human samples is limited, the bovine system allows for a better standardisation. In the present study, the undesirable IVD deformation due to swelling pressure was prevented by applying static loading with the cell culture inserts. Thus, this system might be comparable to the other studies using IVD with CEP to prevent deformation. In addition, the present organ culture system provides unimpaired nutrient supply from all sites to the IVD.

The present organ culture system was tested for its suitability for cell injection experiments. In the present study, small volumes of a cell suspension or a cell-seeded albumin-hyaluronan hydrogel could be injected and cells as well as the hydrogel were detectable afterwards. In this first approaches, the cell-seeded hydrogel showed probably positive effects on cells close to the injection site. The more intense alcian blue staining in the cells' periphery provides an indication of a possibly increased matrix formation. However, the injected cells or the albumin-hyaluronan hydrogel did not influence the amount of GAG released into the cell culture medium. The injected volumes were probably too small to influence

the whole IVD punch. Roberts *et al.* reported on the same problems and used enzymatical digestion with trypsin and papain to create defects of about 2 ml volume. Defects of this size can also be seen in clinical practice [216]. However, others were able to inject a cell suspension of articular chondrocytes overexpressing BMP7 into an organ culture of whole rabbit IVD [291]. In this model, cell viability was maintained for two months and a positive effect on matrix synthesis by the genetically altered chondrocytes was demonstrated. A recent study of Peroglio and co-workers characterised an injectable hyaluronan-based hydrogel in an ovine organ culture [188]. A good cytocompatibility of the hydrogel and cell survival within this carrier in the IVD tissue was reported. However, effects of the hydrogel on the IVD tissue were not evaluated.

Otherwise, no study exists reporting about cell therapy simulations in *in vitro* organ culture systems using cell suspensions or cell-seeded carrier materials. Thus, the present system provides great potential for future studies as it allows for a standardised simulation of normal *versus* degenerated conditions regarding glucose supply, oxygen concentration and pH value. The influence of these factors on different biomaterial-based approaches can be investigated at well-controlled *in vitro* conditions. However, further investigations and characterisation of the histologically observed features are needed to evaluate influences of cell therapies on the IVD tissue. This will be part of future studies.

5. Summary

As disc degeneration is often associated with back pain, it has a high clinical relevance. The reasons leading to disc degeneration are as multifactorial as its consequences are variable. The present work addressed two major topics: the understanding of the influence of environmental stress on intervertebral disc (IVD) cells (part A) and the development of biological repair strategies (part B).

In part A, glucose and oxygen concentration as critical nutritional factors were analysed since with proceeding disc degeneration and calcification of the cartilaginous endplates the main route of transport of nutrients and metabolites is impaired.

Regarding the impact of glucose concentration, it was investigated how glucose deprivation influences bovine nucleus pulposus (NP) cells after long-term culture in alginate beads and if this glucose deprivation also influences mechanosensitivity. Despite a successful differentiation of NP cells, subsequent glucose deprivation revealed a strong down-regulating impact on gene expression of matrix molecules and an increase in matrix metalloproteinase 13 expression. Application of hydrostatic pressure did not affect these results. However, in high-glucose chondrogenic medium supplemented with transforming growth factor β_1 (TGF-β_1), hydrostatic pressure increased expression of matrix molecules and decreased expression of catabolic enzymes. These findings may indicate an interaction of glucose supply, presence of TGF-β_1 and mechanical loading.

Furthermore, questions on the impact of oxygen concentration on long-term differentiation of NP cells and on the comparison of disc-hyperoxic 21% oxygen (group A) to disc-normoxic 6% oxygen (group B) were addressed. It should thereby be clarified if it is important to culture NP cells at reduced oxygen conditions. Additionally, the effect of oxygen reduction to 1% which might occur during disc degeneration was investigated starting from the two different pre-culture conditions at 21% (group A) or 6% oxygen (group B). Regarding long-term cultivation, the different oxygen concentrations had only minor influence on the results although some differences existed in the expression of several genes between 6% and 21% oxygen. However, the changes observed after transfer to 1% oxygen were less intense in cells adapted for a long period to 6%

oxygen. Human NP cells were thereby rather insensitive to alterations in oxygen level. The overexpression of the angiogenic factor vascular endothelial growth factor in all cases after a reduction of oxygen level was demonstrated, probably representing a tissue response to counteract oxygen deprivation during disc degeneration.

Part B focussed on the development of strategies for cell-based therapies in case of disc regeneration, which is a growing research field.
First, influences of medium supplementation with platelet-rich plasma (PRP) and mechanical loading during tissue engineering using human mesenchymal stem cells (MSC) and NP cells were investigated to clarify if it is possible to induce a disc-like phenotype with these factors. For both, MSC and NP cells, human PRP was not able to replace TGF-β_1 with regard to chondrogenic differentiation. The mixture of growth factors in PRP appeared to promote proliferation rather than chondrogenic differentiation. Mechanical stimulation did not appear to enhance chondrogenic differentiation. Thus, mechanical loading as promising approach for the development of more functional biological implants needs further investigation. However, the variability of the results implies limitations indicating that the individual background of the patients might influence the application of this potential form of therapy.
Secondly, the fate of disc cells during cell therapy approaches in a degenerative environment was investigated. As no adequate animal model for disc degeneration exists and as the availability of complete human IVD is very limited, a standardised model system that simulated the degenerative disc environment was established and characterised to test effects on and survival of injected cells. The present findings on a decreased intradiscal proteoglycan concentration due to glycosaminoglycan (GAG) release into the culture medium and the formation of cell clusters suggest that this organ culture system is suitable for *in vitro* simulation of a degenerative disc environment at standardised conditions. The detection of green fluorescent protein-labelled cells appearing surrounded by intense GAG staining indicated that the injected cells might have survived and stayed metabolically active within the disc organ culture environment. Thus, this organ culture model can be used for *in vitro* testing of cell-based therapy strategies by simulation of a degenerative disc environment.

6. References

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Curriculum vitae

For reasons of data protection the curriculum vitae is not included in the online version.