Engineering Human Nasal Chondrocyte Spheroids, Towards Regeneration of the Intervertebral Disc

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2. Summary

Chronic low back pain (LBP) is a leading cause of disability worldwide and is strongly associated with degeneration of the intervertebral disc (IVD) [1, 2]. Current therapies for the treatment of discogenic LBP (termed degenerative disc disease, DDD) include conservative or surgical methods which have their limitations and drawbacks. These may include discectomy, fusion surgery and disc arthroplasty [3]. Nevertheless, in recent years, scientific knowledge on human physiology and cell biology as well as interactions has greatly increased creating a tremendous impact in the advancement of tissue engineering and prominently regenerative techniques for IVD repair [4]. Cell therapy has shown promising results for IVD regeneration.

Regenerative IVD therapies target minimal-invasive application of autologous cells amongst others that should ideally differentiate to IVD-like cells and/or secrete trophic and anti-inflammatory factors with the aim to repair the IVD [3, 5-7]. A typical approach taken is to combine cell suspension with biocompatible materials which are supposed to provide mechanical stability and protection of the cells after implantation. Nevertheless, none of these strategies have been widely accepted for clinical implementation. Several publications have indicated that therapeutic cells suffer within the harsh microenvironment of the DDD [5, 8-12]. Human nasal chondrocytes (NC) are a promising cell source for the treatment of DDD. NC can be harvested from nasoseptal cartilage biopsy obtained under local anaesthesia and with minimal donor site morbidity [13-15]. They were shown to possess high proliferation and post-expansion differentiation capacity and can adapt to the heterotopic transplantation sites [16]. Moreover, they showed superior viability over articular chondrocytes (AC) and mesenchymal stromal/stem cells (MSC) in simulated DDD microenvironment thus represent a robust cell population with the potential of survival in the IVD post injection [17].

Within other tissue engineering fields, it has been demonstrated that spheroid-based strategies might help to overcome hurdles associated with DDD due to their superior regenerative performance and/or resistance compared to single cells [18, 19]. Spheroids can self-assemble by forming intercellular contacts on non-adhesive substrates [20-23]. Their 3D organization mimics closer the physiological conditions and allows the cells to differentiate toward target tissue. The 3D structure allows the cells to accumulate ECM that might protect them from a surrounding harsh micromilieu [1, 24]. Furthermore, by priming the spheroids using biomimetic environment [25-27], genetically modifying the cells [28, 29], and/or combining the spheroids with an instructive biomaterial [30] is hypothesized to further increase their performance within a harsh microenvironment. Therefore, taking a spheroid-based therapeutic approach for IVD repair could be a promising strategy.

Currently applied *in vitro* models to study cell treatment strategies for IVD repair use 2D and 3D culture systems among others [31]. The use of an *in vitro* 3D model mimicking the degenerated IVD environment, would allow to study the regenerative performance of the therapeutic cells. The harsh IVD microenvironment is characterized by avascular, hypoxia, low glucose level, acidic, inflamed, high osmolality and non-physiological biomechanics [32]. However, it has been shown that less than 15% of the in vitro studies include either one of these parameters thus not mimic the harsh IVD microenvironment properly [31].

In my PhD thesis I give an introduction into the field of my research (Chapter 1: Introduction) and highlight the areas of future research in spheroid-based regeneration of IVD in the form of a review (Chapter 2: Spheroid-Based Tissue Engineering Strategies for Regeneration of the Intervertebral Disc). I discuss cell sources and methods for spheroid fabrication and characterization, mechanisms related to spheroid fusion, as well as enhancement of spheroid performance in the context of the IVD microenvironment.

I report my findings on the suitability of nasal chondrocyte derived spheroids (NCS) for use as grafts in cell-based and scaffold-free regeneration of the NP. In particular, I investigate whether human NCS (1) can be generated in a feasible and reproducible way (2) possess the biomechanical and biochemical properties relevant for the target NP tissue, and (3) can be injected into the IVD via a spinal needle and engraft within a DDD-mimicking microenvironment (Chapter 3: Nose to Spine: spheroids generated by human nasal chondrocytes for scaffold-free nucleus pulposus augmentation).

Furthermore, I introduce a novel 3D *in vitro* degenerative NP micro-tissue (NPμT) model that allows researchers to study the reaction of NCS to the harsh DDD microenvironment. I investigate how pre-conditioning of the NCS with FDA approved drugs could enhance their performance within DDD microenvironment (Chapter 4: Human 3D nucleus pulposus micro-tissue model to evaluate the potential of pre-conditioned nasal chondrocytes for the repair of degenerated intervertebral disc).

3. Chapter 1: Introduction

In this section, the structure of the IVD and its function is described. Furthermore, the development of degenerative disc disease (DDD) and the current therapies for DDD treatment are depicted.

Lower back pain is a common musculoskeletal disorder concerning more than 600 million people around the world making it the leading disability in the world [33]. It creates a significant socioeconomic burden among active ageing populations. In total ~40% of the world population is experiencing LBP during their lifetime [34]. Low back pain is a leading purpose for physician visits, hospitalization and use of other health care services [35]. IVD degeneration is considered to be the most common and primary cause of low back pain. Discogenic LBP (termed degenerative disc disease, DDD) is referred to as the association of IVD degeneration with pain and inflammation [36, 37].

3.1 Intervertebral Disc (IVD)

The IVD is built as a tough tissue structure in between the vertebral bodies with three distinctive parts (Figure 1): the nucleus pulposus (NP), the annulus fibrosus (AF) and of cartilaginous end plate (CEP). This complex structure allows the spine to perform flexible 3D motions. The IVD needs to fulfill three important functions. It must be able to bond and hold the vertebrae bodies of the spine together, then it is required to act as a shock absorber and lastly by taking the role of a "pivot point" it must offer the spine the possibility to bend, rotate and twist [38, 39].

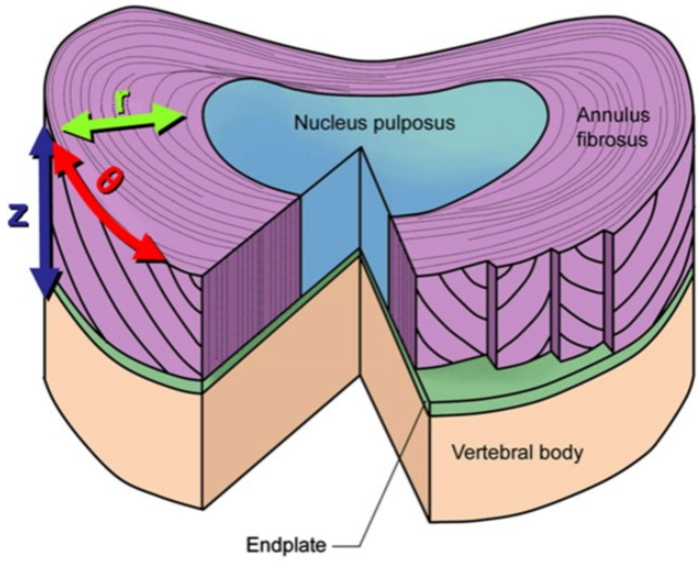

Figure 1: Schematic representation of the multi-scale architecture of the intervertebral disc with the indication of the primary geometric axes [40].

The NP and AF have a number of structural, cellular, and molecular differences, which explain their distinctive biomechanical properties (Table 1).

*Table 1. Composition of healthy nucleus pulposus (NP) and annulus fibrosus (AF) [41]. *Percentage of wet weight; ‡Percentage of dry weight of the IVD. PG = proteoglycans.*

	Water	**Collagens**	**PG**	**Other proteins**	**Cells**
NP (inner core, highly hydrated and jelly tissue)	70-90%*	15-20%‡ mainly collagen type II	65%‡	20-45%‡	Minor
AF (outer IVD ring, elastic and fibrous tissue)	60-70%*	50-60%‡ mainly collagen type I	20%‡	10%‡	Minor
Function	*Hydrostatic pressure*	*Tensile strength*	*Osmotic pressure*	*Support of matrix and cells*	*Homeostasis*

3.1.1 Nucleus Pulposus

The nucleus pulposus (NP) is a large hydrated inner core of the IVD and it contains mainly water, collagens, and proteoglycans (Figure 2) [42, 43]. 77% of the wet weight in NP consist of water and together with collagen type II, the NP is capable to elastically deform under stress [44]. The primary collagen in the NP is collagen type II (4% of the wet weight) while collagen of type VI, IX, XI are present in smaller amounts. Proteoglycans constitute around 14% of the wet weight in the NP and contributes mainly to the tensile strength of the disc hence plays a key role in resisting compression and providing resilience. The most abundant proteoglycan in the NP is aggrecan (ACAN) with lesser amounts of versican, biglycan, decorin, and fibromodulin [45]. The major macromolecules are chondroitin sulfate A and C, which are strongly hydrophilic and promote disc viscosity by binding water. When the NP is experiencing axial compression, it diverts the forces evenly towards the AF therefore acting as a shock absorber. Morphologically, the nucleus pulposus of a child is translucent and gel-like but during adulthood it becomes more whitish and fibrous due to an increase in concentration and diameter of the collagen fibrils [46].

The cellularity of the NP reaches an average cell density of 3000 cells/mm^3 which remains three times lower than the cellularity found in the AF (9000 cells/mm^3) [47]. In early childhood, the cells within the NP have a similar phenotype (25 − 85μm diameter, vacuole-like) as the cells within the embryonic notochord. During the first decade of life, the amount of these cells decreases and are slowly replaced by smaller (10μm diameter), round and non-vacuolated cells [48]. These mature NP cells portray a morphology termed as "chondrocyte-like cells" because of the large similarity to articular chondrocytes. The distinction remains with a small portion of cells still expressing notochordal biomarkers and carrying a different phenotype [49].

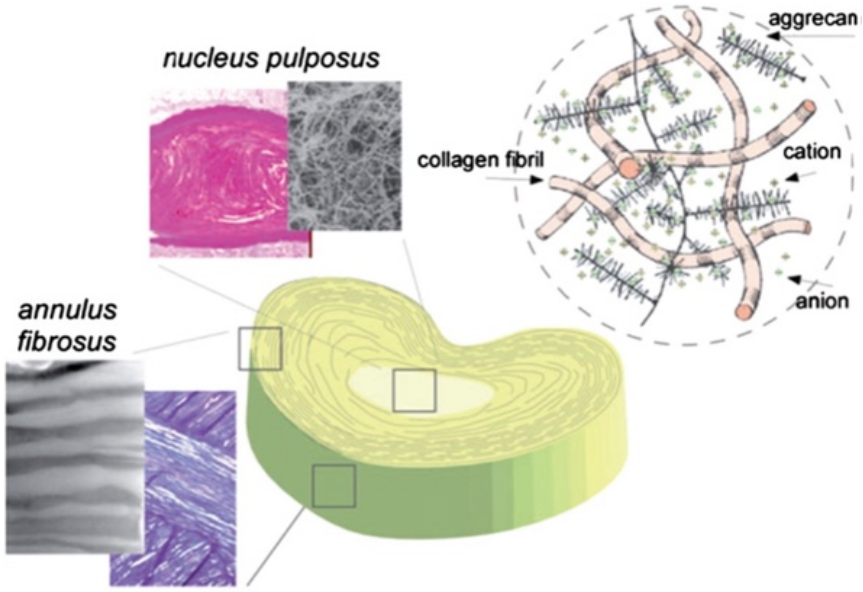

Figure 2: Schematic demonstrating the different regions of an IVD with their composition and structure [50].

3.1.2 Annulus Fibrosus

The AF is formed of 15-25 parallel layers, namely the lamellae, which are each approximately 0.05-0.5 mm in thickness. This thickness also increases from outer to inner part among other characteristics that follow such trend. Less than half of these lamellae are circumferentially incomplete and this number escalates with age [47]. Similarly, to NP, the inner section of AF has a higher content of proteoglycan, water and collagen type II but reduced in collagen type I. Collagen type II create a meshed network that hold proteoglycans and thus retaining water. Just like a hyaline cartilage, this empowers the inner AF to cope with large compressive forces [48]. Strong collagen type I fibers are constituents of each layer with an orientation that alternates throughout subsequent sheets between ±45° to the transverse plane (Figure 3c). Towards the inner wall of the AF, this angle increases to approximately 60° [49]. This type of network provides an increased shear resistance between neighboring layers. The AF allows the NP to withstand the tensions enabling an elastic deformation.

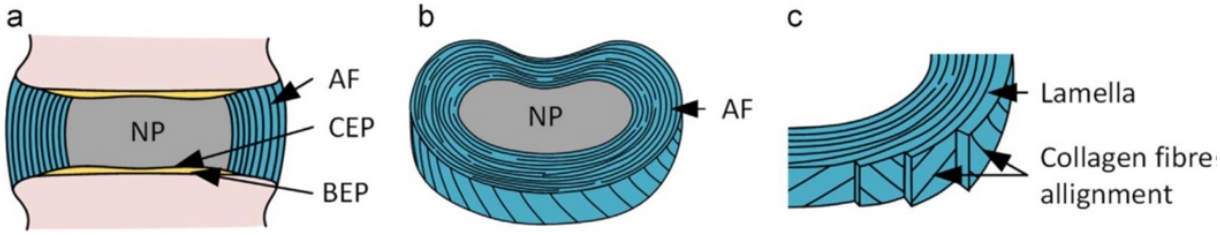

Figure 3: Anatomy of a disc. (a) Cross section of a disc in the coronal plane, (b) diagram of a transversely sliced IVD and (c) diagram showing the alternating fiber alignment in successive lamellae. AF: annulus fibrosus; CEP: cartilaginous end plate; BEP: bony end plate; NP: nucleus pulposus [39].

3.1.3 Cartilaginous end plate

The cartilaginous end plate (CEP) is usually around 0.6 mm thick. CEP are made of hyaline cartilage that is attached to the adjacent bony end plates (BEP), a thick layer of perforated cortical bone [51]. The thickness of the IVD decreases with age. Major components at dry weight of the CEP are collagen type II and proteoglycans. The entire unit contains about 60% water. The collagen content is higher and PGs content lower, at the brink of the disc [52]. The stable 3D collagen type II network of the CEP prevents swelling which also reduces the discharge of water from the NP when heavily pressured while still allowing nutrients and oxygen to diffuse into the disc from the vertebral body [52]. While some of the compressive load on the NP is transmitted towards the AF, the underlying layer of CEP and BEP are capable of bulging by about 1 mm into the vertebral body [53].

3.2 Degenerative disc disease (DDD)

3.2.1 Disc degeneration

The association of disc degeneration with inflammation and chronic lower back pain is referred to as degenerative disc disease (DDD). IVD degeneration and subsequent imbalance of anabolic/catabolic activities could arise due to aging. In fact, it involves the loss and destruction of tissue over time that may very well be asymptomatic for certain people [38]. At the early stages of IVD degeneration, the NP first begins to dehydrate and become more fibrous [54]. The dehydration is directly linked to a drastic decrease in size and content of PGs, collagen type II and water concentration in the NP. It causes the pressure to drop in the NP leading to approximately 50% in reduction of the longitudinal diameter [55]. The NP cannot execute anymore its function properly namely to distribute axial compression evenly towards AF which results in some cases in AF rupture. The accumulation of these circumstances results in further disc matrix degeneration as well as loss of hydration and disc height causing a significant impediment on the ability of the IVD to function properly (Figure 4). With increasing age the calcification of the CEP by calcium phosphate crystalline deposits influence the IVD degeneration [56]. Reduced CEP permeability could lead to reduced vascular connections in the subchondral bone and hinders oxygen, nutrition and waste diffusion as well as hampers cell metabolism and biosynthetic function causing or accelerating IVD degeneration [57-59]. Subsequently, the decrease in oxygen concentration leads to an increase in lactate production and a decrease in pH as well as affect adversely the cellular metabolism [60, 61].

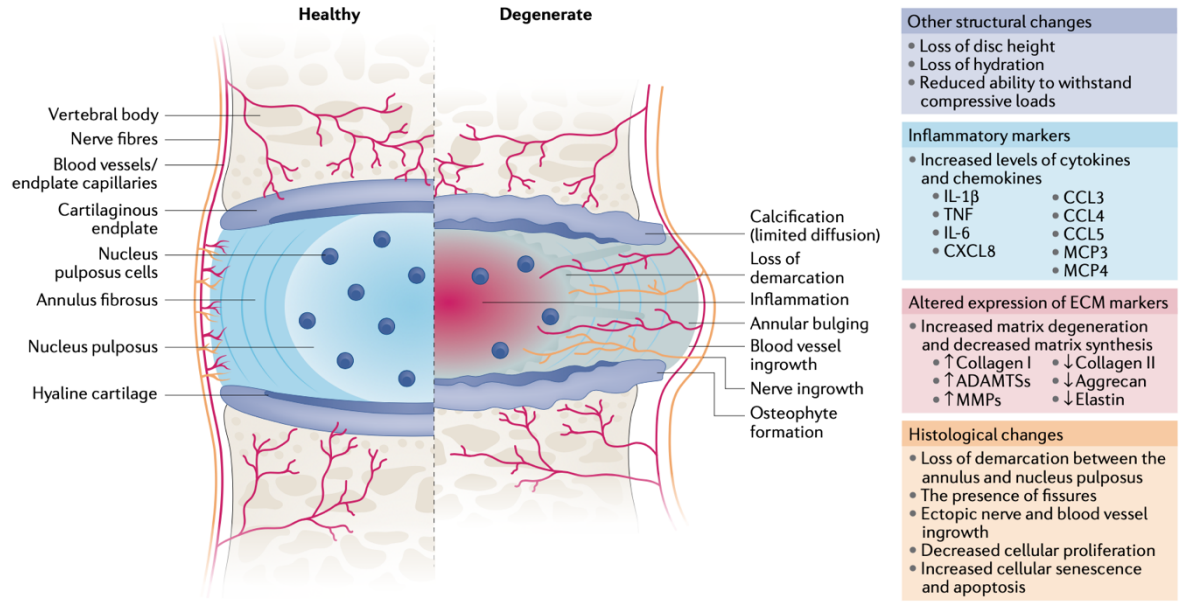

Figure 4: IVD anatomy and physiology in health (left) and disease (right) [59]. *IVD degeneration could lead to altered ECM marker expression, loss of disc height and subchondral bone sclerosis. CEP calcification combined with limited blood flow compromise disc nutrition, oxygenation, and removal of metabolic waste leading to unfavorable effects on the activity and viability of IVD cells. Development of ectopic sensory nerve fibers and blood vessels lead to discogenic pain.*

3.2.2 Pain and inflammation

The onset of discogenic LBP is characterized by the migration of immune cells into the degenerating disc and the ingrowth of nerve fibers into an otherwise aneural IVD [62-64]. The degenerating IVD releases chemokines and cytokines which enhance the activation and infiltration of leukocytes into the IVD which secrete their own cytokines, further amplifying the inflammatory response [65, 66]. Infiltration of the immune cells is accompanied by neovascularization and the appearance of nociceptive nerve fibers that arise from the dorsal root ganglion (DRG) within the herniated IVD [62-64]. Consequently, neurogenic factors produced by the herniated disc as well as invading leukocytes induce the expression of pain-associated cation channels such as acid-sensing ion channel 3 (ASIC3) and transient receptor potential cation channel subfamily V member 1 (TrpV1) in the DRG [67-69].

The involvement of low-grade inflammation and metabolic disturbances in IVD degeneration has been extensively scrutinized, directing the focus of research to the immune-metabolic features of disease pathophysiology and the severe alterations in metabolism [57, 70-72]. The progression of IVD degeneration has been associated with the abnormal production of pro-inflammatory molecules secreted by both NP and AF cells as well as macrophages, T cells, and neutrophils [73-75]. These molecules could activate signaling pathways that promote autophagy, senescence or apoptosis [76-78]. Pro-inflammatory mediators associated with IVD degeneration and ECM-degrading factors such as metalloproteinases (MMPs) and a disintegrin and metalloprotease with thrombospondin motifs (ADAMTS) include TNF, IL-1α, IL-1β, IL-6, IL-8, IFN- γ, and PGE_2 among others [79-82]. For example, IL-1α and IL-1β could trigger signal transduction via binding to the receptor of IL1 (IL1-R1) which activates a cascade of events downstream of the IL-1R complex (Figure 5). As a result, signaling

proteins such as mitogen-activated kinases (JNK, p28, ERK1/2), transcription factors including NFκB (p65 and p50 subunits) and c-Jun (a subunit of AP-1) will be activated which control the expression of inflammatory and catabolic genes such as MMPs and ADAMTS [83].

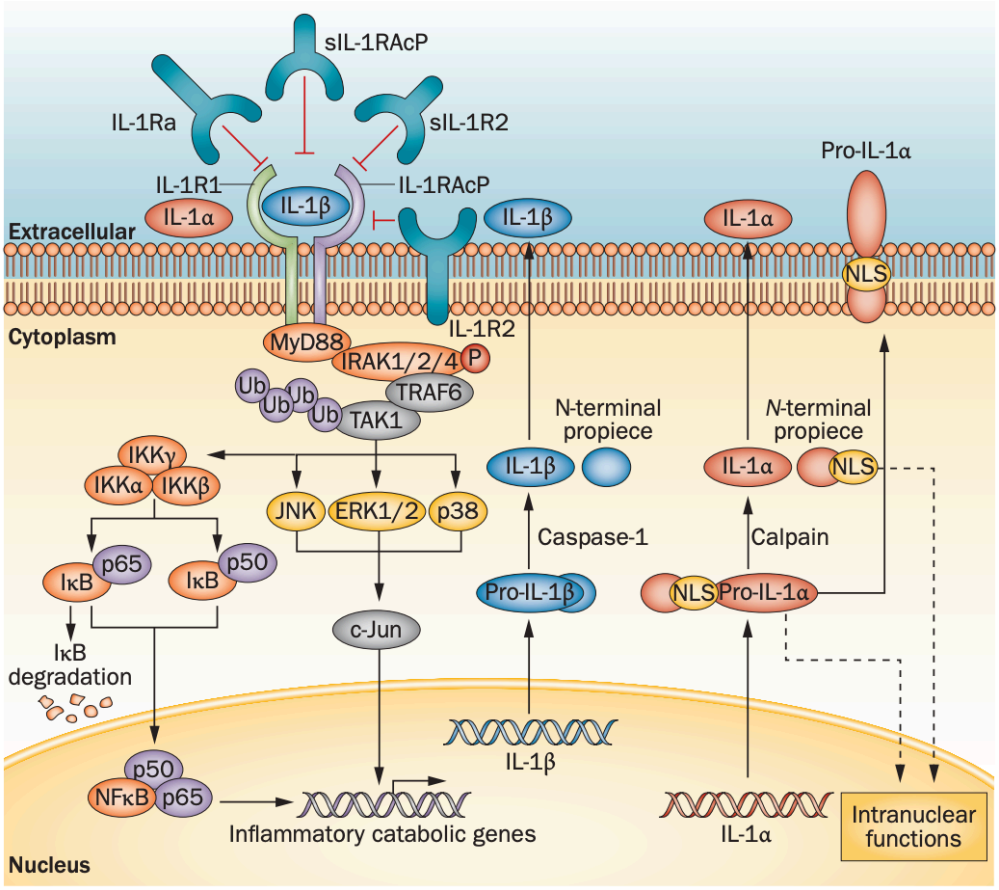

Figure 5: IL-1α and IL-1β synthesis and signal transduction pathway [83]. *IL-1α and IL-1β are synthesized as precursor proteins (pro-IL-1α and pro-IL-1β), which then undergo proteolytic cleavage by calpain and caspase-1, respectively, to produce the mature active forms. Pro-IL-1α, IL-1α and IL-1β can all bind to IL-1R1, which enables recruitment of the IL-1RAcP co-receptor. A cascade of events downstream of the IL-1R complex results in the activation of important signalling proteins, such as mitogen-activated kinases (JNK, p38, ERK1/2), as well as transcription factors, including NFκB (p65 and p50 subunits) and c-Jun (a subunit of AP-1), which control expression of a number of inflammatory and catabolic genes. Signalling through the IL-1R complex is modulated by inhibitory actions of IL-1R2, sIL-1R2, sIL-1RAcP and IL-1Ra. Abbreviations: AP-1, activator protein 1; ERK1/2, extracellular signal-regulated kinase 1/2; IκB, inhibitor of nuclear factor κB; IKK, IκB kinase; IL-1R1, IL-1 receptor 1; IL-1R2, IL-1 receptor 2; IL-1Ra, IL-1 receptor antagonist; IL-1RAcP, IL-1 receptor accessory protein; IRAK, IL-1 receptor-activated protein kinase; JNK, c-Jun N-terminal kinase; MyD88, myeloid differentiation primary response gene 88; NFκB, nuclear factor κB; NLS, nuclear localization sequence; sIL-1R2, soluble IL-1R2; sIL-1RAcP, soluble IL-1RAcP; TAK1, transforming-growth-factor-β-activated protein kinase 1; TRAF6, TNF receptor-associated factor 6.*

3.2.3 Diagnosis of DDD

The ideal methodology to diagnose a degenerated disc remains complex and difficult. An IVD degeneration is graded on the basis of published criteria such as magnetic resonance imaging (MRI) [84], radiographic imaging [85], or by directly visualizing of IVDs dissected in the longitudinal or transverse plane [86]. Unfortunately, these methods are found to be imperfect as they do not necessarily deal with the entirety of the disc or simply turn out to be ambiguous as they are unable to reliably characterize all stages of the degeneration [86]. Currently the most efficient clinical method is to perform an MRI giving the possibility to investigate each portion of the IVD and consequently assess the disc degeneration and disc height [87]. With such imaging the disc degeneration is analysed based on water content, height, as well as searching for tears or disparities within the tissue.

For grading lumbar disc degeneration, criteria were published by Pfirrmann et al. in 2001 [88]. In their study, the authors developed a classification system for disc degeneration based on routine T2 weighted MRI. The signal loss of the disc on T2-weighted MRIs has been shown in previous study to correlate with progressive degenerative changes of the intervertebral disc [89]. The grading system and algorithms are based on MRI signal intensity, disc structure, distinction between NP and AF, and IVD height (Figure 6) which is now being routinely used by surgeons for scoring IVD of patients.

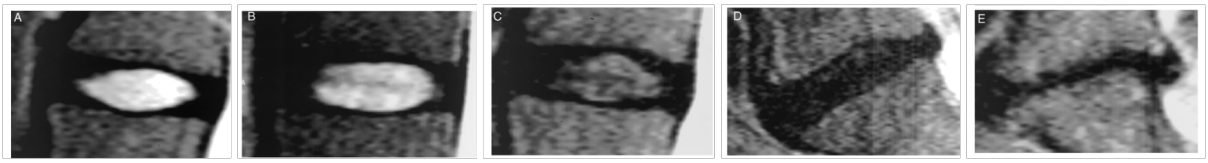

Figure 6: A–E, Grading system for the assessment of lumbar disc degeneration [88]. Grade I: The structure of the disc is homogeneous, with a bright hyperintense white signal intensity and a normal disc height. Grade II: The structure of the disc is inhomogeneous, with a hyperintense white signal. The distinction between nucleus and anulus is clear, and the disc height is normal, with or without horizontal gray bands. Grade III: The structure of the disc is inhomogeneous, with an intermediate gray signal intensity. The distinction between nucleus and anulus is unclear, and the disc height is normal or slightly decreased. Grade IV: The structure of the disc is inhomogeneous, with an hypointense dark gray signal intensity. The distinction between nucleus and anulus is lost, and the disc height is normal or moderately decreased. Grade V: The structure of the disc is inhomogeneous, with a hypointense black signal intensity. The distinction between nucleus and anulus is lost, and the disc space is collapsed. Grading is performed on T2-weighted midsagittal (repetition time 5000 msec/echo time 130 msec) fast spin-echo images.

3.3 Current approaches for DDD treatment

3.3.1 Nonsurgical treatments

According to the guidelines of the global spine care initiative, patients with DDD lasting >3 months without serious pathology are recommended non-pharmacological options, exercise and yoga amongst others for treatment, in combination with psychological therapies such as cognitive behavioral therapies and progressive relaxation (Table 2) [90]. DDD patients experience persistent symptoms and which are often accompanied with functional limitation thus the main target of non-pharmacological treatment is to restore function but also to address psychological and social factors.

Table 2. Non-pharmacologic recommendations for chronic non-specific low back pain [90].

Intervention	Benefits	Harms	Resources	Feasibility	Recommendation
Exercise	Moderate	Small	Low	High	R
Yoga	Small–moderate	Small–moderate	No evidence	Moderate	R
Patient education	Small	Small	Low	High	R
Acupuncture	Moderate	Small	Moderate	Moderate	RC
Biofeedback	Small	Low	Moderate	Moderate	RC
Low-level laser	Small	Low–moderate	No evidence	Moderate	RC
Clinical massage[a]	Small–moderate	Small	Low	Moderate–high	RC
Manual therapy	Small–moderate	Small–moderate	Moderate	Moderate	RC
Multidisciplinary/multimodal rehabilitation	Small–moderate	Small	Low	Low–moderate	RC
Progressive relaxation	Small	Small	Moderate	Moderate	RC
Psychological therapies	Small	Small	Low–moderate	Moderate	RC
Electrical muscle stimulation	None	No evidence	Moderate	Moderate	RA
Electroacupuncture	None	Small	–	–	RA
Moist heat	None	No evidence	–	–	RA
Short-wave diathermy	None	Small	–	–	RA
Relaxation massage[b]	None	Small	–	–	RA
Standalone course of relaxation training	None	Small	–	–	RA
Strain–counterstrain	None	Small	–	–	RA
TENS	None	Small	–	–	RA
Kinesio taping	Uncertain	Small	Low–moderate	High	I
Lumbar support	Uncertain	Small	Low	High	I
Ultrasound	Uncertain	Small	Moderate	Moderate	I
Traction	Uncertain	Small	Moderate	Moderate	I

R recommended, *RC* recommendation for consideration, *RA* recommended against, *I* inconclusive, – no data

[a]Clinical massage aims to address clinical concerns (e.g., myofascial trigger point therapy, myofascial release)

[b]Relaxation aims to promote relaxation and wellness (e.g., swedish massage, spa massage)

Pharmacological treatments attempt to reduce pain rather than repair the damaged IVD. The treatments used presently are mainly conservative and are aimed at returning patients to work. They range from bedrest to use of nonsteroidal anti-inflammatory drugs (NSAIDs), skeletal muscle relaxants, and antidepressants amongst others (Table 3). They are frequently prescribed for the patients with DDD and in some cases, opioids are used but in caution due to the risk of addiction and consequences resulting out of it such as overdosing, and in worst case to death [91, 92].

Table 3. Pharmacologic interventions for chronic non-specific low back pain [90].

Intervention	Benefits	Harms	Resources	Feasibility	Recommendation
Acetaminophen	Small	Small	Low–high	High	RC
Antidepressants (serotonin norepinephrine reuptake inhibitors, tricyclic antidepressants)	Small–moderate	Small–moderate	Low–moderate	High	RC
NSAIDs	Small–moderate	Small	Low	High	RC
Opioids	Small–moderate	Moderate–high	Low–moderate	High	RC[a]
Benzodiazepines	None	Moderate	Low	High	RA
Botulinum toxin injection	None	Small	High	Low	RA
Gabapentin/pregabalin	Uncertain	Small–moderate	Low–moderate	High	I
Skeletal muscle relaxants	Uncertain	Small–moderate	Low	High	I

R recommended, *RC* recommendation for consideration, *RA* recommended against, *I* inconclusive

[a]Short-term use with caution

3.3.2 Surgical treatments

For patients at severe stages, surgical interventions are considered. For example, patient with herniated or bulging discs, with signs of compressed spinal nerves, would most likely undergo discectomy. Alternatively, in the case of complete IVD replacement, fusion surgery or total disc arthroplasty will be attempted [3]. In addition, discectomy and fusion procedures, which aim at removing the pain source and restoring the kinematical function, are largely palliative in nature and may lead to re-operation and instability. These surgeries do not intend to restore the IVD, but instead immobilizes the joint and which could further induce degeneration in the neighboring discs [93]. Replacement of the degenerated discs by artificial discs has mixed clinical outcomes, and many unanswered questions regarding the design, choice of materials, indications of disease, salvage procedures, and economic issue need to be clearly addressed [94].

3.3.3 Cell- and scaffold-based approaches for IVD repair

Translation of new approaches for IVD repair faces several critical challenges, mainly related to (i) creating more hospitable IVD microenvironment, (ii) selecting ideal therapeutic cell source for DDD treatment (iii) physiological properties of the cells/constructs (iv) implementation of proper *in vitro* models [1, 31, 95].

3.3.3.1 Creating more hospitable IVD microenvironment

Several *in vitro*, *ex vivo*, and *in vivo* studies have investigated the effect of anti-catabolic or anti-inflammatory factors for IVD repair [96-98]. The aim was to either directly suppress the expression of catabolic enzymes (i.e., MMPs and ADAMTS) or by downregulating pro-inflammatory mediators (i.e., TNF-α, IL-1) [99, 100]. Clinical studies demonstrated that the injection of anti-TNFα compounds could reduce pain in patients suffering of severe sciatic pain or radicular pain [101, 102]. However, these therapies could not eliminate the need of surgical procedures [103-105]. The injection of the anti-IL-6 compound Tocilizumab in patients with discogenic LBP and spinal stenosis lead to reduced LBP [106, 107]. Another approach is to inhibit signaling pathways promoting inflammation. For example, IL-1 receptor antagonist (IL1-Ra) has been reported to reduce *in vitro* anti-inflammatory and anti-catabolic factor release of NP cells by inhibiting the p38 MAPK activity [108]. Furthermore, it has been demonstrated in an *ex vivo* study that IL1-Ra delivered directly and by gene therapy inhibits matrix degradation in the intact degenerate human intervertebral disc [100]. Although these approaches showed promising anti-catabolic and anti-inflammatory effects, they are not sufficient enough to regenerate the IVD function since the resident cells fail to restore ability to synthesize ECM [109].

Pro-anabolic strategies using growth factors (i.e., GDF-5 or TGFβ) have been also investigated to induce anabolic activities within the NP tissue with encouraging *in vitro*, *ex vivo* and *in vivo* results [110-114]. Growth and differentiation factor-5 (GDF-5) is known to downregulate the expression of catabolic factor matrix metalloproteases (MMPs) to inhibit ECM catabolism and upregulates the expression of major ECM components , aggrecan and collagen type II, to enhance the ECM anabolism [115]. Clinical studies investigated the safety, tolerability and efficacy of injection of GDF-5 into degenerating IVD and the results indicate no major adverse events directly related to GDF-5 injection as well as the patients reported moderate improvement of their pain and disability (https://clinicaltrials.gov; NCT01158924, NCT00813813, NCT01182337, and NCT01124006). However, these pro-anabolic approaches are limited by the little amount of healthy cells in the NP. Therefore, a single intradiscal injection of biological factors with anti-catabolic, anti-inflammatory and pro-anabolic effects combined with healthy therapeutic cells, which survive and produce ECM within the DDD microenvironment, could be a better approach to repair the NP tissue.

3.3.3.2 Selecting ideal therapeutic cell source for DDD treatment

To repopulate the NP tissue with cells, endogenous stem/progenitor cell recruitment by injecting chemokine ligands (i.e., CCL5 or CXCL12) [116-118] or exogenous cell injection strategies have been explored over the last two decades [119]. In our studies, we focus on the latter therapeutic approach. Currently, differentiated cell sources such as NP, AF, notochordal cells, nasal chondrocytes (NC), articular chondrocytes (AC) as well as stem/stromal cells such as bone marrow-derived mesenchymal stem/stromal cells (BMSC), adipose-derived mesenchymal stromal/stem cells (ADSC), umbilical cord stem/stromal cells (UCSC), nucleus pulposus-derived progenitor cells (NPPC) and induced pluripotent stem/stromal cells (iPS) (Table 4) were scrutinized even in preclinical and/or clinical trials for the IVD repair, also in combination with instructive biomaterials or growth factors [120-134]. However, each cell source has its own limitations. Autologous IVD (especially NP) cells are very sparse in the adult, and their phenotype is changing with aging [135]. The slow expansion rate and the loss of native phenotypic features during the expansion in monolayer are also potential drawbacks [136]. NPPC have been a challenge to implement in clinics due to their extremely low yield and the fact that their numbers decrease with age and degeneration [125, 126]. iPS have shown to exhibit features of teratoma formation and uncertain differentiation capability [124]. Even though MSC have demonstrated promising results in preclinical and pilot clinical trials for IVD repair, MSC-based IVD therapy is not yet in routine clinical use [137-139] possibly due to their high inter-individual variability in their differentiation capacity [140, 141] and/or the fact that these cells are not performing as expected in a harsh degenerated microenvironment which negatively affects their survival, function, and differentiation [5, 17, 142-146]. For both endogenous and exogenous cell supplementation, healthy cells are either not available in excess for treatment, donor site morbidity arises, and/or cell survival within the harsh DDD microenvironment is limited [5, 141, 144, 145, 147].

Table 4. Cell sources for disc regeneration.

Cell types	Source	Advantages	Disadvantages
MSCs			
BMSCs	*Bone marrow*	Strong self-renewal ability, multiple differentiation potential, with homing ability, and technology for solation and expansion is mature	The way obtaining BMSCs is invasive
ADMSCs	Adipose	Abundance, ease to harvest, low immunogenicity	Poor ability to differentiate into chondrocytes
UCMSCs (WJMSCs)	Umbilical cord	Pluripotent, with no ethical barriers, strong proliferation ability, extensive differentiation ability, low immunogenicity and no tumorigenicity	Almost impossible to obtain autologous cord cells, and the experimental cost of WJMSCs is high
IVDSCs	IVD	Can be stimulated to proliferate and differentiate in situ	Low yield in number, decreased viability, and expression of proteoglycan and COL II in IDD, and the curative effect is not obvious
PSCs			
IPSCs	Artificially derived from somatic cells by reprogramming with transcription factors	High capacities of self-renewal, proliferation, and differentiation	Safety problems, especially potential tumorigenicity
ESCs	Early-stage embryo	High capacities of self-renewal, proliferation, and differentiation	Ethical barriers

ADMSCs adipose mesenchymal stem cells, ***BMSCs*** bone marrow mesenchymal stem cells, ***COL II*** collagen type II, ***ESCs*** embryonic stem cells, ***IDD*** intervertebral disc degeneration, ***IPSCs*** induced pluripotent stem cells, ***IVD*** intervertebral disc, ***IVDSCs*** intervertebral-derived stem cells, ***MSCs*** mesenchymal stem cells, ***PSCs*** pluripotent stem cells, ***UCMSCs*** umbilical cord mesenchymal stem cells, ***WJMSCs*** Wharton's Jelly mesenchymal stem cells

It has been demonstrated that AC may represent a better alternative in terms of resilience in harsh microenvironments [133, 134]. However, AC might retain their phenotype and produce ECM with hyaline cartilage-like composition, possibly affecting the biomechanical properties of the IVD *in vivo* [128]. NC (i.e., cells that can be isolated from nasoseptal cartilage biopsy obtained under local anaesthesia and with minimal donor site morbidity [13-15]) were shown to possess high proliferation capacity and moreover, the dedifferentiated NC are capable to form cartilage post-expansion and can adapt to the heterotopic transplantation sites [16]. Moreover, these cells showed superior viability in simulated DDD microenvironment (compared to AC and MSC) thus they might represent a robust cell population with a likelihood of survival post injection [17, 134, 148]. Furthermore, in the degenerative IVD condition, NC produced a ratio of low collagen to high GAG content whereas AC produced less favorable high collagen content [134]. There are no long-term survival results of NC in an *in vivo* degenerative IVD setting available. However, NC have been implemented in clinical trials for the reconstruction of nasal alar lobules [13] and the repair of articular cartilage defect in the knee [149]. Furthermore, Acevedo Rua et al. has recently demonstrated that tissue-engineered cartilage graft using NC (N-TEC) maintained its cartilaginous properties when exposed to *in vitro* inflammatory stimuli present in osteoarthritic joints [150], which has similarities with degenerating IVD environment [57]. In the same study, preclinical results demonstrated that the graft survived and engrafted in sheep articular cartilage defects that mimic degenerative settings. The authors also tested the safety of autologous N-TEC was tested in clinical setting for the treatment of osteoarthritic cartilage defects in the knees of two patients with advanced OA (Kellgren and Lawrence grades 3 and 4) who were otherwise considered for unicondylar knee arthroplasty. No adverse reactions were recorded and patients reported reduced pain, improved joint function, and life quality 14 months after surgery.

3.3.3.3 Spheroids for DDD treatment

Spheroid-based approaches for tissue repair has been implemented in other fields of tissue engineering and could support to overcome the challenges within the IVD field due to their superior regenerative properties comparted to single cells [18, 19]. Cells self-assemble to spheroids and mature by forming intercellular contacts and producing extracellular matrix (ECM) [20-23]. The 3D spatial organization simulates physiological conditions, and controls the cell phenotype, function, and the cell response to loading. Due to increased ECM accumulation in spheroids, their adhesion properties also increase and protect at least the inner cells more from harsh conditions [1, 24]. The size of the spheroids should not be above 500 µm in diameter to prevent necrotic core formation [151]. The intrinsic ability of spheroids to fuse with the tissue of interest allows them to integrate structurally and functionally into the new tissue environment [152]. They have been used combined with biomaterials or without as scaffold-free products for tissue repair. In fact, spheroids-based technology is in clinical use for articular cartilage repair and is being investigated for regeneration of skin and blood vessels among others, supporting the idea of using spheroids for IVD regeneration [19, 153]. In Figure 7, different possibilities of spheroid-based regeneration methods for IVD repair are illustrated [41].

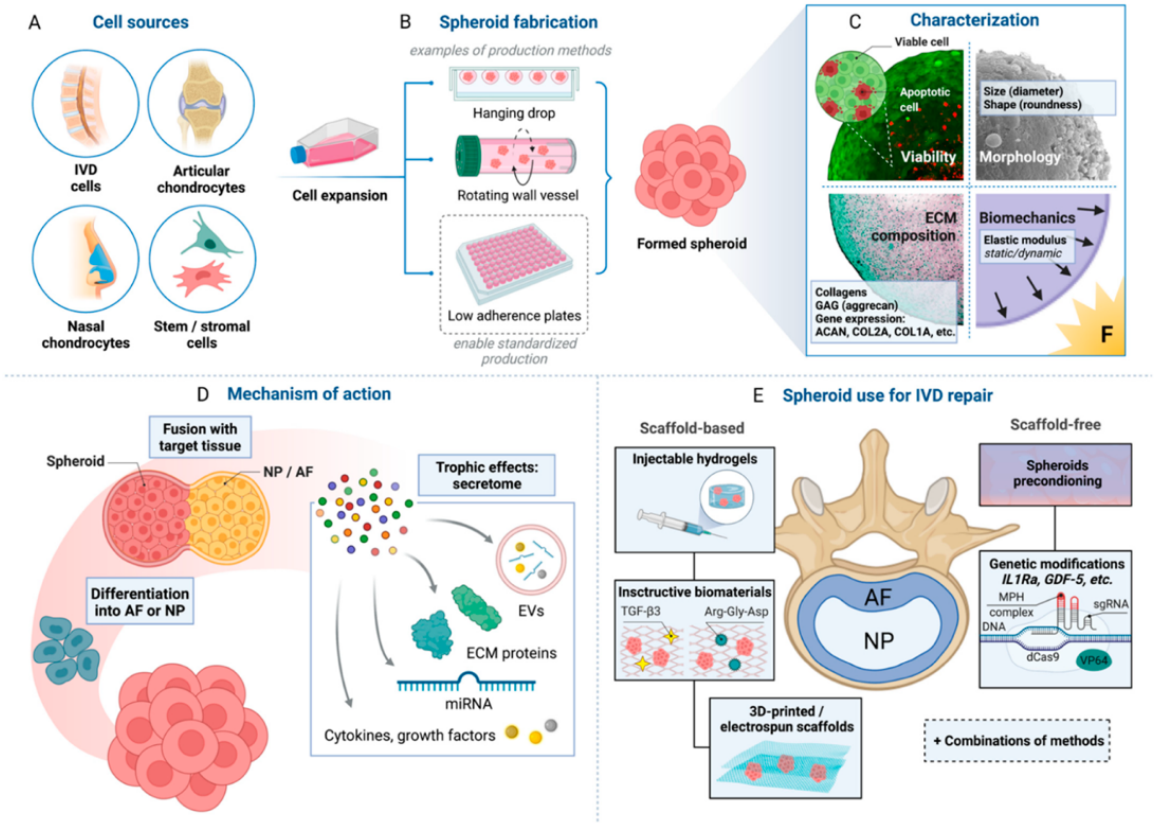

Figure 7: Spheroid-based regeneration of the intervertebral disc (IVD) [41]. (A) Several cell sources have been proposed for IVD repair. (B) While variety of spheroid fabrication methods are suitable for research purposes, low adherence plates facilitating large-scale standardized spheroid production might be the first-choice technology for clinical application. (C) The systematic characterization of the produced spheroids (e.g. cell viability, spheroid geometry, extracellular matrix (ECM) composition, biomechanical properties) is essential to ensure their applicability for IVD repair. (D) Spheroids can exhibit several modes of action to support the IVD, including rapid fusion with target tissue and mechanical support, differentiation of the constituent cells into IVD-like cells, and by secretion of biomolecules (cytokines, growth factors, extracellular vesicles (EVs), ECM proteins, etc.). (E) Both scaffold-based and scaffold-free approaches demonstrate the potential for the regeneration of the IVD. IVD = intervertebral disc, ECM = extracellular matrix, GAG = glycosaminoglycans, NP = nucleus pulposus, AF = annulus fibrosus, EVs = extracellular vesicles. Created with BioRender.com

Injected spheroids into the NP tissue should ideally adhere to the NP tissue enabling the surface cells to migrate and the spheroid itself should undergo remodelling. Spheroids are expected to accumulate NP-like matrix within the degenerating NP tissue allowing biochemical integration of the spheroid cells into the surrounding NP tissue. The regeneration of the NP tissue should lead to IVD height restoration and IVD function [24, 154]. The 3D organization of the therapeutic cells result in increased paracrine effects compared to 2D culture. Trophic factors produced by the therapeutic cells induce their differentiation leading to accumulation of cell-produced ECM and encapsulation of these cells in native ECM [41, 152, 155]. AC-based spheroids were implemented clinically for the repair of traumatic cartilage defects in the knee and demonstrated to produce hyaline-like structure and potential to synthesize articular cartilage-specific matrix [24, 154]. Furthermore, it was demonstrated that ECM and biomechanical properties of spheroids derived from human nasal chondrocytes are tuneable by cell culture supplements, possibly to match properties of target tissue (NP)

and that spheroids of less than 600µm are injectable into a (bovine) IVD through a clinically relevant spinal needle, without their mechanical damage [155]. Cell-produced ECM within spheroids retain growth and tropic factors and builds a physical barrier between the harsh micro-milieu of the damaged tissue and the therapeutic cells [18]. For the scaffold-free tissue repair the number of cells required to maintain the same 3D shape as scaffold-based constructs are higher. Since the native NP tissue contains proportionally very low cell numbers compared to ECM, the use of biomaterial might help to maintain the implant volume.

3.3.3.4 Enhancement of spheroid function

The therapeutic cells can support IVD repair not only by differentiating toward target structures and accumulating ECM but also via paracrine effect which could counteract the effects of inflammation present in DDD and promote anabolic activities. Therefore, choosing a therapeutic strategy that enhances the anabolic, anti-catabolic, and anti-inflammatory effects of the spheroids could lead to increased performance and resistance of spheroids within the harsh DDD condition. Compared to single cells, cells in 3D configuration have been shown to release significantly more growth and anti-inflammatory factors [156-158]. The secreted biomolecules could be entrapped in the cell-produced ECM and control downstream biological processes thus becoming crucial for molecular signalling mechanisms involved for tissue regeneration. By using particular culture supplements, the paracrine effect of the spheroids could be further enhanced providing improved function even without the implementation of stimulative growth factors [156, 159].

A part of the secreted factors from spheroids is composed of extracellular vesicles. Recently, the therapeutic effects of extracellular vesicles on IVD repair was reviewed [160]. For example, extracellular vesicles secreted by MSC were shown to increase regenerative properties and exhibit anti-inflammatory and anti-apoptotic effects in the IVD. Spheroids could be used to improve the quality and increase the yield of therapeutic vesicles.

Using spheroid-conditioned medium containing therapeutic secreted molecules could represent an alternative cell-free treatment of DDD with less problems in regard to clinical translation [157, 158]. In fact, applying pre-conditioning during spheroid generation *in vitro* showed to increase tissue-specific functions such as improved chondrogenesis and resistance to harsh conditions. For example, spheroids formed under hypoxia upregulated the expression of collagen II and aggrecan at mRNA and protein levels as well as increased ECM deposition resulting in higher quality of cartilage formation [25, 161]. In regard to IVD repair, the implementation of hypoxia on MSC showed beneficial effects by inducing the hypoxia-inducible factor (HIF) signalling pathway which in also known to be involved in maintaining cell phenotype, metabolism and homeostasis of the IVD [25-27]. The results of hypoxia on spheroids formed with MSC has not yet been investigated.

Recently the priming of adipose-derived MSC (ACS)-spheroids with Matrilin-3, a non-collagenous ECM adaptor protein, has been scrutinized [162]. These spheroids upregulated gene and protein expression of growth factors and downregulated the secretion of hypertrophic ECM components. Moreover, the priming lead to the induction of SOX9, collagen II and aggrecan mRNA expression and resulted in enhanced chondroitin sulphate accumulation in NP cells (indirect co-culture). Matrilin-3-primed ASC spheroids could also support the repair of an AF puncture-induced IVD degeneration [162]. Altogether, there is evidence for the usefulness of spheroid pre-conditioning to improve the ability of spheroids to repair the IVD.

Genetic modifications could also enhance the chondrogenic properties and resistance of spheroids to the harsh DDD microenvironment [28, 29]. For example, interleukin-1 receptor antagonist (IL1-Ra) and growth differentiation factor-5 (GDF-5) represent promising targets [163]. Moreover, genetically modified MSC spheroids with upregulated Runx2 were demonstrated to overcome negative effects associated with harsh microenvironment (not in IVD tissue) and promoted regeneration in bone tissue engineering [164]. However, there are major limitations if human genetic engineering strategies are chosen, mostly related to viral vectors and low expression of transgenes. Instructive biomaterials were designed allowing to regulate physical and chemical signals, modulate cellular behaviour and promote specific spheroid phenotypes [30]. The encapsulation of growth factors promotes spheroid fusion while materials with/without Arginylglycylaspartic acid (RGD) peptides would regulate spheroid spreading [165]. A combination of biomaterial and MSC spheroids which allows slow release of growth factors showed promising outcome when implanted in sheep osteochondral repair [165]. Altogether, spheroid-based approaches to restore disc function represent a promising future and has to be investigated further. More information on spheroid is given in chapter 2.

3.3.3.5 Physiological properties of the cells/constructs

The use of biomaterial for DDD treatment has been investigated thoroughly. In regard to spheroids, biomaterials could be used already during or after spheroid formation. For example, hydrogels allow to reconstruct the 3D structure of the NP tissue thus create volume. Furthermore, it supports cell adhesion, migration, and integration. Biological material such as collagen, hyaluronan, chitosan, or fibrin are being implemented to simulate native ECM thus to mimic *in vivo* environment. It has been shown that MSCs encapsulated injectable colloidal gelatine hydrogels support NP-like differentiation, increase cell adhesion within the tissue preventing cell leakage, and increase therapeutic cell survival in a rabbit model [166]. Synthetic polymers such as poly(lactide) (PLA), poly(glycolide) (PGA), and poly (ε-caprolactone) (PCL) are also being used since they contain properties allowing facilitated processing, tuneability of mechanical properties and degradation patterns, and low immunogenicity [167, 168].

In the field of IVD research, the combination of hydrogels with spheroids has to be tested. But for cartilage repair it has already been tested. For the functional regeneration of articular cartilage in sheep, hydrogel was used to embed MSC-spheroids in bi-layered osteochondral implants [165]. For better performance of the spheroids, it might be helpful to encapsulate them into an injectable biomaterial which allows to keep the spheroids in place, protect them from harsh micromilieu and promote cell differentiation [169, 170]. Besides modulating the spheroid function, the biomaterial needs to allow spheroid spreading and fusion with damaged NP tissue. However, it has been suggested that delayed spheroid spreading can lead to superior chondrogenic effects *in vitro* and *in vivo* [171]. In regard to NP tissue, it has to be investigated if delaying the spheroid spreading using biomaterials might have beneficial effects for the NP repair. Due to the viscous nature of hydrogels, cell migration and the exchange of nutrients and waste products into and out of the hydrogel can be hindered thus the synthesis of newly formed ECM can be impaired [172-174]. An approach to overcome this limitation was taken by creating highly porous silk fibroin scaffold with interconnected macropores, leaving space for NP cells to infiltrate, increase, and deposit newly synthesized ECM and the results has shown positive outcome [173].

By including biomaterial already during spheroid fabrication allows to form composite spheroids. For example, it has been shown that forming composite multicellular spheroids using MSC and synthetic biodegradable nanofilaments improves adipogenic potential compared to homotypic spheroids. The size of the spheroids could also be controlled depending on the quantity of nanofilaments. Furthermore, the biomaterial allowed to release over time bioactive drugs such as growth factors in order to modulate the properties of the target tissue [175]. Altogether, the idea to combine spheroids with injectable instructive biomaterial might lead to better results for the NP repair. It has to be defined in the future which biomaterial could have potential for spheroid encapsulation and biomechanical stability.

3.3.3.6 Implementation of proper In vitro co-culture models

The first step for developing new therapeutic cell-based strategies for IVD repair includes testing in *in vit*ro models. For the proper design of the *in vitro* models, the selection of ideal cell source, culture system and culture condition are of importance. The right choice of species from which therapeutic cells are being isolated for *in vitro* culture has to be considered because it is known that species-specific responses could lead to different outcomes [176, 177]. Therefore, for initial proof of concept experiments, the data collected using non-human cells has to be interpreted cautiously. The differences could result from species-specific proteoglycan accumulation capacity, energy metabolism, or the progression of DDD within the tissue from which the cells of interested are being isolated [178-180]. Initial experimental studies use animal cells isolated from healthy young adult animals but these cells are not representing the senescent and catabolic phenotype present in a degenerating adult human IVD [181, 182]. Nevertheless, studies using human cell sources have increased around 50% over the past 15 years [31].

For *in vitro* IVD cell and therapeutic cell co-culture studies, 2D and 3D culture systems are commonly being used. 2D culture is well established thus more comparative literature is available and it is easier for cell observation and general analysis compared to complex 3D cell culture systems. For example, it has been reported that direct cell-cell contact in monolayer co-culture of NP and MSC cells upregulated cell viability, proteoglycan synthesis and cell proliferation of NP cells [183]. Another study demonstrated that the monolayer NP-MSC co-culture (ratio: 3:1) cause differentiation of the MSC cells to NP-like phenotype [184]. To investigate the bi-directional exchange of cellular components and how it affects the phenotype of the NP cells, studies have been performed in 2D NP-MSC cultures (ratio: 1:1) and the results indicate that not only cell-cell interactions play a role for intercell communication but also the bi-directional exchange of cellular components such as extracellular vesicles affect the cell phenotype [185]. Even though these findings are essential for the fundamental understanding of cell function and communication, the 2D culture system does not represent proper real cell environment enabling to mimic the *in vivo* situation of the host tissue. Besides unnatural elongated and flat shape of the cells [186], the 2D culture could lead to changes in cellular phenotype as well as de-differentiation of the cells (e.g., loss of ECM synthesis) which could lead to failure in clinical translation [187].

3D culture systems have shown to restore the cell phenotype and allow to reproduce in vivo spatial distribution of the cells [188]. They represent a more relevant biomimetic tissue models which makes them more physiologically relevant and predictive than 2D cultures thus facilitating the translation to *in vivo* applications. Furthermore, the 3D structure of the cells allows to create complex organization of the cells which models closer the cell interactions taking place in a tissue [188]. In the field of IVD repair, 3D models have been widely used to study NP cell behavior, function, differentiation. The alginate-gel matrix system was extensively used to study production, organization and turnover of proteoglycans [189-191] as well as biochemical characteristics of AF and NP cells [192, 193].

Different *in vitro* 3D co-culture systems (i.e. insert system, alginate bead system, pellet system) were used to study the interaction of therapeutic cells with the IVD cells. By using indirect co-culture transwell insert system, it has been demonstrated that co-culturing NP monolayer cells and ADSC pellets (ratio: 1:2) promotes the differentiation of ADSC into NP like cells [194]. Indirect co-culture of porcine NP and bone marrow cells (ratio: 1:1) in 3D alginate hydrogels showed that MSC acts as supporting cells enhancing NP proliferation and subsequent matrix production by NP cells [195]. The crosstalk of MSC and NP cells through trophic factors has already been investigated and reported to play a key role for intercellular communication [196-198].

The direct co-culture of therapeutic cells with NP cells has also been widely performed. For example, it has been shown that co-culture of MSC with NP cells (ratio: not indicated) using calcium alginate gel balls lead to the reduction of senescence associated β-galactosidase expression, increased cell proliferation, decreased catabolic activity and reduced NF-κB signaling in senescent NP cells [199]. Direct co-culture of human BMSC with bovine NP cells (ratio: 1:1) encapsulated in alginate beads revealed that hypoxia and co-culture could lead to BMSC differentiation into NP-like phenotype [200]. With the aim to overcome several major disadvantages of the alginate bead system such as lack of reproducibility, uniformity of quality and size of the microspheres [201], the pellet culture system was used extensively used for co-culture studies between therapeutic cells with NP cells. For example, it has been demonstrated that co-culture of MSC with NP cells in pellet could modulate the gene expression of the MSCs towards chondrogenesis and matrix deposition [202, 203]. In a serum free pellet system containing high concentration of TGFβ the co-culture of synovium-derived MSCs with NP cells (ratio: 1:1) lead to similar differentiation state and matrix production as NP pellets alone [204].

Tissue explant and organ culture model are excellent to test local tissue responses, integration and delivery of therapeutic cells into the IVD tissue and also in regard to biological and cellular functions [205-208]. However, due to the handling and complexity of *in vitro* tissue/organ culture, they are not ideal for initial fundamental studies. For large scale drug testing experiments, organ-on-chip systems derived from microfluidic technologies have recently gained interest for biomedical research. It allows to mimic dynamic tissue microenvironments, while controlling crucial tissue parameters such as flow rates, molecular gradients, and biophysical cues [209]. Recently, it has been proposed to investigate pathological alterations of IVD cells/tissues (reviewed by Mainardi et al. [210]). Even though it represents an ideal platform to mimic *in vivo* conditions with high throughput, it has some drawbacks such as classical readouts within IVD research (i.e., histological staining) are challenging or fabrication and handling of the device is difficult.

Selecting the ideal culture condition to mimic the DDD microenvironment is essential to study the potential of therapeutic cell for IVD regeneration. The harsh IVD microenvironment is characterized by avascular, hypoxia, low glucose level, acidic, inflamed, high osmolality and non-physiological biomechanics [32]. Several studies have assessed the performance of the therapeutic cells within *in vitro* models simulating some of the parameters present in the DDD microenvironment (Table 5) [145]. For example, hypoxic culture condition has revealed to increase HIF-1 and 2 expression in nucleopulpocytes which lead to increased cell proliferation and matrix production [211]. Acidity significantly decreased cell proliferation and synthesis of

ECM-associated component such as aggrecan in BMSC [144]. IL1-β treatment of NP derived MSC revealed to reduce aggrecan and SOX expression while improving their neurogenic differentiation which might play a role in IVD neoinnervation [212]. However, it has been shown that less than 15% of the *in vitro* studies include either one of these parameters thus not mimic the harsh IVD microenvironment properly [31].

Table 5. Different responses of therapeutic cells to degenerative microenvironment [145].

	NPCy	NP-MSCs	BM-MSCs	ADSCs
Hypoxia and low glucose concentration	NPCy survive by relying on anaerobic glycolysis [71]. Low O_2 concentration increases the expression of HIF-1 and 2, which upregulate cell proliferation and matrix production [72].	Low O_2 concentration is associated with a higher viability and proliferative capacity of NP-MSCs compared to ADSCs [33, 34].	Hypoxia increases BM-MSCs CFU, reduces cell senescence and maintains cell stemness [74–77].	Low glucose slightly increases cell apoptosis and inhibits cell proliferation while enhancing aggrecan production [36, 73].
Acidity	NPCy survival at low pH is mediated by ASICs [84–86], even if critically acidic pH has been associated with decreased cell viability and upregulation of metalloproteinases and proinflammatory cytokines [87–89].	Low pH leads to reduced cell proliferation, enhanced apoptosis, and diminished expression of stemness-related and ECM genes [91]. However, overall performance was better than BM-MSCs and ADSCs [34].	Acidic pH significantly decreases cell proliferation, aggrecan, and type I collagen production [90].	Low pH promoted cell necrosis, reduced the proliferation rate, and diminished aggrecan production, while increasing type I collagen synthesis [36].
Hyperosmolarity	NPCy respond to hyperosmolarity through TonEBP activation [92, 93], which increases ECM gene expression [96]. However, excessive hyperosmolarity results in upregulation of proinflammatory cytokines and cell apoptosis [97–100].	Hyperosmolarity has been demonstrated to induce progenitor cell differentiation towards a mature NP phenotype [104].	Hypertonic conditions reduced BM-MSC proliferation, anabolism, and chondrogenic differentiation [73].	IVD-like hyperosmolarity significantly reduced ADSC viability and proliferative capacity and abated aggrecan and type I collagen synthesis [36].
Mechanical loading	Physiological loadings promote cell anabolism while abnormal mechanical stimuli cause ECM breakdown and reduced cell viability [46, 105].	Cyclic mechanical loading favours the differentiation of NP-MSCs towards mature NPCy [37], while static prolonged loading diminished cell viability, migration, differentiation, and stemness [110].	Cyclic mechanical loading enhances BM-MSC chondrogenic differentiation and cell anabolism [26].	ADCs may protect NPCy from apoptosis and promote the synthesis of ECM genes under prolonged loading [111].
Inflammation	Proinflammatory cytokines induce NPCy apoptosis, senescence, and autophagy and upregulate the synthesis of metalloproteinases, thus resulting in ECM breakdown [51, 112].	IL-1β may reduce aggrecan and SOX expression by NP-MSCs while improving their neurogenic differentiation, which may have a role in IVD neoinnervation [49].	BM-MSCs may support resident cells by secreting anti-inflammatory cytokines, anticatabolic, and growth factors [79, 114].	Under inflammatory conditions, ADSCs have been shown to increase proliferation, proinflammatory cytokine production, and osteogenic differentiation [125].

NPCy = nucleopulpocytes; NP-MSCs = nucleus pulposus-derived mesenchymal stem cells; BM-MSCs = bone marrow-derived mesenchymal stem cells; ADSCs = adipose tissue-derived mesenchymal stem cells; HIF = hypoxia-inducible factor; CFU = colony-forming units; ASIC = acid-sensing ion channels; ECM = extracellular matrix; TonEBP = tonicity enhancer-binding protein; NP = nucleus pulposus; IVD = intervertebral disc; IL = interleukin.

4. Aims and outline

The overarching goal of my work is to assess the potential of engineered human nasal chondrocyte spheroids for intervertebral disc regeneration.

Towards that goal, the specific aims of this thesis are to:

I. Propose and discuss the use of cellular spheroids for regeneration of nucleus pulposus (NP) and annulus fibrosus. Chapter 2 is thus dedicated to review

a. Cell sources and methods for spheroid fabrication and characterization.
b. Mechanisms related to spheroid interaction with a target environment.
c. Spheroid-based cell therapies for degenerative disc disease (DDD).
d. Strategies to enhance spheroid function in a DDD microenvironment.

A review article was published (first-authorship) in International Journal of Molecular Sciences - IF: 6.2

Kasamkattil, J., A. Gryadunova, I. Martin, A. Barbero, S. Scharen, O. Krupkova, and A. Mehrkens. "Spheroid-Based Tissue Engineering Strategies for Regeneration of the Intervertebral Disc." *Int J Mol Sci* 23, no. 5 (Feb 25 2022). https://dx.doi.org/10.3390/ijms23052530.

II. Assess the suitability of autologous spheroids formed with nasal chondrocytes (NCS) as a graft for cell-based, scaffold-free regeneration of the NP. Chapter 3 thus addresses whether human NCS

a. can be generated in a reproducible way with characteristics compatible with minimally-invasive delivery in the NP.
b. possess the biomechanical and biochemical properties relevant for the target NP tissue.
c. can be injected into native intervertebral disc (IVD) ex vivo via a spinal needle and engraft within a DDD-mimicking microenvironment.
d. can be regulated in their properties (i.e., elastic modulus) using different media composition.

Results were published (co-first-authorship) in Acta Biomaterialia - IF: 10.6

Gryadunova, A., **J. Kasamkattil**, M. H. P. Gay, B. Dasen, K. Pelttari, V. Mironov, I. Martin, S. Scharen, A. Barbero, O. Krupkova, and A. Mehrkens. "Nose to Spine: Spheroids Generated by Human Nasal Chondrocytes for Scaffold-Free Nucleus Pulposus Augmentation." *Acta Biomater* 134 (Oct 15 2021): 240-51. https://dx.doi.org/10.1016/j.actbio.2021.07.064.

III. Investigate whether pre-conditioning of nasal chondrocytes (NC) can enhance their capacity to repair IVD tissue, using a 3D *in vitro* human NP micro-tissue model. Chapter 4 thus reports on

a. the feasibility to develop a 3D *in vitro* degenerative NP micro-tissue model capturing features of a DDD microenvironment.
b. the response of NC suspensions as well as NC spheroids (NCS) when exposed to the DDD microenvironment mimicked by the model.
c. the effect of NCS pre-conditioning on their function when exposed to the DDD microenvironment.

Results are being prepared for publication in Frontiers in Bioengineering and Biotechnology – IF: 6.1

J. Kasamkattil, Gryadunova, A., M. H. P. Gay, B. Dasen, H. Hilpert, R. Schmid, K. Pelttari, I. Martin, S. Scharen, A. Barbero, O. Krupkova, and A. Mehrkens. Potential title: "Development of 3D *in vitro* NP micro-tissue model to study the reaction of nasal chondrocytes to degenerative disc microenvironment." Envisioned journal for submission: *Frontiers in Bioengineering and Biotechnology*.

5. Chapter 2: Review - Spheroid-Based Tissue Engineering Strategies for Regeneration of the Intervertebral Disc

Results were published (first-authorship) in International Journal of Molecular Sciences - IF: 6.2

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Review

Spheroid-Based Tissue Engineering Strategies for Regeneration of the Intervertebral Disc

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Abstract: Degenerative disc disease, a painful pathology of the intervertebral disc (IVD), often causes disability and reduces quality of life. Although regenerative cell-based strategies have shown promise in clinical trials, none have been widely adopted clinically. Recent developments demonstrated that spheroid-based approaches might help overcome challenges associated with cell-based IVD therapies. Spheroids are three-dimensional multicellular aggregates with architecture that enables the cells to differentiate and synthesize endogenous ECM, promotes cell-ECM interactions, enhances adhesion, and protects cells from harsh conditions. Spheroids could be applied in the IVD both in scaffold-free and scaffold-based configurations, possibly providing advantages over cell suspensions. This review highlights areas of future research in spheroid-based regeneration of nucleus pulposus (NP) and annulus fibrosus (AF). We also discuss cell sources and methods for spheroid fabrication and characterization, mechanisms related to spheroid fusion, as well as enhancement of spheroid performance in the context of the IVD microenvironment.

Keywords: nucleus pulposus; annulus fibrosus; cell therapy; regenerative medicine; intervertebral disc degeneration

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1. Introduction

A long-lasting episode of low back pain (LBP) affects 80% of people at least once in their lifetime. The major cause of LBP is degenerative disc disease (DDD), an age-related pathology of the intervertebral disc (IVD) [1]. During DDD, the affected IVD suffers from progressive loss of cells and the functional extracellular matrix (ECM), leading to spinal instability and the development of chronic pain [1]. Although discogenic LBP significantly affects the quality of life by causing disability, practically no effective treatments exist. A surgical standard of care for DDD is spine fusion, which permanently connects two or more vertebrae in the spine. Spine fusion carries risks of slow recovery and adverse effects, e.g., accelerated degeneration of adjacent IVDs or implant instability, to name a few [2,3]. In fact, ~20% of spine fusion surgeries fail to improve symptoms (long-term pain) for various reasons [4,5].

Emerging regenerative IVD therapies (recently reviewed in [6,7]) envision the minimally invasive application of autologous cells that are expected to support the IVD by differentiating into IVD-like cells and/or by secreting trophic and anti-inflammatory factors [7–10]. Usually, cells are injected in suspension or combined with a biocompatible

material, to provide initial mechanical stability and protection after implantation. However, despite substantial advances, none of these strategies have been widely adopted clinically. The challenges for IVD cell therapies are related to the unique anatomical structure, biochemical composition, and microenvironment of human IVD [7,8,10]. Numerous publications have suggested that therapeutic cells may suffer due to lack of nutrients and oxygen and high levels of lactate, inflammation, and complex (often non-physiological) loading [8,11–15].

Recent developments in other fields of tissue engineering demonstrate that spheroid-based approaches could help overcome some of these challenges due to their superior regenerative performance and/or resistance when compared to single cells [16,17]. Spheroids are self-assembling living microtissues based on multicellular aggregates that mature by the formation of intercellular contacts on non-adhesive substrates [18–21]. Their 3D architecture controls cell phenotype and function and enables cells within spheroids to favorably respond to loading. It is believed that 3D configuration provides spheroids with a higher ability to adhere and protects cells from harsh conditions [22,23]. However, the size and cell density of spheroids should be precisely controlled due to the possibility of necrotic core formation resulting from impaired fluid exchange, as shown in spheroids with a size above 500 µm in diameter [24]. Spheroids have the intrinsic capacity to fuse, which allows for structural and functional integration of spheroid-based grafts. Spheroids have been used in combination with biomaterials or alone as scaffold-free products [17]. Spheroid-based technology is in clinical use for articular cartilage repair and under investigation for regeneration of skin, blood vessels, and other tissues, supporting the feasibility and translational potential of this strategy for IVD regeneration [17,25].

Alone or in combination with injectable biomaterials, spheroids represent a promising, minimally invasive IVD treatment. Although the concept of spheroids is not new, less attention has been given to testing and reviewing this strategy in the IVD field. The main goal of this review is to outline our perspective on possible developments in spheroid-based tissue engineering of the main IVD tissues: nucleus pulposus (NP) and annulus fibrosus (AF) (Table 1). We also provide a brief overview of cell sources and methods for spheroid fabrication and characterization, describe mechanisms underlying spheroid fusion/integration with target tissues, and discuss the enhancement of spheroid functions in the context of the IVD microenvironment.

Table 1. Composition of healthy nucleus pulposus (NP) and annulus fibrosus (AF) and function of these components [26–32].

Tissue Function	Water Hydrostatic Pressure	Collagens Tensile Strength	PG Osmotic Pressure	Other Proteins Support of Matrix and Cells	Cells Homeostasis
NP (inner core, highly hydrated tissue)	70–90% *	15–20% ‡ mainly collagen type II	65% ‡	20–45% ‡	4000 cells/mm^3
AF (outer IVD ring, elastic, and fibrous tissue)	60–90% *	50–70% ‡ mainly collagen type I	10–20% ‡	10% ‡	3000–9000 cells/mm^3

* Percentage of wet weight. ‡ Percentage of dry weight of the IVD. PG = proteoglycans.

2. Cell Sources for IVD Tissue Engineering

Cell sources for IVD repair were recently reviewed [33–36]. Autologous IVD cells are commonly isolated from specimens removed during surgeries, expanded, and transplanted back to augment IVD cell population. NP and AF cells showed promise in preclinical settings and clinical trials, leading to the emergence of clinical products that support autologous cell supplementation [37–40]. Although these cells are likely affected by pre-existing degeneration [41,42], they are able to survive in an IVD-specific microenvironment.

Unlike most other cell types, NP cells constitutively express hypoxia-inducible factor 1-alpha (HIF1α), which makes them well equipped to manage the limited nutrient supply to a certain extent [39,43]. However, autologous IVD (especially NP) cells are very scarce in adults, and their phenotype changes with aging [44]. The slow expansion rate and the loss of native phenotypic features during the expansion in the monolayer are also potential drawbacks [45]. Multipotent tissue-specific NP progenitor cells (NPPC) were recently identified and investigated for their enhanced regenerative capacity [46–48]. NPPCs are characterized by a spheroid colony forming (in contrast to fibroblastic colony forming) and positivity for the cell surface marker angiopoietin-1 receptor (Tie2) [46,47]. However, it has been a challenge to implement NPPC in clinics due to their extremely low yield and the fact that their numbers decrease with age and degeneration [46,47], but not as drastically as notochordal cells—a unique sub-population with stem cell properties originating from embryonic notochord [33]. The transition of notochordal cells into chondrocyte-like cells before adulthood, and the subsequent decrease in proteoglycan content, is believed to cause NP degeneration [49].

Given the limited availability of healthy autologous IVD cells, there has been an interest in other cell types (Figure 1A). Induced pluripotent stem cells (iPS) emerged as an adult cell source with great potential to address existing limitations of sparsity and donor site morbidity. Although iPSs still bear the concern of teratoma formation and uncertain differentiation capability, it is possible to avoid these features by pre-differentiation into committed cell lineages, e.g., notochordal cells, as recently demonstrated by regenerative effects in vivo in a porcine model [50].

To date, the most extensively investigated cell type for IVD repair has been adult mesenchymal stem/stromal cells (MSCs) [45,51], e.g., bone marrow-derived [52,53], adipose tissue-derived [54], or muscle-derived [55]. Implantation of MSCs has resulted in the restoration of the IVD height, IVD-like phenotype expression, ECM synthesis, and improvement in MRI signals [42,51,56]. An ideal therapeutic cell type should differentiate toward NP/AF cell-like phenotypes in vivo. A number of NP and AF phenotypic markers have been identified (e.g., Brachyury, FOXF1, PAX1, and KRT19) and MSCs showed good differentiation capacity towards IVD cell types in an IVD-specific microenvironment [33]. Notably, MSCs are effective at repairing large lesions (6 × 20 mm) in the partial-thickness AF-injury sheep model [56] with the recovery of disc height and functional biomechanical properties of repaired IVD tissues [57,58]. Although promising outcomes were found in animal and pilot clinical trials [59], MSC-based IVD therapy is not yet in routine clinical use [6,60,61], possibly due to variable differentiation capacity leading to inferior constructs [62] and/or the fact that these cells are not performing as expected in a harsh, degenerated microenvironment, which negatively affects their survival, function, and differentiation [8,62–66].

It has been demonstrated that adult chondrocytes from other locations may represent a better alternative in terms of resilience in harsh microenvironments [15,67]. In contrast to MSCs, articular chondrocytes (AC) (particularly from younger donors) show an enhanced ability to survive and produce ECM in porcine IVD defects in vivo [68]. However, chondrocytes might retain their phenotype and produce ECM with cartilage-like composition, possibly affecting the biomechanical properties of the IVD in vivo [33]. Although autologous AC are clinically used for IVD repair [6], they might also be influenced by pre-existing degeneration and donor site morbidity remains an issue. Notably, cells derived from other locations, namely nasoseptal chondrocytes (NC) originating from hyaline cartilage of the nasal septum, demonstrated superior ability over MSCs and AC to survive in osteoarthritic and DDD conditions but their ability to form functional ECM still remains impaired [69,70]. NC recently emerged as a promising cell source for cartilage pathologies. NC exhibit features of self-renewal and adapt to heterotopic transplantation sites, including osteoarthritic knee cartilage defects [71–73]. Engineered NC cartilage grafts are already being used to repair cartilage defects in patients [74,75]. Neuroectoderm-derived NC can be distinguished from mesoderm-derived AC by lack of expression of specific HOX genes

and reprogrammed to stably express HOX genes typical for AC, as shown in goat articular cartilage defects [71]. In view of the IVD, adaptation of NC to NP/AF phenotypic features remains to be investigated.

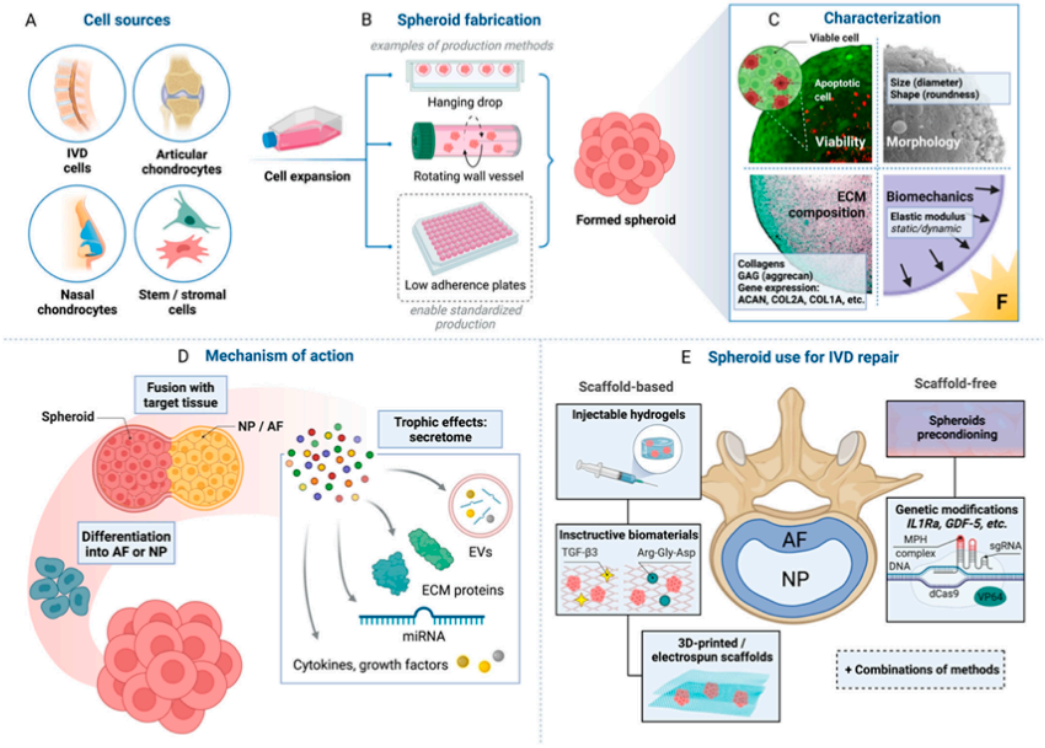

Figure 1. Spheroid-based regeneration of the intervertebral disc (IVD). (**A**) Several cell sources have been proposed for IVD repair. (**B**) While a variety of spheroid fabrication methods are suitable for research purposes, low adherence plates facilitating large-scale standardized spheroid production might be the first-choice technology for clinical application. (**C**) The systematic characterization of the produced spheroids (e.g., cell viability, spheroid geometry, extracellular matrix (ECM) composition, and biomechanical properties) is essential to ensure their applicability for IVD repair. (**D**) Spheroids can exhibit several modes of action to support the IVD, including rapid fusion with target tissue and mechanical support, differentiation of the constituent cells into IVD-like cells, and by secretion of biomolecules (cytokines, growth factors, extracellular vesicles (EVs), ECM proteins, etc.). (**E**) Both scaffold-based and scaffold-free approaches demonstrate the potential for the regeneration of the IVD. IVD = intervertebral disc, ECM = extracellular matrix, GAG = glycosaminoglycans, NP = nucleus pulposus, AF = annulus fibrosus, EVs = extracellular vesicles. Created with BioRender.com.

Spheroid Configuration of Therapeutic Cells

Spheroids can be derived from somatic as well as stem/stromal cells. In IVD repair, spheroid cultures could reduce the rate of dedifferentiation and enable rapid reimplantation of autologous NP/AF cells. Recently, spheroid-formation assay was successfully applied to the expansion of NPPC. Zhang (2020) demonstrated that this culture significantly enriched the percentage of Tie2-positive NP progenitors (vs. monolayer), possibly bringing NPPC closer to clinical application [76]. Spheroid configurations were also shown to improve the ability of MSCs to differentiate into chondrogenic and osteogenic lineages [64]. Further-

more, human MSCs spheroids performed better than single cells and differentiated into NP-like cells scaffold-free [77,78] and in hydrogels [79]. Similarly, iPS were shown to form microtissues differentiating into NP-like cells [80], with the capacity to be implanted (or injected) into the IVD. Notably, autologous AC-based spheroids were extensively investigated in preclinical settings and then implemented in patients suffering from traumatic or degenerative injuries of articular cartilage, leading to clinical benefit [81–83]. Compared to human AC spheroids and/or monolayer, NC spheroids manifest higher viability, more uniform morphology, and higher expression of COL2A1 and SOX9 [84]. Spheroids generated from human nasoseptal cartilage progenitors (i.e., a subpopulation of chondrocytes derived from superficial zone of human nasal septal cartilage samples shown to display progenitor cell features [85,86]) exhibit increasing biomechanical stability, synthesis of TGF-β1/2/3, and enhanced expression of ECM components over time even without a chondrogenic stimulus (compared to monolayer) [84]. Moreover, our group has recently demonstrated that NC spheroids (NCS) not only accumulate ECM without chondrogenic stimuli but also survive and fuse with NP spheroids in the DDD-mimicking microenvironment and are compatible with spinal needles for minimally-invasive application [87]. The potential use of spheroid-based regeneration methods for IVD repair is depicted in Figure 1.

3. Spheroid Formation and Characterization

3.1. Mechanisms of Spheroid Formation

In the first stage during chondrogenesis, known as pre-cartilage condensation, MSCs aggregate, increase cell density, and upregulate their hyaluronidase activity [88]. Consequently, MSCs stop proliferating, express ECM-related adhesion molecules, slow down the cell movement due to decreased hyaluronan in the ECM and promote close cell-cell interactions, which triggers signal transduction pathways involved in chondrogenic differentiation [89]. Following similar principles, three crucial steps are involved in the spheroid formation. First, dispersed cells begin to aggregate through establishing loose integrin-ECM bonds followed by upregulated cadherin expression and accumulation due to cell-cell contact. Consequently, compact spheroids form due to the homophilic cadherin-cadherin interactions [90]. The classic cadherins represent calcium-dependent, homophilic, cell-cell adhesion molecules found in nearly all solid tissues [91]. Prior studies have noted the importance of cadherins in both spheroid formation and fusion [92,93]. The spheroidal organization of the cells induces the synthesis of endogenous ECM which can affect the cell behavior in various ways. The cell-ECM interaction through receptor-mediated signaling might directly regulate the cell functions or ECM-associated growth factors can control the cell proliferation and differentiation thus regulate the cell phenotype [94]. For example, integrins were demonstrated to physically bridge the ECM to the network of cytoplasmic actin microfilaments providing an appropriate molecular scaffold for signaling components, resulting in changes of cell shape and actively regulating cell proliferation and differentiation [95]. Moreover, the integrins were proposed to act as direct mechanotransducers controlling many aspects of cell function including cytoskeleton remodeling and migration through physical forces [96]. On the other hand, the ECM proteins can regulate the growth factor bioavailability by establishing stable gradients of growth factors such as FGFs, HGF, and VEGFs [97]. They might be bound by ECM-associated proteins like fibronectin, by collagens and proteoglycans, or in combination with heparin and heparin sulphate [98]. This could lead to the activation of MAPK/ERK pathway resulting in modifications of intracellular signaling [99], which has already been described to play a key role in chondrogenesis [100]. Recently, it has been shown that increasing the actomyosin contractility could protect NP cells from TNFα-induced ECM loss thus demonstrating the relation between inflammation triggered ECM loss and mechanotransduction [101]. Therefore, the accumulation of ECM in spheroids could potentially support the cells to regulate their biophysical properties thus instruct them to cope in the harsh IVD microenvironment. The ECM also acts as a physical barrier that could protect the cells within the inner layer of the spheroid from direct exposure to harsh conditions such as inflammation or acidity [102].

Furthermore, since the self-assembly of the cells to a spheroid increases cell adhesion properties [18], the potential of the spheroids to reside within the disc after injection increases preventing cell leakage and with it associated problems such as osteophyte formation [103].

3.2. Spheroid Formation Methods

Standardized large-scale spheroid formation methods are essential to obtain cost-effective and uniform spheroids for research and clinical application. The spheroid formation must be compliant with good manufacturing practice (GMP) for advanced therapy medicinal products (ATMP) guidelines and must respect the specifications and release criteria defined in the investigational medicinal product dossier (IMPD) [104,105]. Sterility, viability, identity, purity and potency tests have to be included to ensure the safety and efficacy of the spheroids. Furthermore, valid release criteria have to be established early in the product development process to ensure smooth clinical translation. For example, it has been demonstrated that clinically relevant FBS substitutes such as human platelet lysates or autologous serum will have the same effect on the cells [106,107]. It is advisable for saving time and costs to check early whether the factors used for the formation of spheroids are translatable into the clinical setting and moreover, if there are corresponding GMP compliant substitutes available for human use.

Several methods are available for the fabrication of spheroids (Figure 1B), among others pellet culture, hanging drop, spinner culture, rotating wall vessel, microfluidics, magnetic levitation, and low-attachment surface culture (reviewed in [108,109]). These methods are suitable for basic research purposes but not all of them are relevant for clinical application, mostly because of the high labor intensity and cost, hindering large-scale production [108,110]. Low adherence plates prevent cell adhesion promoting cell aggregation and spheroid formation on a large scale [111,112] and ensure reproducible formation of spheroids with ~500 µm in size and the roundness score above 0.70–0.95, indicating compatibility with injectable IVD therapy [113–115]. A lower roundness score is typical for an ellipsoid shape and/or formation of satellites, which indicates lower reproducibility in the spheroid formation process [116,117] and might result in injectability issues.

3.3. Spheroid Characterization

During spheroid generation, cell-produced ECM will be accumulated, which allows to mimic in vivo spatial distribution and physiological microenvironment of the cells. Furthermore, cell-cell interactions and cell-ECM interactions will be favored inducing the secretion of differentiation factors [118]. The systematic characterization of the produced spheroids is essential to ensure the reproducibility of spheroids geometry (regular spherical shape) and their injectability into the damaged IVD using standard surgical equipment, i.e., 30G to 22G spinal needle [92].

The size of the spheroids should be smaller than the inner diameter of the needle to be used for the injection of the spheroids into the target tissue. For example, a clinically relevant spinal needle (22G, BD, 405149) for the injection of spheroids into the degenerating nucleus pulposus tissue has a diameter of 600 µm thus the spheroids should be <600 µm to lower the risk of being damaged during injection [87]. The size of the spheroids also influences their viability, whereas smaller ones show better viability than larger spheroids [119]. The metabolic waste removal and nutrition diffusion to the core of the spheroids are relative to their size, thus a larger spheroid size (>500 µm) could lead to a necrotic core formation [120,121]. The cell viability of the produced spheroids should be monitored closely, since both necrotic and apoptotic cell death may initiate and maintain the degenerative process of the IVD by the induction of matrix metalloproteinases, MMP-1 and MMP-13, among other factors [122] (Figure 1C). Appropriate extracellular matrix content (high proteoglycan to collagen ratio, an abundance of aggrecan and collagen type II for NP; collagen types I and II for AF) and biomechanical properties (elastic modulus close to that of the

native NP or AF) are further requirements for cellular aggregates designed for application in IVD repair [123–125].

The most common methodologies for spheroid characterization include conventional morphometric analysis for size and shape quantification, assay staining protocols (e.g., calcein AM, ethidium homodimer, etc.) to measure cell viability, histological and immunohistochemical analysis to evaluate ECM composition and cell organization, atomic force microscopy and controlled compression to identify biomechanical properties, as well as more advanced mathematical modelling and computational simulation to predict spheroids' post-implantation behavior [126–129].

A direct evaluation of the fusion kinetics in vitro is also advisable in order to simulate the integration of the implanted spheroids into the host tissue. When placed in close proximity to each other, spheroids merge over time to form one larger structure, providing a simplified model that can be easily quantified, e.g., to evaluate the influence of the DDD microenvironment on spheroid integration [93,130]. As an example, the angle between the chondrocyte spheroid and NP spheroid increases to a maximum of 180° in 96 h of their fusion; therefore the integration of therapeutic spheroids into the defect area in native NP could be expected to take ~4 days [87]. Interestingly, it has been recently shown that fusion culture has contributed to increased collagen type II synthesis and accumulation in human chondrocyte spheroids compared to single aggregates, thereby providing evidence of newly formed, self-made ECM typical for native hyaline cartilage [131]. Since NP shares a similar collagen phenotype to that of cartilage, further studies on spheroid fusion are of particular significance to IVD repair.

4. Spheroid Interactions with Target Environment

For long-term functionality, the structural and functional integration of the spheroids into the damaged tissue is essential. The spheroids should (i) adhere to the target site, (ii) loosen up their compact 3D organization, while surface cells migrate into the damaged area, and finally (iii) complete integration into the defect area and synthesize and secrete injured site-specific proteins to support the tissue repair [111].

Spheroids exhibit liquid-like behavior and undergo coalescence similarly to liquid droplets [132] as explained by the differential adhesion hypothesis (DAH), which claims that the cells rearrange in order to increase the number of cadherin adhesive bonds and reduce free energy [133]. The first stage of spheroid integration, or adhesion to the target site, relies upon various types of cell adhesion molecules; the cadherins, however, are crucial for this process [134,135]. Pre-cartilage condensation, a process of particular relevance to chondrogenesis, is mediated by cellular condensation through N-cadherins [136]. While the extracellular domain of N-cadherin forms interactions between opposing cells, the intracellular domain is anchored to the actin cytoskeleton by α-catenin, β-catenin, and other signaling molecules [137].

Migration of surface cells into the damaged area is a driving factor for the second integration stage. Previous studies have examined the assembly and fusion of spheroids containing various cell types [138,139], and cell migration during the fusion process has been shown to involve cytoskeletal dynamics [140]. The lipid bilayer of a cell membrane is draped over the actin cortex, and they deform simultaneously, being tightly bound to each other by anchoring proteins, such as ezrin, radixin, and moesin [141–143]. During the initial steps of fusion, the actin cytoskeleton produces multiple thin, finger-like protrusions that push into the cells on the adjacent damaged surface. The invasive protrusions, or filopodia, are generated through the formation of parallel actin bundles by actin polymerization. At the apex of the filopodia, the plasma membranes of the neighboring cells make contact when the cell–cell recognition molecules form an intercellular adhesion complex that eventually develops into the fusion pores [144]. Over time, this zone expands to form a stable contact region between cellular aggregates and the defect area. The realignment of the actin cytoskeleton is required for the fusion of spheroids produced from primary chondrocytes, and the disruption of microfilaments inhibits the process completely [145].

The complete integration into the defect area is characterized by the synthesis and secretion of site-specific proteins to support the tissue repair. When placed on the damaged articular cartilage, chondrocyte spheroids have shown to cover the entire surface of the degenerated cartilage within 3 weeks [21]. The cells not only migrate out of spheroids but also synthesize new ECM composed primarily of collagen type II and proteoglycans (PG) [146,147]. ECM in articular cartilage is reconstructed and remodeled upon spheroid implantation since the new chondrocytes replace matrix macromolecules lost through degradation [148]; the underlying molecular mechanisms, however, have yet to be investigated.

Understanding the role of biomolecules involved in spheroid integration into the IVD is a step towards fine-tuning the abovementioned interactions and accelerated healing of the damaged tissue. It has been recently shown that the physiological dynamic compression of 0.4 MPa up-regulates N-cadherin expression in NP cells compared to static compression [149,150]. Spheroid preconditioning under dynamic mechanical loading therefore appears to be an appealing option to enhance the integrative potential by improving their adherence to the target site. Since chondrocyte migration is impaired by inflammatory stress typical for DDD condition, anti-TNF-α bioactive molecules, such as etanercept and adalimumab as well as anti- IL1β drugs, such as anakinra, could be incorporated into microparticles and integrated into the chondrocyte spheroids or used as medium additives prior to implantation [151].

5. Spheroid-Based Cell Therapies for Degenerative Disc Disease

IVD contains distinct anatomical regions, namely the nucleus pulposus (NP), annulus fibrosus (AF), and cartilaginous endplates [152,153], which are all substantially different and unique structurally, mechanically, and biochemically, and present challenges for IVD tissue engineering. Ideally, an engineered construct should closely resemble the ECM architecture of the target tissue and rapidly integrate within a defect. Numerous studies have investigated the use of various cell-laden scaffolds and hydrogels. Despite strong efforts, scaffold-based approaches are still limited in terms of reduced cell viability, inconvenient manipulations, and unwanted degradation patterns. A less explored way to generate IVD-like neotissue is a scaffold-free, spheroid-based approach when therapeutic cells build their own support ECM from the beginning, which might enhance biomimetic functions and fasten the regulatory approval process in clinical translation. However, as spheroid ECM can be considered immature compared to native tissue, a combination of spheroids with biomaterials could be a practical alternative for the regeneration of NP and AF (Figure 1E).

5.1. Nucleus Pulposus

The NP is a hydrated structure predominantly composed of a loose network of highly hydrated proteoglycans (PG) and collagen type II, with PG/collagen ratio 26:1 in healthy IVD [154]. Notably, the microenvironment of degenerated NP contains low levels of oxygen and glucose, acidic pH, high osmolarity (relative to other tissues), and complex loading [12,155]. These harsh conditions were shown to induce a cellular catabolic shift that accelerates the degradation of ECM and negatively influences the function of therapeutic cells [13,14]. The catabolic shift is characterized by upregulation of pro-inflammatory cytokines and ECM degrading enzymes, as well as downregulation of inflammation antagonists and inhibitors [156,157]. The survival rate of therapeutic cells in the NP is also affected by reduced nutrient supply due to large IVD size and endplate calcification [152]. Altogether, these conditions limit the numbers of therapeutic cells to be used. Strategies to regenerate NP should consider the specific anatomy, limited diffusion rate, and harsh microenvironment while providing resistance to the compressional and torsional stresses within the spinal column [158]. An ideal therapy for NP regeneration would be liquid before application (injectable) and rapidly solidify and/or integrate upon injection to ensure correct distribution and retention in the NP [62,159].

The intrinsic ability of spheroids to rapidly fuse with target tissue is believed to be crucial for regeneration [160]. In order to prevent their extrusion from NP, the adhesion of spheroids and migration of surface cells into NP, followed by spheroid remodeling, must take place. Consequently, spheroids are expected to secrete an NP-like matrix into the defect cavity, leading to restoration of IVD height, gap filling, and biochemical integration of spheroid cells into the surrounding NP tissue [22,112].

In scaffold-free conditions, a supportive material is not used, thus there is no need to consider long-term effects of an implanted scaffold [161]. 3D configuration and increased paracrine effects of spheroids (compared to 2D) are thought to enhance the differentiation potential of therapeutic cells. The ability of spheroids to synthesize their own ECM results in the encapsulation of cells in native ECM, the composition of which is driven by the original cell type and culture conditions [87,160]. In the clinical repair of cartilage defects in the knee, AC-based spheroids (Spherox) generated a hyaline-like structure and showed the potential to synthesize an articular cartilage-specific matrix [22,112]. We have recently demonstrated that ECM and biomechanical properties of spheroids derived from human NC are tuneable by cell culture supplements, possibly to match properties of target tissue (NP) and that spheroids of less than 600 µm are injectable into an (bovine) IVD through a spinal needle, without their mechanical damage [87]. A self-produced ECM of spheroids is also believed to retain growth and trophic factors and constitute a physical barrier between harsh target tissue and therapeutic cells [16]. However, in scaffold-free tissue repair, the cell numbers required to maintain the same 3D architecture as constructs that are scaffold-based, are higher. Importantly, native NP tissue contains, proportionally, very low cell numbers compared to the amount of ECM; thus the use of a supportive biomaterial might be warranted to maintain the graft volume.

A biomaterial could be present after spheroid fabrication or already at the stage of spheroid assembly. Hydrogels have several advantages for NP repair, such as a 3D structure that generates volume and promotes cell adhesion, migration, and integration. Natural materials, such as collagen, hyaluronan, chitosan, or fibrin, mimic an in vivo environment, as they bear similarities with the native ECM. For example, injectable colloidal gelatine hydrogels with encapsulated MSCs support the NP-like differentiation, reduce cell leakage, and improve the survival of therapeutic cells in a rabbit model [162]. On the other hand, synthetic polymers, such as poly(lactide) (PLA), poly(glycolide) (PGA), and poly(ε-caprolactone) (PCL), offer easier processing, tuneability of mechanical properties and degradation patterns, and low immunogenicity [163,164].

While the combination of spheroids with hydrogels has yet to be investigated in the IVD field, spheroid-based constructs have already been tested in cartilage repair. As an example, an alginate/hyaluronic acid (HA) hydrogel was used to embed MSC spheroids in bi-layered osteochondral implants that supported the functional regeneration of articular cartilage in sheep [165]. An encapsulation of spheroids in an injectable biomaterial might help to hold them in place, protect them further from unfavorable microenvironments, and instruct them towards differentiation [166,167]. It would be expected that a biomaterial will not impair the ability of spheroids to spread and fuse with NP tissue but rather modulate these functions. In cartilage repair, it was suggested that delayed spheroid spreading, achieved by the use of PLGA/chitosan (CS)-containing constructs, can provide superior chondrogenic effects in vitro and in vivo due to the fact that spheroid 3D architecture is preserved longer [168]. Whether delaying spheroid spreading/fusion by the use of a biomaterial would produce beneficial effects in NP repair has yet to be investigated. It should be noted that spheroids rely exclusively on diffusion to transport nutrients and eliminate waste, so their interior might start suffering from a lack of nutrients, oxygen, and excess waste products, if spreading is inhibited for longer periods. Nevertheless, these negative effects and consequent onset of necrosis could be partially regulated by spheroid size and the total number of therapeutic cells in the NP.

Recent developments expanded the possibilities for modulating spheroid spreading (and other parameters) by generating composite spheroids, with a biomaterial included

already during spheroid fabrication. In adipose tissue engineering, composite multicellular spheroids formed by MSCs and synthetic biodegradable nanofilaments showed enhanced adipogenic potential compared to homotypic spheroids. It was also demonstrated that the size of these spheroids could be readily controlled with the integrated amount of nanofilaments. Moreover, the material part of the spheroids could be used to sustainably release bioactive drugs (e.g., GFs) in order to fine-tune the properties of target tissue [169]. Including biomaterials during spheroid fabrication process was also shown to influence spheroid roundness in ligament tissue engineering [170].

Combining spheroids with an injectable instructive biomaterial is an attractive possibility for the regeneration of the NP. In the future, it will be necessary to define the best biomaterial for spheroid encapsulation with regard to their fusion kinetics (with target NP tissue and with each other) and biomechanical stability.

5.2. Annulus Fibrosus

Approaches to regenerate NP are likely to have limited success without sufficient repair of the AF, i.e., the outer ring of the IVD. The AF is composed of circumferential layers of lamellae formed by closely arranged fibers of collagen type I. AF provides load-bearing function, tensile resistance, and adequate support to maintain NP pressure [28]. During IVD degeneration, non-physiological loading and catabolic shift reduce ECM turnover, leading to the development of microdamage, clefts, and tears in the AF [171]. Due to the loss of PG and inflammation-associated upregulation of specific growth factors (NGF, VEGF), nerves and vessels from adjacent tissues grow deeper into the IVD [172], which causes nerve irritations and aggravates pain [172,173]. Strategies to regenerate AF thus focus on filling structural defects and rapidly restoring physiological ECM structure and function (collagen lamellae) to support AF's tensile resistance and prevent excess nerve ingrowth. Persistent AF defects increase the risk of recurrent IVD herniations, which then require reoperations [174].

Strategies to mechanically repair AF were developed (sutures, patches) but none of these techniques significantly altered annular healing in animal models nor demonstrated long-term benefits in clinical trials [175]. It is crucial that AF implants maintain adhesion to target tissue, especially under strain. Novel AF sealants have been generated and showed promising results [175–178]. Compared to acellular therapies, cell-based implants improve ECM deposition and organization in animal studies and show more successful AF remodeling in the long-term [178]. Nevertheless, neither biological/mechanical properties similar to AF tissue nor native-like ECM organization were fully reproduced to date [178]. Importantly, regeneration of inner AF has been a challenge, as current implants fail to fully bridge inner AF defects.

Due to their intrinsic ability to adhere, spheroids could serve as building blocks for a living AF patch that fuses to larger 3D structures in situ or before implantation. Spheroid-based architecture could achieve successful defect bridging and fix the implanted material in place [178], especially if combined with a biomaterial [16]. Recent advances make it possible for spheroids to be seeded into biomaterials during or after the fabrication process, immobilized on pre-fabricated scaffolds, or embedded between scaffold layers in a patterned manner, possibly achieving the typical lamellar structure of the AF [179]. Combining spheroids with injectable hydrogels could efficiently fill irregularly shaped defects in a minimally-invasive and rapid manner as well as instruct the behavior of therapeutic cells [180,181].

A sufficiently porous biomaterial is needed to seed spheroids randomly or into a specific structure. Although spheroids were not widely explored in AF tissue engineering, recent developments expanded the possibilities for spheroid-biomaterial seeding in related areas of musculoskeletal repair. In bone tissue engineering, foaming/freeze-drying techniques were used to produce scaffold microporosity that promoted spheroid penetration into the scaffold and fixed them in place [16,17]. Spheroids were also generated in situ in a novel porous PLGA/CS scaffold obtained after lyophilization. ASC spheroids formed

in these scaffolds promoted hyaline cartilage-specific chondrogenesis in vitro and structural/functional regeneration in vivo (rabbit model). This method reproducibly yielded spheroids of smaller sizes (diameter less than 200 µm), which facilitated the penetration of oxygen and nutrients into spheroids [182]. In situ generation of spheroids directly within an implantable scaffold might reduce culture time and lab manipulation, supporting the applicability in clinical AF repair. However, the exact pore size and porosity of scaffolds produced by methods like foaming or lyophilization might be difficult to control.

3D printing can reproducibly control the internal pore size (50–800 µm), porosity, pore interconnectivity, and mechanical performance of tissue-engineered scaffolds. Huang (2013) used a solid freeform fabrication method to prepare PLGA-CS scaffolds that delayed spheroid spreading in cartilage repair. Their scaffold showed a fully interconnected macroporous structure and controlled geometry, maintained the 3D microenvironment of MSC spheroids, and a showed superior ability to regenerate chondral defects in a rabbit model when combined with spheroids (vs. single cells) [168]. 3D printing also holds promise to generate scaffolds that precisely fit the geometry of interest, allowing for guidance of the spheroid placement into specific shapes and geometries [179]. However, the automated seeding of spheroids onto 3D-printed scaffolds to produce a complex 3D construct has not yet been largely explored [16]. To precisely replicate AF structure using spheroids as building blocks, patterned micro- and nano-structures could be produced, e.g., by an innovative "lockyball" approach, where ASCs were immobilized into solid synthetic microscaffolds (lockyballs) fabricated by two-photon polymerization and designed with hooks and loops to enhance the retention and integration at the implantation site [183].

The generation of functional double-layered AF patches with one side promoting integration with inner AF and the other side sealing the defect from outside is an attractive proposition. In cartilage repair, Favreau (2020) developed compartmentalized, multi-layered implants seeded with spheroids to treat osteochondral defects. The first compartment was based on therapeutic collagen membranes associated with BMP-2 to provide structural support and promote subchondral bone regeneration, while the second compartment contained BMSC spheroids dispersed in alginate hydrogel to support the regeneration of the articular cartilage [165]. These implants showed promising results in a sheep model. With modifications relevant for AF tissue, a spheroid-laden part could be used to bridge AF tears while a cell-free layer could possibly serve for AF sealing.

In order to recapitulate natural ECM structure and facilitate interactions between living AF patches and resident cells, electrospinning could be the fabrication method of choice. Electrospinning and its modifications can generate randomly organized or aligned fibers that mimic the natural ECM and provide wide cell adhesion surfaces and adjustable porosity. This allows spheroid immobilization and modulation of spreading as well as cell migration and differentiation. An alignment of electrospun fibers was shown to regulate ASC spheroid functions. Non-aligned nanofibrillar structures demonstrated a heterogeneous dispersion of ASC spheroids, preventing efficient cell colonization of the nanofibers' surface [184]. On the other hand, ASC spheroids seeded on aligned nanofibrillar structures (produced by jet-spraying) showed rapid and homogeneous cell dispersion, high viability, chondrogenic differentiation, and fused with each other, increasing the cell contact of the surface of the nanofibers. Therefore, fiber alignment that mimics the lamellar AF structure could produce a patch that integrates with target tissue more rapidly.

A combination of the above-mentioned fabrication methods may enhance the desired properties of (hybrid) living AF patches and/or immobilized spheroids in the scaffold. In skin tissue engineering, Lee (2020) seeded ASC spheroids onto a 3D-printed alginate-based mesh, which was followed by electrospinning of alginate/polyethylene oxide fibers directly onto the spheroids. The alginate scaffolding structure clearly retained the characteristics of the spheroids and maintained their superior regenerative capacity over scaffolds without the mesh [185].

The main function of spheroid-based living AF sealants would be to sustain tension generated by the NP and thus prevent NP extrusion until the defect is healed. Mechanical

properties of AF sealants can be increased by crosslinking agents (genipin, glutaraldehyde, riboflavin), which also promote their attachment to native tissue. Genipin crosslinked (cell-free) hydrogels achieve biomechanical properties of AF tissue [186–188], even possibly outperforming FDA-approved materials under loading [188]. However, their failure to adhere to AF tissue at higher strains of 15–30% (typical for degenerative overloading) was also reported [186]. As some crosslinking agents might negatively influence cell viability, adhesion, and spreading, preliminary tests should be performed to select an appropriate agent/concentration for each material and application [186,187]. Nevertheless, recent advances in other fields of tissue engineering clearly demonstrate the potential of the synergistic scaffold-based and scaffold-free strategies for AF repair.

5.3. Enhancement of Spheroid Functions

Besides degradation of ECM, DDD is characterized by sterile tissue injury and unresolved inflammation. The evidence suggests that therapeutic cells can mediate tissue repair not only by differentiation towards target structures but also via the secretion of soluble factors that enhance tissue repair (Figure 1D). Directing therapeutic spheroids towards paracrine trophic, anabolic, and anti-inflammatory functions is an exciting strategy to potentiate their performance and resistance. It is known that spheroids already release higher amounts of growth factors and anti-inflammatory factors, compared to single cells [189–191]. The secreted biomolecules are entrapped in ECM and readily control a range of biological processes, becoming a source of relevant regenerative cues. Thanks to recent developments, the secretion of beneficial factors from spheroids can be further enhanced by specific 3D-culture conditions, providing superior functions even without the use of stimulative growth factors [189,192].

In addition to secretome, therapeutic properties of spheroids are mediated (at least partially) via exosomes, the nanometer-size type of extracellular vesicles (EVs) that carry RNAs, proteins and lipids from the parent cell [193,194]. Recently it was shown that inhalation treatment of lung spheroid-derived exosomes (as well as secretome) provided anti-inflammatory properties and improved lung regeneration in two animal models of pulmonary fibrosis [195]. The therapeutic potential of EVs in IVD regeneration was recently reviewed [196]. For example, MSC-derived EVs are believed to promote regeneration and proliferation and reduce inflammation and apoptosis in the IVD, possibly via miRNAs and other (yet unknown) mechanisms. The use of spheroids could improve the quality and possibly increase the yield of therapeutic vesicles. The application of spheroid conditioned medium containing therapeutic secretome and/or EVs could be considered as a cell-free alternative for the treatment of DDD, with a lower regulatory burden [190,191].

Tissue-specific functions were shown to be promoted by a biomimetic environment applied during spheroid generation in vitro. Biomimetic spheroid priming enhances their chondrogenic capacity and/or resistance in harsh conditions. In cartilage tissue engineering, scaffold-free chondrocyte spheroids generated under hypoxia, upregulated the expressions of collagen II and aggrecan at mRNA and protein levels, increased ECM deposition, and generated a higher quality of cartilage [83,197]. In IVD repair, preconditioning of MSCs with hypoxia is known to provide beneficial effects by activating a hypoxia-inducible factor (HIF) signaling pathway found to be involved in phenotype maintenance, metabolism, and homeostasis of the IVD [43]. It remains to be seen whether preconditioning of (MSC) spheroids with hypoxia (or other microenvironmental conditions) further augments their effects on IVD-specific phenotype and function.

Recently Muttigi et al. (2020) described the promising effect of spheroid priming with Matrilin-3, a noncollagenous ECM adaptor protein. Matrilin-3-primed ASC spheroids increased gene/protein expression of growth factors and reduced the secretion of hypertrophic ECM components. Furthermore, Matrilin-3-primed ASC spheroids induced the stable mRNA expression of SOX9, collagen type II, and aggrecan, and enhanced chondroitin sulphate accumulation in NP cells (indirect co-culture). Matrilin-3-primed ASC spheroids also facilitated IVD repair in a rabbit model with AF puncture-induced IVD degenera-

tion [77], highlighting preconditioning as a useful approach to promote the regenerative capacity of spheroids for IVD repair.

Genetic modification could also enhance the chondrogenic capacity and resistance of spheroids. Genetic engineering to resist in a harsh IVD microenvironment has been widely considered [198,199]. Specifically, inflammation antagonists (e.g., IL1Ra) and IVD-related growth factors (e.g., GDF-5) appear to be promising targets [200]. Although not yet investigated in the IVD, genetically modified MSC spheroids with upregulated Runx2 were shown to overcome negative effects of a harsh microenvironment and promote regeneration in bone tissue engineering [201]. Major limitations of human genetic engineering are related to viral vectors and low (transient) expression of transgenes. Recently it was shown that the expression of non-viral transgenes could be maintained much longer in spheroids transplanted in vivo versus single cells. In hepatic regeneration, such a genetically modified spheroid system contributed to significantly higher therapeutic effects of transplanted hepatocytes in the host tissue [202]. Genetically modified spheroid systems might thus contribute to the maintenance of non-viral transgenes in the IVD and enrich anti-inflammatory and/or anabolic functions.

Instructive biomaterials providing physical and chemical signals required to modulate cellular behavior and reinforce particular spheroid phenotypes were designed [203]. Materials, e.g., with/without RGD peptides, would modulate spheroid spreading, while encapsulation of growth factors promotes spheroid fusion [165]. While not yet applied to spheroids, cell/growth factor-loaded particles ensured sustained release and chondrogenic differentiation of encapsulated therapeutic cells, aiding IVD regeneration in animal models [204,205]. Similarly, cell-free siRNA complexes encapsulated in injectable HA hydrogels retained release/activity over a prolonged period of time in vitro and in vivo [206]. Continuous supply of bioactive material combined with 3D cell configurations might enhance differentiation into chondrocyte-like NP cells as well as rejuvenate resident IVD tissue [204]. An implant combining MSC spheroids and a biomaterial with slowly released growth factors showed promise in sheep osteochondral repair [165].

Although single spheroids might lack in ECM organization being mechanically inferior to native tissue, spheroids are mechanosensitive, potentially enhancing the right interaction between an implant and target tissue upon loading [207,208].

6. Conclusions

In this review, we highlighted the potential of spheroid-based tissue engineering strategies for the repair and regeneration of the IVD. We first recapitulated the known aspects of spheroid tissue engineering and emphasized how promoting cell-ECM interactions in spheroids might be beneficial for IVD repair. We also introduced cell sources for IVD tissue engineering, with specific focus on 3D spheroid configurations, and enlisted fabrication and biomechanical/biochemical characterization methods to be used to reproducibly generate spheroids compatible with IVD tissue. Furthermore, mechanisms of spheroid integration into damaged tissues, including adhesion, migration of cells, and synthesis and secretion of site-specific proteins, were described to aid scientific understanding of underlying biological cues for tissue engineering purposes.

Spheroids can be applied in NP and AF both in scaffold-free and scaffold-based configurations, possibly providing advantages over cell suspension, as the 3D organization enables the cells to differentiate and synthesize endogenous ECM. In NP regeneration, spheroid-based strategies might prevent or delay a catabolic shift in therapeutic cells and/or accelerate graft integration in the DDD microenvironment. The use of scaffold-free spheroids might limit issues associated with biomaterials, such as inadequate degradation properties, thus simplify regulatory approval process. However, to generate volume within the NP tissue, the combination of spheroids with an injectable biomaterial should not be excluded from future investigations. In AF tissue engineering, spheroids could serve as building blocks for living AF patches, together with various biomaterials to seal the AF. A biomaterial could regulate the rate of spheroid fusion with AF, define spheroid position

in a patch, and/or release factors that regulate spheroid functions. Spheroids' capacity for rapid fusion might aid in filling deeper and irregular AF defects. The enhancement of spheroid functionality using a biomimetic and/or bio-instructive environment, as well as genetic modifications, are further measures to increase spheroid functionality in IVD regeneration.

From a clinical point of view, spheroid-based tissue engineering strategies for regeneration of the intervertebral disc represents an ideal minimal invasive treatment for a disease with a great burden for the patients and society in general. Unlike current surgical treatment options (most frequently fusion surgery) this kind of treatment could, once established, be performed as an outpatient procedure, which would further add to patient comfort. Ideally, this treatment will restore the function of the affected disc/motion segment and hopefully acceleration of adjacent segment degeneration (which often occurs after fusion surgery) will be avoided. In cases with more than one affected level, one could even treat several levels at the same time or add a level that may not seem "healthy" anymore but still seems to have sufficient function and may become symptomatic in the future. Adding such levels in the currently established treatment with fusion surgery is out of the question, as each added level would increase the potential for complications and adjacent segment degeneration.

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6. Chapter 3: Nose to Spine: spheroids generated by human nasal chondrocytes for scaffold-free nucleus pulposus augmentation

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Nose to Spine: spheroids generated by human nasal chondrocytes for scaffold-free nucleus pulposus augmentation

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ABSTRACT

Cell-based strategies for nucleus pulposus (NP) regeneration that adequately support the engraftment and functionality of therapeutic cells are still lacking. This study explores a scaffold-free approach for NP repair, which is based on spheroids derived from human nasal chondrocytes (NC), a resilient cell type with robust cartilage-regenerative capacity. We generated NC spheroids (NCS) in two types of medium (*growth* or *chondrogenic*) and analyzed their applicability for NP repair with regard to injectability, biomechanical and biochemical attributes, and integration potential in conditions simulating degenerative disc disease (DDD). NCS engineered in both media were compatible with a typical spinal needle in terms of size (lower than 600μm), shape (roundness greater than 0.8), and injectability (no changes in morphology and catabolic gene expression after passing through the needle). While *growth* medium ensured stable elastic modulus (E) at 5 kPa, *chondrogenic* medium time-dependently increased E of NCS, in correlation with gene/protein expression of collagen. Notably, DDD-mimicking conditions did not impair NCS viability nor NCS fusion with NP spheroids simulating degenerated NP *in vitro*. To assess the feasibility of this approach, NCS were injected into an *ex vivo*-cultured bovine intervertebral disc (IVD) without damage using a spinal needle. In conclusion, our data indicated that NC cultured as spheroids can be compatible with strategies for minimally invasive NP repair in terms of injectability, tuneability, biomechanical features, and resilience. Future studies will address the capacity of NCS to integrate within degenerated NP under long-term loading conditions.

Statement of significance

Current regenerative strategies still do not sufficiently support the engraftment of therapeutic cells in the nucleus pulposus (NP). We present an injectable approach based on spheroids derived from nasal chondrocytes (NC), a resilient cell type with robust cartilage-regenerative capacity. NC spheroids (NCS) generated with their own matrix and demonstrated injectability, tuneability of biomechanical/biochemical attributes, and integration potential in conditions simulating degenerative disc disease.

To our knowledge, this is the first study that explored an injectable spheroid-based scaffold-free approach, which showed potential to support the adhesion and viability of therapeutic cells in degenerated NP. The provided information can be of substantial interest to a wide audience, including biomaterial scientists, biomedical engineers, biologists and medical researchers.

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1. Introduction

Chronic low back pain (LBP) is a leading cause of disability and the main reason for non-cancer opioid prescriptions in western countries [1,2]. LBP is strongly associated with the degeneration of the intervertebral disc (IVD) [3]. Discogenic LBP (termed degenerative disc disease, DDD) often arises from a damaged nucleus pulposus (NP), the inner part of the IVD [3]. The affected NP suffers from inflammation and progressive loss of cells and the functional extracellular matrix (ECM), leading to spinal instability and the development of chronic pain [3]. However, there is currently no therapy available that can efficiently stop or reverse the progression of DDD. DDD is treated either by common pain medication or invasive surgeries, with risks of adverse effects, slow recovery, and high rates of recurrence [4,5]. In fact, ~20% of spinal fusion surgeries (surgical standard of care) fail to improve patient's symptoms for various reasons [6,7]. As conventional treatments are focused on the alleviation of symptoms, they often have an inadequate long-term efficacy [1].

Innovative NP tissue engineering strategies for long-term pain relief have showed promise in preclinical studies, but their clinical use is still limited [8-10]. The challenges in cell-based NP regeneration are related to the unique anatomical and biochemical NP microenvironment, which limits the cell type, carrier, as well as the delivery route to be used. The commonly investigated cell type for NP repair are mesenchymal stromal cells (MSC). It is thought that MSCs are capable of supporting NP by differentiating into NP-like cells and by secreting trophic and anti-inflammatory factors [11,12]. However, MSCs are not performing as expected in the harsh NP microenvironment, which is associated with hypoxia, inflammation, acidity, and low nutrient levels [12-14]. We have previously shown that DDD-mimicking conditions significantly reduce long-term survival of MSCs [15]. Notably, nasal chondrocytes (NC) derived from adult hyaline cartilage of the nasal septum, have demonstrated superior ability over MSCs (and articular chondrocytes) to survive/perform in harsh microenvironments [15,16]. Apart from their resilience, NC exhibit features of self-renewal, can adapt to heterotopic transplantation sites, and possess robust cartilage-repair capacity *in vivo* [17,18]. Recently, our group developed scaffold-based engineered NC grafts that are successfully used to repair nasal and articular cartilage in patients [19,20].

To broaden the applicability of NC for IVD repair, we propose a 3D scaffold-free approach. Clinical translation of cell-laden biomaterials for NP repair can be challenging due to the regulatory requirements related to these complex medical products: although numerous cell carriers/scaffolds were investigated, clinical evidence on their efficacy is still limited [21,22]. It is essential that a carrier/scaffold is compatible with NP tissue and ensures cell retention [23]. Scaffold-free conditions overcome limitations associated with exogenous carriers/scaffolds including damage, degradation, or immunological responses to a biomaterial [24]. Additional limitations in cell-based NP repair are leakage of therapeutic cells into the surrounding tissues [25] and loss of cell viability caused by the DDD microenvironment [15]. In order to bypass these challenges and retain the possibility of an injectable solution, here we propose an intradiscal delivery of self-assembling, living microtissues, hereafter referred to as spheroids. Spheroids are multicellular aggregates that mature by the formation of intercellular contacts on non-adhesive substrates and possess a high adhesion capacity compared to a cell suspension [26-29]. It was shown that cell-synthesized 3D matrix provides spheroids with superior regenerative properties over single cell suspensions [30]. Notably, autologous transplantation of articular chondrocyte-based spheroids was previously implemented in patients suffering from traumatic or degenerative injuries of articular cartilage, which lead to clinical benefit [31,32]. Application of NC-based spheroids (NCS) in NP repair might provide important advantages in terms of regulatory compliance, tissue adhesion, and biomechanics, possibly supporting clinical applicability [1,33].

With the final goal to assess whether autologous NCS represent a suitable graft for cell-based scaffold-free regeneration of the NP, we investigate whether human NCS (1) can be generated in a feasible and reproducible way with regard to minimally-invasive delivery in the NP, (2) possess the biomechanical and biochemical properties relevant for the target NP tissue, and (3) can be injected into the IVD via a spinal needle and engraft within a DDD-mimicking microenvironment. To reduce potential regulatory burdens during clinical translation, we test the possibility of NCS cultured without additional growth factors vs. NCS cultured in the presence of chondrogenic supplements.

2. Materials and methods

2.1. Experimental design

The study design that includes fabrication and characterization of NCS is displayed in Fig. 1.

2.2. Cells and cell sources

Written informed consent was obtained from all donors. Tissues were collected following local ethical committee approval (EKNZ-2015305, University Hospital Basel). Nasal septal cartilage tissue was harvested from patients undergoing rhinoplasty or from cadavers (total $n = 5$) (Table S1). NC were isolated using 0.15% collagenase type II (Worthington, 4176) for 22 h and expanded in *NC expansion medium* for up to three passages [15] (composition in Suppl. Material). For fusion and viability analysis (Section 2.6), human NP tissue ($n = 2$, Table S1) was acquired from patients undergoing surgery for DDD. NP and AF tissues were carefully separated by an experienced surgeon and NP cells were isolated from only the NP tissue by an established protocol using 0.15% collagenase type II for 22 h [15]. After digestion, NP cells were expanded in *NP expansion medium* (composition in Suppl. Material) up to 2 passages.

2.3. Fabrication of chondrospheres

NCS were formed in Corning spheroid microplates (Corning, 4520) according to the manufacturer's protocol. Briefly, passage two/three NC were harvested, resuspended in *growth* or *chondrogenic culture media* (composition in Suppl. Material), and dispensed at 12.5×10^3 cells/well to form NCS. Microplates were incubated at 37 °C in a humidified atmosphere with 5% CO_2 for 1, 3, or 7 days. Five to ten biological replicates per donor were used for each type of analysis. For fusion and viability analysis, passage two NP cells were resuspended in *NP expansion medium* (Suppl. Material) and dispensed into each microplate well (Corning, 4520) at 25.0×10^3 cells/well to form the nucleus pulposus spheroids (NPS). After 7 days of incubation in standard conditions (37 °C, 5% CO_2), NPS were exposed to DDD-mimicking conditions (37 °C, 5% CO_2 and 2% O_2, hypoxia), and the medium was changed to DDD-mimicking medium (Suppl. Material). Microplates with NPS were then incubated for an additional 7 days.

2.4. Potential for injectability

2.4.1. Morphology of nasal chondrospheres

To determine morphology and uniformity, images of NCS were obtained during a 7-day culture period using light microscopy (Nikon Eclipse Ti-E, Japan; acquisition software: Nikon NIS; camera: Nikon DS-Qi2; objective: 10x). Subsequently, image analysis of

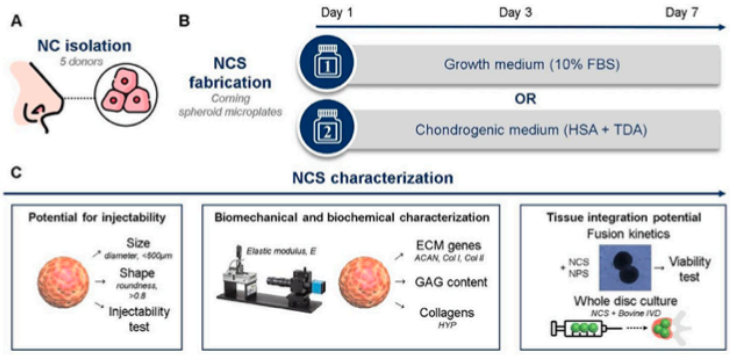

Fig. 1. Experimental design. (A) Isolation of nasal chondrocytes (NC) from human nasal septal cartilage tissue, **(B)** fabrication of NC chondrospheres (NCS) on non-adherent plates in growth or chondrogenic medium, and **(C)** characterization of NCS incubated for 1, 3, or 7 days in each medium. NPS = nucleus pulposus spheroid, FBS = fetal bovine serum, HSA = human serum albumin, TDA = TGFβ*3 (T)*, dexamethasone (D), ascorbic acid (A).

NCS diameter and roundness was performed using Image J 1.48v software (NIH, Bethesda, MD, USA). An upper size limit for NCS diameter was set at 600 µm, based on a reference spinal needle (22Gx7IN, Becton-Dickinson, 405149), which represents commonly used products. Feret's diameters of the detected NCS edges were measured initially as pixels and converted to micrometers by comparison to a reference length. Roundness was calculated as $4 \times \text{area} / (\pi \times \text{major axis}^2)$. The accepted shape coefficient was set between 1 and 0.8 [34].

2.4.2. Shear resistance of nasal chondrospheres

The effects of injection-related shear stress on morphology and gene expression of NCS were verified using NCS cultured in growth or chondrogenic medium for 3 days ($n = 3$ per group). NCS were passed through a spinal needle (22G, Becton-Dickinson, 405149) at an approximate speed of 200 µL/min, imaged with the EVOS XL Core Imaging System (Invitrogen™ AMEX1000, USA), placed again into Corning spheroid microplates, and cultured for a further 24 h. Control NCS were not passed through the needle. After 24 h, NCS were collected and gene/protein expression of stress-associated targets (COX-2, IL-6, caspase 3, MMP13; ***Table S2***) was analyzed as described in Section 2.5.2.

2.5. Biomechanical, biochemical and molecular characterization

2.5.1. Elastic modulus assessment

To evaluate the elastic modulus (E) of the whole spheroid, an unconfined compression test was performed using a micro-scale, parallel-plate compression testing system (Microsquisher, CellScale, Canada) supported by SquisherJoy software. During unconfined compression the sample bulges radially subjected the tissue to both compressive (in the direction of loading) and tensile (in the radial direction) deformations. Single NCS were placed in a container filled with PBS at 37°C and controlled compression of NCS between two parallel plates was performed. NCS were compressed to a 50% deformation in 20 seconds. The microbeam with diameters 304.8 µm (recommended max force 917 mN) or 406.4 µm (recommended max force 2897 mN) was employed with regard to the stiffness and sensitivity required to measure biomechanical properties of NCS. The force-displacement data obtained from the compression test were converted into stress-strain curves, and the lower portion of the curve (0-20% strain) was used to obtain a linear regression line and to estimate the elastic moduli (E, kPa).

2.5.2. Gene expression analysis

Eight NCS per donor were combined for total RNA extraction using the RNeasy Mini Kit (Quiagen, 74106), according to the manufacturer's protocol. The RNA yield and purity were measured on a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, USA). SuperScript™ III Reverse Transcriptase kit (Invitrogen, 18080093) was used to reverse-transcribe 0.5 µg of RNA into cDNA in a 30 µL volume. Ten ng of cDNA/well was mixed with TaqMan™ Universal PCR Master Mix (Applied Biosystems, 4304437), RNase-free water, and primers (Table S2) in a total volume of 10 µL and used for quantitative real-time polymerase chain reaction (RTqPCR) performed on a 7300 Real-time PCR System (Applied Biosystems, USA). For each sample, Ct values of the target were subtracted from the Ct values of a housekeeping gene (human GAPDH, Hs02758991, Applied Biosystems) to derive the ΔCt. Gene expression was quantified relative to the housekeeping gene ($2^{-\Delta Ct}$).

2.5.3. Biochemical content quantification

NCS were digested for 16 h at 56 °C in 1 mg/mL proteinase K solution [1 mg/ml proteinase K (Sigma-Aldrich, P2308) in 50 mM Tris (Sigma-Aldrich, A5456-3) with 1 mM EDTA (Fluka, 03680), 1 mM iodoacetamide (Sigma-Aldrich, I-1149) and 10 mg/mL pepstatin A (Sigma-Aldrich, P5318)]. Glycosaminoglycan (GAG) content was determined spectrophotometrically using Blyscan Glycosaminoglycan Assay (Biocolor, B1000). DNA content was measured using the CyQuant Cell Proliferation Assay Kit (Invitrogen, C7026), with bacteriophage λ DNA as a standard, according to the manufacturer's protocol. Total collagen content was quantified using the Hydroxyproline (HYP) Assay Kit (Sigma-Aldrich, MAK008) according to manufacturer's protocol. Both GAG and HYP contents were normalized either to single NCS or to DNA content.

2.5.4. Histology and immunohistochemistry

NCS were fixed in 4% paraformaldehyde, washed in PBS and embedded in Richard-Allan Scientific™ HistoGel™ Specimen Processing Gel (Thermo Scientific, HG-4000-012). The gel was warmed to a liquid state, then retained at 65 °C in a water bath. NCS were embedded in 30 µL gel droplets and processed using Tissue Processing Center TPC 15 Duo (Medite, Germany). Samples were embedded in paraffin, cut in 5 µm-thick sections using the sliding microtome Microm HM 430E (Thermo Scientific, Germany), and mounted on poly-L-lysine coated glass slides. After dehydration, Safranin-O/fast green (SafO/FG; All Sigma-Aldrich; SafO: 84120, FG: F-7252) staining with hematoxylin (J.T. Baker, MFCD00078111) nu-

clear counterstaining was performed to analyze chondrogenic tissue formation according to the standard protocol. Widefield microscopy (Nikon Ti2, Japan; acquisition software: Nikon NIS; Camera: Nikon DS-Ri2; Objective: 40x; NA: 0.95) was applied for imaging. Images were processed using Fiji/ImageJ software (NIH, Bethesda, MD).

For immunohistochemistry, sections were subjected to enzymatic epitope retrieval, blocked with 1% bovine serum albumin (BSA, Sigma, SLBW6770) supplemented with Triton X-100 (1:1000; VWR Life Science, 97062-208), and the following primary antibodies were used: rabbit anti-type I collagen (Col1) human-specific, (1:200; Abcam, ab34712); mouse anti-type II collagen (Col2) human-specific (1:200; Abcam, ab23446); rabbit anti-cleaved caspase-3 (cCas3) human-specific (1:500; Cell Signalling, 9661); rabbit anti-aggrecan (1:100; Abcam, ab3778). Respective matching, secondary antibodies Alexa Fluor 647- or 546-conjugated (1:500; Invitrogen, A21245 and A11030) were used, with DAPI as a nuclear counterstain. Widefield fluorescence microscopy (Nikon Ti2, Japan; acquisition software: Nikon NIS; Camera: Photometrics Prime 95B; Objective: 40x; NA: 0.95) was applied for imaging. Images were processed using Fiji/ImageJ software (NIH, Bethesda, MD).

2.5.5. Image quantification

Semiquantitative histological scoring using the Bern Score (BS) was carried out on Safranin O-stained histological images as previously described [35]. Briefly, three parameters (staining intensity, distance between cells, cell morphology) were assessed and each category received a score between 0 and 3. The resulting values were then summarized to calculate final BS between 0 and 9. Three independent scorers were involved.

Immunofluorescence images were quantified using ImageJ. Immuno-positive areas containing NCS were used as areas of interest. For each image, the mean gray value representing each target was obtained.

2.5.6. ELISA

IL-8 and MMP13 release into a NCS culture medium was analyzed using ELISA. SensoLyte plus MMP13 Assay Kit (AS-72019, AnaSpec) was used for fluorometric detection of active MMP13, performed according to the manufacturer's protocol. Human IL-8 ELISA Set (555244, BD) with ELISA reagent set B (550534, BD) were used to detect IL-8 according to the manufacturer's instructions. Culture medium collected from NC treated with a low-grade pro-inflammatory cocktail (100 pg/ml of TNFα, IL1β, IL6) was used as a positive control (PC).

2.5.7. Western blot

After the washing step with PBS, 32 NCS, matured for 3 days in growth or chondrogenic medium were pooled into Lysing Matrix tubes (MP Biomedicals) containing 300ul lysis buffer (25 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 1 mM EDTA, 5% glycerol and supplemented with a protease inhibitor cocktail (Roche)) and mechanically disrupted with 3 rounds of homogenization (6m/s, 30sec) using the Fastprep instrument (MP Biomedical). After centrifugation (5 min, 20000 rcf, 4 °C), the supernatants were collected and protein concentration was determined using a BCA protein assay kit (ThermoFisher). Protein extracts from each sample were subjected to 8% SDS/PAGE and then transferred onto PVDF membranes using the Trans-blot turbo blotting system (Bio-Rad). After blocking (5% fat-free powdered milk in TBS with 0.1% Tween for 1 h), membranes were probed with primary antibodies against Collagen I (1/1000, ab138492, Abcam) or Collagen II (1/1000, ab188570, Abcam). Anti-GAPDH antibody (1/10000 Origen) was used as a loading control. Blots were then exposed to appropriate peroxidase-coupled, secondary antibodies (Southern Biotech). Protein detection was done using Immobilon Western Chemiluminescent HRP Substrate (Millipore) and a signal was acquired with the Fusion FX7 Imaging system (Vilber Lourmat). According to the manufacturer, ab188570 detects the C-terminal propeptide of collagen type II at low kDa. Size of this band may vary due to post-translational modifications, cleavage or assay conditions. The amount of C-terminal propeptide is proportional to collagen II synthesis [36].

2.6. Potential for integration with nucleus pulposus

2.6.1. Fusion kinetics assay

To simulate the situation after NCS injection into degenerated NP, the fusion kinetics between NCS-NPS and NCS-NCS was evaluated in ***DDD-mimicking medium*** (***Suppl. Material***), with ***control fusion medium*** (***Suppl. Material***) used in parallel. NCS incubated in growth or chondrogenic medium for 1, 3, and/or 7 days were used for this assay. The spheroids were placed in pairs in close proximity to each other on the bottom of each well in order to form a doublet. For NCS-NPS fusion, NPS pre-formed in DDD mimicking conditions (Section 2.3) were placed together with 1-, 3- or 7-day-old NCS. The pairs of NCS-NCS and NCS-NPS were incubated at 37 °C with 5% CO_2, 2% O_2 (hypoxia) or 20% O_2 (standard) for 96 h [37]. Images of spheroid doublets were used for quantitative analysis of the fusion process. The images were obtained with phase-contrast, time-lapse microscopy (Olympus IX81, Japan; acquisition software: Olympus BSW; camera: CoolSNAP HQ2 CCD; objective: 10x) at 0, 4, 8, 24, 48, 96 h. Intersphere angle, contact length, and doublet length were measured using Fiji/ImageJ software (NIH, Bethesda, MD) and plotted as a function of time. Doublet length was normalized to initial doublet length.

2.6.2. Viability assay

The viability of fusing NCS-NCS and NCS-NPS was assessed using the CellTiter-Glo Luminescent Cell Viability Assay (Promega, G7570) according to the manufacturer's protocol. Briefly, the microplate with spheroids was left at room temperature for 30 minutes prior to examination. Four empty wells were filled with 100 µl of the corresponding medium to obtain a value of background control. A volume of CellTiter-Glo Reagent equal to the volume of the medium was added to each well and mixed well to induce cell lysis. The plate was incubated at room temperature for one additional hour to stabilize the luminescent signal. Luminescence was recorded using a SPARK Multimode Microplate Reader (Tecan, Switzerland). Spheroids after 0h, 24h, and 96h of fusion were used for a viability assessment. In parallel, viability of single NCS and NPS was measured for the same time points (control). Luminescence signal, proportional to cellular ATP generation, was expressed in relative light units (RLU).

2.6.3. Intervertebral disc (IVD) organ culture model

Fresh bovine tails (n = 2, 5-6 months old) were obtained from a local slaughterhouse within 24 h after sacrifice. The tails were sterilized in betadine solution (Mundipharma, 780239, 1:10) for 30 min and processed under sterile conditions to remove the surrounding soft tissues. Exposed caudal spines were cut at each growth plate using a custom-made guillotine. Six IVDs from 2 tails were washed with betadine solution and PBS, and pre-cultured in ***NP expansion medium*** (***Suppl. Material***) under static conditions for 24 h. IVDs were randomly divided into three groups: healthy control, degraded ECM, and degraded ECM + NCS (generated for three days in ***growth medium***). To induce ECM degradation, NP was enzymatically digested by injection of 3 U/mL papain, as described before [38]. After seven days of papain digestion, selected IVDs were injected with 16 NCS using a spinal needle (22G, BD, 405149) and incubated at 37 °C in an humidified atmosphere with 5% CO_2 and 20% O_2 for another 24 h. Sixteen NCS were selected

for injection to reach the total of 2 × 10^5 theoretically injected cells [39]. The samples were fixed in 4% paraformaldehyde at room temperature for seven days, processed for 28 h using Tissue Processor (TPC 15 DUO, Medite, Germany) and embedded in paraffin. Samples were cut in 4 µm-thick sections using sliding microtome Microm HM 430E (Thermo Scientific, Germany), and mounted on poly-L-lysine coated glass slides. The structural integrity of NCS within IVD tissue was evaluated by imaging the sections using a widefield microscope (Nikon Ti2, Japan; acquisition software: Nikon NIS; Camera: Nikon DS-Ri2; Objective: 4x; NA: 0.2). Images were processed using Fiji/ImageJ software (NIH, Bethesda, MD).

2.6.4. In situ Hybridization (Alu staining)

In situ Hybridization with DNA-probes was performed according to Kasten (2005) [40]. A digoxigenin-labelled probe for human-specific Alu repetitive sequence was prepared by PCR containing 1 × PCR buffer (M792A Promega), 1.5 mM MgCl2, 1 mM dATP, 1 mM dCTP, 1 mM dGTP, 0.65 mM dTTP, 0.35 mM digoxigenin-11-dUTP (11093088910, Roche), 0.2 uM of Alu-specific primers, 3.75U Taq DNA Polymerase (M830B, Promega) and 50 ng of human genomic DNA in a total volume of 50 µl. The following primers were used: forward 5′-cgaggcgggtggatcatgaggt-3′, reverse 5′-ttttttgagacggagtctcgc-3′. Sections were deparaffinized in XEM-200, rehydrated in ethanol, incubated for 10 min at room temperature in 0.2 n HCl and treated with 0.5 mg/mL pepsin in 0.01N HCl for 10 min at 37 °C and washed with PBS. Sections were then prehybridized for 30 min h at 42 °C in a hybridization buffer containing 4 × SSC (85635, Sigma), 50% deionized formamide, 1 × Denhardt's solution, 5% dextrane sulfate and 1 µg/ml salmon sperm DNA. The Hybridization buffer was replaced by a fresh buffer containing 0.2 ng/µl digoxigenin-labelled ALU probe, target DNA+probe was denatured for 3 min at 95 °C cooled down. Hybridization was carried out for 2 h at 42 °C. Slides were washed twice for 5 min in 2 × SSC at room temperature and twice for 10 min at 42 °C in 0.1 × SSC. Signals were detected using anti-digoxigenin AP (11093274910, Roche) incubated 1h at RT and NBT/BCIP (1168145100, Roche) as a substrate. Counterstaining was performed using Nuclear Fast Red (N3020 Sigma). Finally, slides were dehydrated and mounted in Pertex and imaged.

2.7. Statistical analysis

All data were analyzed using GraphPad Prism software ver. 8.0.1 (GraphPad Software, Inc., La Jolla, Ca) and reported as mean ± SD. The following tests were used to assess the statistical significance: for normally distributed data, analysis of variance (ANOVA) followed by Tukey's *post-hoc* test (column analysis) or Sidak's *post-hoc* test (group analysis); for non-normally distributed data, Kruskal-Wallis test with Dunn's *post-hoc* test (column analysis) or the Mixed-effects model with Sidak's *post-hoc* test (group analysis). The correlation assessment was carried out by calculating Person's correlation coefficients and p values between relevant sample groups. Correlation between the following properties was tested: E, DNA/spheroid, GAG/spheroid, GAG/DNA, HYP/spheroid, HYP/DNA, GAG/HYP, gene expressions of ACAN, COL1A1 and collagen type II (COL2A1). Numerical values of probability smaller than 0.05 were considered as statistically significant.

3. Results

3.1. Morphology and injectability of nasal chondrospheres

NCS were generated from human NC ($n = 5$) in growth or chondrogenic medium for up to 7 days, and characterized in terms of size, shape, and morphology. These parameters are crucial for injectability via spinal needle and determine the reproducibility of the production process. Although inter-donor variability was detected for the size parameter (Table S3), the majority of NCS passed the size threshold (diameter <600 µm; 100% of NCS generated in growth medium, 87% of NCS generated in chondrogenic medium) (Fig. 2A) as well as the shape threshold (roundness >0.8) (Fig. 2B). Only 1.3% of NCS cultured in growth medium and 9.3% of NCS cultured in chondrogenic medium contained shape irregularities such as elongation and satellites (Fig. S1). NCS with similar morphology were formed in both growth and chondrogenic medium (Fig. 2C, D). Collectively, size and shape of all NCS, except day 1 NCS cultured in chondrogenic medium, satisfied the target requirements. Given the similar performance of NCS at most time points, injectability of NCS was verified using NCS cultured at the intermediate maturation time point (i.e. three days). Injection of NCS through the spinal needle did not alter NCS morphology (Fig. 2F, G) nor gene expression of COX-2, IL-6, caspase 3 and MMP13, as indicators of shear-stress, inflammation, apoptosis, and catabolism, respectively [41,42] (Fig. 2E). The release of the inflammation/catabolic mediators IL-8 and active MMP13 was unchanged upon NCS injection (Table S4). Cleaved caspase 3 (cCas3) immunostaining was similar between injected and non-injected NCS, confirming gene expression data (Fig. S2).

3.2. Biomechanical and biochemical characterization

Elastic modulus (E) of native NP tissue in unconfined compression was reported to be ~5-10 kPa for healthy and ~10-60 kPa for aged and degenerated NP [43,44]. Biomechanical properties within these scales are expected to facilitate physiological interaction of spheroids with NP tissue. We showed that the E of single NCS cultured in growth medium reached ~5 kPa at all maturation time points, while chondrogenic medium time-dependently increased the E of NCS (Fig. 3A), with low inter-donor variability in this parameter (Table S3). It is known that mechanical characteristics of tissues (e.g. stiffness) are commonly associated with their ECM components [45,46]. Gene expression data revealed that collagen type I (COL1A1) expression time-dependently increased only in chondrogenic medium, while aggrecan (ACAN) gene was upregulated in both types of medium (vs. day 0) (Fig. 3D-F). To further evaluate the structural/functional NCS components during 7-day maturation, additional genes were analyzed. The expression of Ki67 (proliferation marker) was significantly upregulated in NCS cultured in chondrogenic medium (vs. growth medium), while no significant differences were found in SOX9, Thrombospondin 4 (COMP), Collagen type X, Versican and IL-6 gene (not expressed) (Fig. S3). The release of inflammation/catabolic mediators IL-8 and active MMP13 was under the detection limit in all conditions (Table S4) Biochemical analyses revealed no significant differences in ECM content between the two medium groups (Fig. 3G, H). Within the groups, NCS showed time-dependent accumulation of HYP/DNA (significant in chondrogenic medium) and GAG/DNA (in both growth and chondrogenic medium), supporting observed gene expression trends (Fig. 3J, K; Table S5). The correlation analysis between E and gene expression/ECM content revealed strong positive association between E of NCS cultured in chondrogenic medium and their HYP content (Pearson's $r_{(E\ vs.\ HYP/DNA)} = 0.9$, $p < 0.0001$) and COL1A1 gene expression (Pearson's $r_{(E\ vs.\ COL1A1)} = 0.8$, $p = 0.015$) (Fig. 4C). An immunofluorescence staining of 7 days-matured NCS (Fig. 4) and Western blot analysis (Fig. S4) confirmed the enhanced accumulation of collagen type I in chondrogenic medium. Histological analysis of NCS indicated prevalent GAG-synthetic effects in NCS cultured in growth medium (Fig. 4) but aggrecan immunofluorescence staining was not significantly different between media (Fig S5),

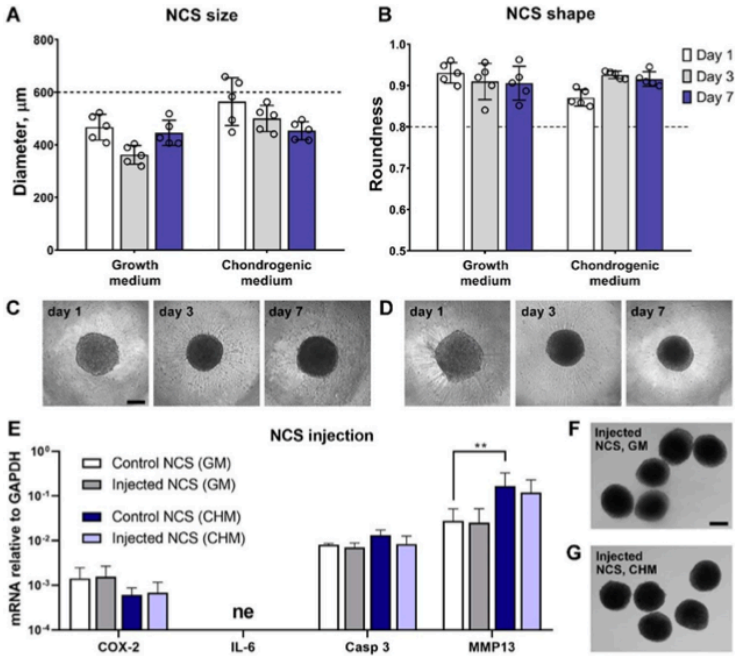

Fig. 2. Injectability of nasal chondrospheres (NCS) generated in growth and chondrogenic medium. (A) Size (diameter) and **(B)** shape (roundness) of NCS ($n = 5$ donors). 600 µm and 0.8 were set as size and shape thresholds, respectively. NCS morphology on day 1, 3, and 7 in **(C)** growth and **(D)** chondrogenic medium. Scale bar = 200 µm. **(E)** Gene expression of NCS 24h after being passed through the spinal needle ($n = 3$ donors, mean ± SD, $^{**}p < 0.01$, 2-way ANOVA). **(F-G)** NCS passed through the spinal needle. GM = growth medium, CHM = chondrogenic medium, ne = not expressed.

3.3. Potential of nasal chondrospheres to integrate with nucleus pulposus tissue

Adequate integration of NCS within NP tissue is expected to facilitate a natural remodelling process. It is envisioned that NCS after injection will integrate with degenerated NP tissue and with each other. To determine the tissue integration potential of NCS in degenerated NP, fusion kinetics between NCS-NCS and NCS-NPS were evaluated in DDD-mimicking conditions *in vitro* [47]. The fusion kinetics assay design is outlined in the Fig. 5A. Three-day matured NCS were used in further tests, as NCS maturation time point (day 1, 3, 7) had no significant effect on the fusion kinetics between NCS-NPS nor NCS-NCS in DDD-mimicking conditions (***Fig. S6***). Importantly, simulated DDD conditions (inflammation, hypoxia, acidity, low glucose) did not impair NCS-NPS fusion kinetics when compared to control condition (normoxia, high glucose) (Fig. 5). In particular, no significant differences were found in doublet length, and intersphere angle during four days of fusion. The contact length of NCS-NPS pre-cultured in growth medium (vs. chondrogenic medium) increased faster, implicating these NCS might have the ability to fuse more rapidly. During the fusion process, we also monitored the spheroid viability. Viability of single NPS and single NCS during four days in DDD-mimicking environment was similar to 0h time point (Fig. 6A, B). Notably, viability of NCS-NPS doublets during four days of fusion in DDD-mimicking conditions tended to increase, with no significant difference between NCS pre-incubated in growth or chondrogenic medium (Fig. 6C). To test whether NCS can be injected via clinically used spinal needle into the IVD without disruption, we used pre-degenerated bovine IVD cultured ex vivo. Twenty-four h after injection, we were able to localize NCS and distinguish them from native NP tissue using human-specific staining. This proof of principle experiment also revealed that the NCS were not damaged during injection and kept their structural integrity (Fig. 7).

4. Discussion

Our study provided *in vitro* evidence of the applicability of NCS for the augmentation of NP. Non-adhesive technology allowed reproducible fabrication of NCS both with and without additional growth factors. We demonstrated that resulting NCS were injectable through the spinal needle with no significant effects of shear stress on gene expression of inflammation, catabolism, and apoptosis markers. While growth medium ensured stable elastic modulus (E), chondrogenic medium time-dependently increased E of NCS in correlation with their gene/protein expression of collagen type I. Importantly, the intrinsic capacity of NCS to fuse was not negatively affected by the DDD-mimicking conditions, supporting their engraftment in harsh microenvironments. This study also provided a proof of concept that NCS can be injected into the IVD via spinal needles without disruption.

Despite the large need for minimally invasive cell-based NP regeneration, no therapy has been adopted clinically yet. Translation of cell-based approaches for discogenic LBP faces several critical challenges, mainly related to (1) cell source and its ability to perform well in the degenerated microenvironment, (2) intradiscal delivery and its compatibility with spinal needles, (3) graft processing/structure and adherence to regulatory standards, and (4)

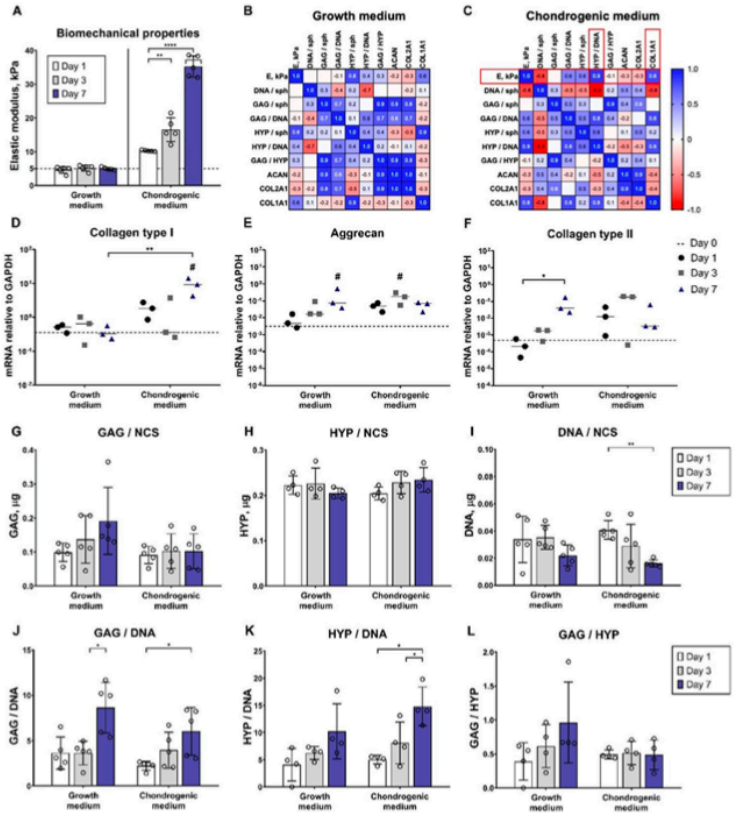

Fig. 3. Biomechanical and biochemical characterization of nasal chondrospheres (NCS) cultured in growth and chondrogenic medium. (A) Elastic modulus ("E") of NCS measured by Microsquisher (n = 5 donors, mean ± SD, $^{**}p < 0.01$, $^{****}p < 0.0001$, 2-way ANOVA). **(B-C)** Correlation (Pearson's r) between E of NCS and other tested parameters for (B) growth medium and (C) chondrogenic medium. **(D-F)** Relative gene expression (2^{-dCt}) of (D) collagen type I, (E) aggrecan, and (F) collagen type II (n = 3, mean ± SD, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{\#}p < 0.05$ vs. Day 0, ANOVA). **(G-L)** Biochemical content of NCS, (G) GAG per NCS, (H) hydroxyproline (HYP) per NCS, (I) DNA/NCS ratio, (J) GAG/DNA ratio, (K) HYP/DNA ratio, (L) GAG/HYP ratio (n = 4-5 donors, mean ± SD, $^{*}p < 0.05$, $^{**}p < 0.01$, 2-way ANOVA).

physiological relevance of suggested treatment in view of NP tissue composition and mechanical properties [1,48]. Successful cell therapy should provide solutions to all these challenges.

MSCs-based therapies for IVD repair showed some clinical success [49]. However, MSCs require supplementation with growth factors for differentiation and phenotypic maintenance. To overcome cost demands and regulatory hurdles, alternative cell sources that are terminally differentiated and retain good potential in forming cartilaginous tissues are justified. NC emerged as a promising cell source for cartilage and IVD tissue engineering and regeneration [16,50]. Unlike autologous NP cells, NC are derived from healthy tissue and accessible with minimal donor-site morbidity. Although NC exhibit some phenotypic differences to native NP cells, their robust cartilage regenerative potential and resilience make them an attractive cell source warranting further investigations [17,18]. Notably, engineered NC cartilage grafts have already been used to reconstitute cartilage defects in patients [19,20].

Primary cells may exhibit different donor-related phenotypic characteristics, possibly linked to their regenerative potential [51]. By using five different human NC donors, we demonstrated the capability to generate uniform NCS with high reproducibility in biomechanical and biochemical properties, despite the batch-to-batch variability commonly associated with primary cells. To reduce burden during the regulatory approval process, we tested the possibility of NCS cultured without additional growth factors (growth medium) vs. specific growth factor supplementation (chondrogenic medium). The growth medium contained FBS, although in clinical settings FBS will be substituted by autologous serum [31] or platelet-based products such as platelet lysate or platelet-rich plasma [52], which showed similar effects [53].

Our envisioned therapeutic strategy is scaffold-free. While injectable cell-carrier systems have improved cell delivery into the NP in preclinical studies, the biomaterial itself poses a challenge for clinical translation [1]. Carrier degradation products might accumulate to levels beyond those found in other (vascular) tissues, possibly causing NP-specific toxicity [54,55]. On the other hand, intradiscal delivery of cell suspensions in a saline solution can lead to cell leakage out of the IVD, hindering the regulatory approval process [25]. We provided evidence that NCS can be delivered into the IVD without damage using spinal needles and rapidly fuse with NPS. Application of NCS with their own ECM might circumvent regulatory issues related to biomaterials as well as increase cel-

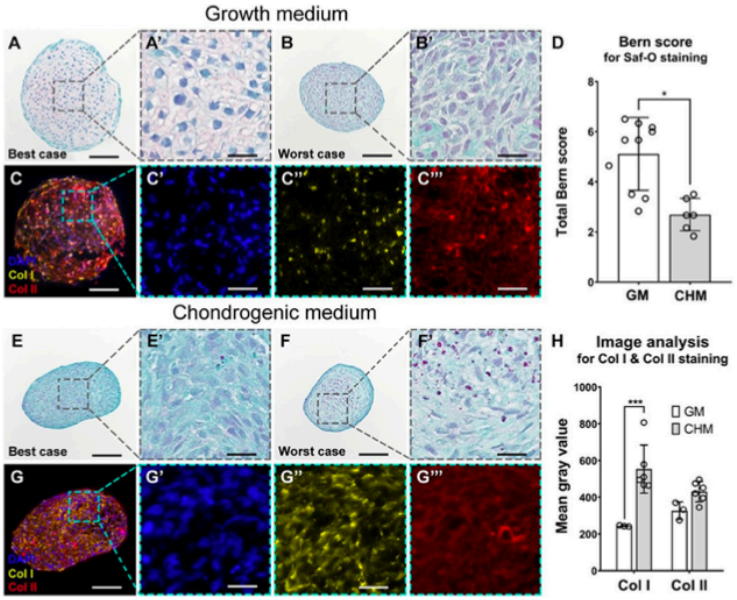

Fig. 4. Histological staining of 7 day-matured nasal chondrospheres (NCS) cultured in growth and chondrogenic medium. Safranin O staining of NCS cultured in growth medium **(A, A')** best case, **(B, B')** worst case) and chondrogenic medium **(E, E')** best case, **(F, F')** worst case). Immunofluorescence staining of collagen type I (yellow) and collagen type II (red) in NCS when cultured in **(C-C''')** growth (GM) and **(G-G''')** chondrogenic (CHM) medium. DAPI (blue) used to stain the nuclei. Scale bars = 200 µm (A-G), 4 µm (A'-G'''). (D) Grading of the NCS performed using the Bern Score. (H) Quantification of collagen type I and II immunofluorescence (n = 2-3 donors, mean ± SD, $^{*}p < 0.05$, $^{***}p < 0.001$, ANOVA).

lular retention in the NP space. The clinical use of spheroid-based scaffold-free technology for articular cartilage repair supports the feasibility and translational potential or this strategy [56].

To generate NCS, we used the cell numbers on a scale of 10^4 as smaller-sized spheroids showed better viability than larger-sized spheroids [57] and metabolic waste removal and nutrition diffusion to the core of spheroids are relative to their size [58]. Furthermore, bigger surface area might increase spheroid fusion kinetics hence promote faster integration in the host tissue. Specific NC and NCS numbers for clinical application will be verified in future *ex vivo* and *in vivo* studies.

An important challenge in NP regeneration is to recapitulate the unique ECM architecture of native NP tissue. Healthy NP tissue is characterized by high proteoglycan to collagen ratio (GAG/HYP 27:1) and abundance of aggrecan and collagen type II, compared to collagen type I [59]. E of healthy NP in unconfined compression was reported to be ~5 kPa, increasing with age and grade of degeneration [44,60]. During degeneration intradiscal pressure in human NP decreases, which is associated with the loss of proteoglycans and leads to a "solid-like" tissue with accumulation of collagens [61-63]. Our group showed previously that biomechanical and biochemical properties of NC-based cartilage grafts are enhanced by pre-culture period, towards attributes required to support long-term stability of the engineered grafts [64]. In this study, pre-culture in chondrogenic medium significantly enhanced biomechanical properties of NCS in correlation with their collagen content. Enhanced synthesis of collagen type I might arise from the ascorbic acid supplementation, which is well known inducer of collagen expression [65]. Increasing E of NCS by chondrogenic medium might help to generate initial stress resistance in degenerated "solid-like" NP *in vivo* by supporting the tensile component of their deformation upon implantation. As ascorbic acid was artificially supplemented, we do not expect collagen accumulation and E to continue increasing upon implantation *in vivo*. However, we cannot exclude this possibility. If collagen type I (and E) will continuously increase *in vivo*, NCS pre-cultured in chondrogenic medium might become unsuitable for DDD therapy. Our planned animal study will focus on this aspect.

The observed decrease of DNA content of NC during the spheroid culture is in line with the results of previous studies demonstrating this phenomenon related to redifferentiation [66-68].

The reaction of therapeutic cells to a degenerative NP microenvironment is critical for the success of cell therapy. Therapeutic (and resident) cells commonly suffer from limited nutrient supply and waste removal *in vivo* due to endplate calcification and/or an excess number of introduced cells [48]. Implanted cells are exposed to the avascular niche with low oxygen and glucose, high concentration of lactic acid, and complex non-physiological loading [69]. Increased concentrations of pro-inflammatory cytokines in degenerated NP tissue cause upregulation of matrix-degrading enzymes and catabolic shift [70], possibly even promoting donor cell death and aggravating the vicious cycle of degeneration. Recently, NC showed superior effects over MSC in terms of viability in simulated DDD microenvironment, suggesting they represent a robust cell population with a likelihood of survival post-injection [15]. In order to illustrate the physiological relevance of our experimental data, we recapitulated the microenvironment of the degenerated human NP by introducing low-grade inflammation, lactic acid, hypoxia, and low glucose into the fusion kinetics tests. Despite existing research on chondrospheres, fusion kinetics analysis has been mostly restricted to tissue spheroids of the same cell type

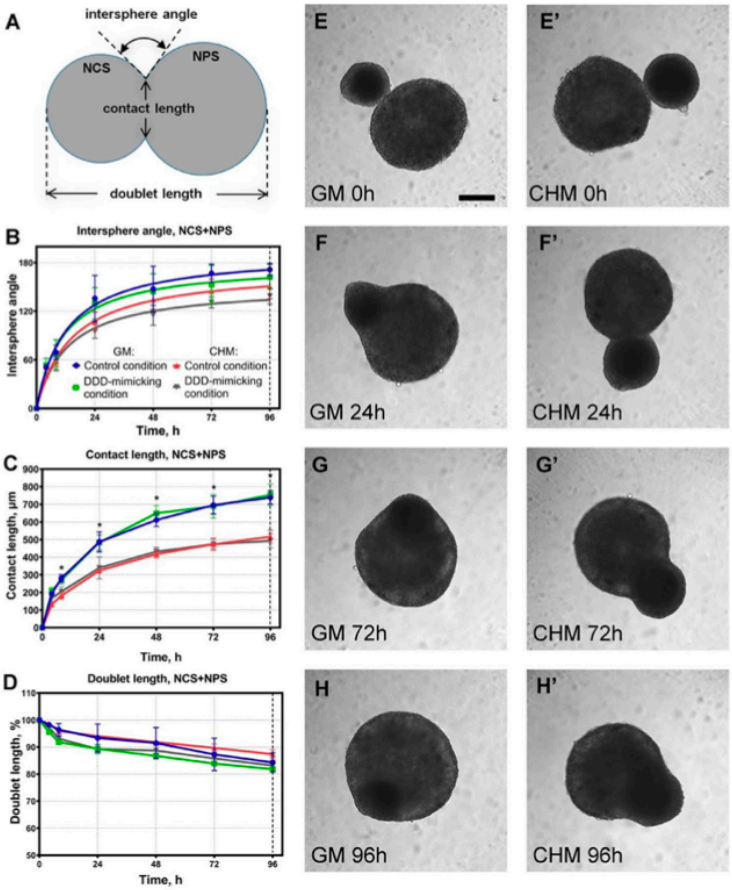

Fig. 5. Fusion kinetics of nasal chondrospheres (NCS) with nucleus pulposus spheroids (NCS+NPS) in DDD-mimicking conditions. **(A)** Schematic representation of the fusion process. Fusion parameters as **(B)** intersphere angle, **(C)** contact length, and **(D)** doublet length, were assessed. Brightfield images of fusing NCS+NPS after NCS pre-incubation in **(E-H)** growth (GM) or **(E'-H')** chondrogenic medium (CHM). GM = pre-incubation in growth medium, CHM = pre-incubation in chondrogenic medium. Scale bar = 200 µm.

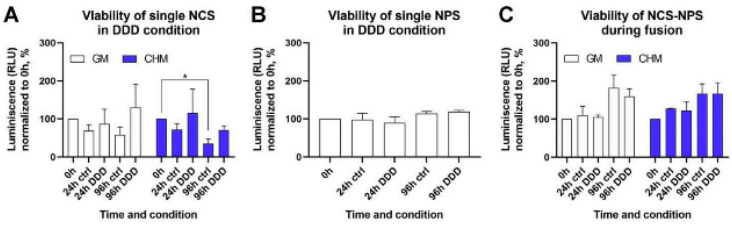

Fig. 6. Viability of nasal chondrospheres (NCS) and nucleus pulposus spheroids (NPS) in DDD-mimicking microenvironment. NCS were pre-cultured in growth (GM) or chondrogenic (CHM) medium for 3 days. NPS were generated in standard conditions (7 days) followed by DDD-mimicking conditions (additional 7 days) to mimic pre-existing DDD. **(A)** Viability of single NCS; **(B)** Viability of single NPS; **(C)** Viability of NCS+NPS during the fusion process ($n = 3$ donors for NCS and 2 donors for NPS, mean ± SD, $^*p < 0.05$, 2-way ANOVA).

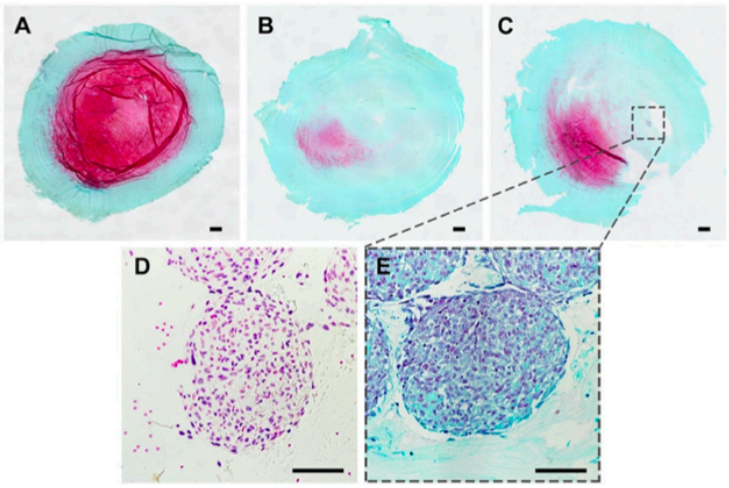

Fig. 7. Injection of nasal chondrospheres (NCS) into bovine intervertebral disc (IVD). Safranin O staining visualizing proteoglycan content within **(A)** healthy IVD, **(B)** papain-degenerated IVD and **(C)** papain-degenerated IVD containing NCS, scale bar = 1 mm. **(D)** Magnification of the NCS within the IVD in image C. **(E)** Alu Staining detecting human cells within the bovine IVD (violet-coloured cells = human cells). Scale bar = 100 µm.

[71,72]. By using NC- and NP-based spheroids co-culture fusion model, we demonstrated that DDD-mimicking environment did not impair NCS-NPS fusion kinetics and viability, suggesting promise for clinical application where faster mobilization of patients in active/functional recovery is preferred. When we compared our data with existing research on the fusion of chondrospheres during the first 24 h of fusion, no significant differences were found between NCS and articular chondrocyte spheroids [71], showing consistency with or our data.

Based on the reported results, NCS matured for 1 day might not be suitable for clinical translation due to their lower reproducibility. NCS performance in DDD-mimicking conditions revealed that NCS generated for 3-7 days (both types of media) are acceptable to enter the follow-up tests compliant with clinical setting (e.g. platelet lysate instead of FBS). Our *ex vivo* experiments provided a proof of concept that injected NCS localize in the NP, with no visible signs of leakage. Further experiments are needed to fully investigate NCS integration potential, survival, and ability to produce ECM in DDD conditions. Before proceeding towards larger size animal studies, we will determine the percentage of NCS localizing in the NP and optimize the total number of NCS to be injected. If negative long-term effects of harsh DDD microenvironment appear, NCS functionality could be further augmented, e.g. by pre-conditioning with specific anti-inflammatory or anabolic factors.

The suggested NCS-based therapy will benefit patients with mild or moderate levels of degeneration, i.e. single-level DDD, with average Pfirrmann Grade III [73]. Optimal NCS dosage (and composition) will likely depend on the stage of degeneration, determined by MRI [48]. The ultimate goal is to alleviate patient's pain (short-term pain relief) and stabilize NP function by reconstituting native tissue structures, to improve long-term quality of life [1]. The proposed mechanism for NCS-based regeneration is both structural (directly supporting NP with their own ECM-forming capacity and stiffness) and trophic (delivering signals to protect or nurture resident NP cells). Future studies will investigate the long-term survival and functionality of NCS within degenerated NP using *ex vivo* and *in vivo* models, also introducing the important variable of mechanical loading.

5. Conclusion

Currently, there is no cell-based therapy able to stop or slow down the progression of DDD. This study analyzed the potential of spheroids derived from human nasal chondrocytes for IVD repair. We provided evidence that NCS can be reproducibly generated *in vitro*, survive, and fuse in harsh DDD-mimicking conditions. Furthermore, NCS are injectable into bovine IVD via a typical spinal needle without losing their structural integrity. Due to their rapid fusion in DDD-mimicking conditions, NCS-based therapy might have the potential to stabilize NP structures with long-term efficacy. Biomechanical function as well as factors determining the survival and function of NCS in the degenerated loaded NP will be analyzed in future studies.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Anna Gryadunova: Data curation, Formal analysis, Investigation, Funding acquisition, Writing – original draft. **Jesil Kasamkattil:** Data curation, Formal analysis, Investigation, Methodology, Writing – original draft. **Max Hans Peter Gay:** Methodology, Writing – original draft. **Boris Dasen:** Methodology. **Karoliina Pelttari:** Resources, Funding acquisition, Writing – review & editing. **Vladimir Mironov:** Methodology, Resources. **Ivan Martin:** Resources, Funding acquisition, Supervision, Writing – review & editing. **Stefan Schären:** Resources, Supervision. **Andrea Barbero:** Conceptualization, Resources, Supervision, Writing – review & editing. **Olga Krupkova:** Conceptualization, Data curation, Formal analysis, Investigation, Funding acquisition, Supervision, Writing – original

draft, Writing – review & editing. **Arne Mehrkens:** Funding acquisition, Resources, Supervision, Writing – review & editing.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.actbio.2021.07.064.

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Supplementary Material

1. Medium composition

NC expansion medium: DMEM (Gibco, 10938-025) containing 10% fetal bovine serum (FBS; Gibco, 12491-015), supplemented with 1 mM sodium pyruvate (SP; Gibco, 11360-039), 10 mM HEPES (Gibco, 15630-056), 100 U/ml penicillin, 100 µg/ml streptomycin, 0.29 mg/ml L-glutamine (PSG; Gibco, 10378-016), 1 ng/ml transforming growth factor (TGF) β1 (R&D, 240-BO-10) and 5 ng/ml fibroblast growth factor (FGF2; R&D, 233-FB-025).

Growth medium: DMEM (Gibco, 10938-025) containing 10% fetal bovine serum (FBS; Gibco, 12491-015), supplemented with 1 mM sodium pyruvate (SP; Gibco, 11360-039), 10 mM HEPES (Gibco, 15630-056), 100 U/ml penicillin, 100 µg/ml streptomycin, 0.29 mg/ml L-glutamine (PSG; Gibco, 10378-016).

Chondrogenic medium: DMEM supplemented with 1 mM SP, 10 mM HEPES, PSG, 10 µg/ml Insulin-Transferrin-Selenium (ITS; Gibco, 51300-044), 5.6 mg/l linoleic acid (LA; Sigma-Aldrich, L9530-5), and 1.25 ml/l human serum albumin (HSA; SL Behring, 43075). 10 ng/ml TGFβ3 (R&D Systems, 243-B3), 1 µM dexamethasone (Sigma-Aldrich, D-2915), and 0.1 mM ascorbic acid (Sigma-Aldrich, A-8960) were added during every medium change.

NP expansion medium: DMEM/F-12 (Gibco, 21331-046) containing 10% FBS, supplemented with 1 mM SP, 10 mM HEPES, PSG (Gibco, 10378-016), and 5 ng/ml FGF2.

DDD-mimicking medium (fusion assay): DMEM, low glucose (Gibco, 11885-084) supplemented with 1mM SP, 10mM HEPES, PSG, 10 µg/ml ITS, 5.6 mg/l LA, 1.25 ml/l HSA. 0,45 mg/ml lactic acid (MP Biomedicals, ICN19022805) and 100 pg/ml of TNFα, IL1β, IL6 (All Sigma-Aldrich, SRP3177, GF331, SRP3096) were added shortly before medium change.

Control medium (fusion assay): DMEM/F-12 (Gibco, 21331-046) supplemented with 1mM SP, 10mM HEPES, PSG, 10 µg/ml ITS, 5.6 mg/l LA, 1.25 ml/l HSA.

2. Supplementary tables

Table S1. *Donor demographics and characteristics of source tissues.*

Donor	Age	Sex	Source
NC1	47	female	cadaver
NC2	53	male	septoplasty
NC3	26	male	rhinoplasty
NC4	34	male	rhinoseptoplasty
NC5	22	male	rhinoplasty
NC6	25	female	rhinoplasty
NC7	26	female	rhinoplasty
NC8	53	female	rhinoplasty
NC9	51	male	rhinoplasty
NP1	47	female	L5-S1 grade 3
NP2	27	male	L3-L4 grade 2-3

Table S2. *Primers used for gene expression analysis (all from Applied Biosystems).*

Target gene	Cat. No	Target gene	Cat. No
Aggrecan, ACAN	Hs00153936	Cyclooxygenase 2, COX-2	Hs00153133_m1
Collagen Type II, COL2A1	Hs00264051	Interleukin 6, IL6	Hs00985639_m1
Collagen Type I, COL1A1	Hs00164004	Caspase 3, CASP3	Hs00234387_m1
Matrix metallopeptidase 13, MMP13	Hs00233992_m1	Ki67	Hs01032443_m1
SOX9	Hs00165814_m1	Collagen type X, COL10A1	Hs00166657_m1
Versican	Hs00171642_m1	Thrombospondin 4, COMP	Hs01561086_g1

Table S3. *Inter-donor variability in size (diameter), shape (roundness), and biomechanical properties (elastic modulus) of NCS cultured for 7 days in growth and chondrogenic medium (n = 5 donors, mean ± SD, $p < 0.05$, 1-way ANOVA). n.s. = not significant.*

	Diameter, p value	Roundness, p value	Elastic modulus, p value
Growth medium	p<0.0001 (most comparisons)	n.s. (most comparisons)	n.s. (most comparisons)
Chondrogenic medium	p<0.0001 (most comparisons)	n.s.	n.s. (day 1, day 7)

***Table S4**. The average release of IL-8 (inflammation marker) and active MMP13 (catabolic marker) from NCS (n = 3) before and after injection through the spinal needle. NC stimulated with low-grade pro-inflammatory cocktail (100 pg/ml of TNFα, IL1β, IL6) were used as positive control (PC). udl = under the detection limit.*

Target	Growth medium		Chondrogenic medium		PC
	Injected	Non-injected	Injected	Non-injected	
IL-8	udl	udl	udl	udl	20 ng/mL
MMP13	udl	udl	udl	udl	46 ng/mL

***Table S5**. Biochemical content of NCS normalized to day 1 (n = 4-5 donors, mean ± SD, $p < 0.05$ vs. day 1, 2-way ANOVA). GAG = glycosaminoglycans, HYP = hydroxyproline.*

Parameter	Growth medium			Chondrogenic medium		
	Day 1	Day 3	Day 7	Day 1	Day 3	Day 7
DNA/NCS	1	1.4 ± 1	0.9 ± 0.7	1	0.7 ± 0.5	0.4 ± 0.1
GAG/NCS	1	1.5 ± 0.7	2.1 ± 1.2	1	1 ± 0.3	1 ± 0.4
HYP/NCS	1	0.8 ± 0.5	0.7 ± 0.5	1	1.1 ± 0.1	1.2 ± 0.1
GAG/DNA	1	1.3 ± 0.9	2.9 ± 2	1	1.9 ± 1	2.7 ± 0.9
HYP/DNA	1	0.8 ± 0.6	1.7 ± 1.8	1	1.7 ± 0.8	3 ± 0.9
GAG/HYP	1	1 ± 0.9	1.7 ± 2	1	1 ± 0.3	1 ± 0.4

3. Supplementary figures

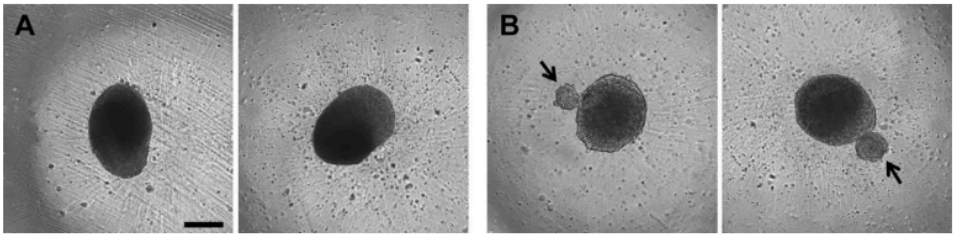

Fig. S1. *Shape irregularities including elongation* ***(A)*** *and satellites formation* ***(B)*** *demonstrated by 1.3% in NCS cultured in growth medium and 9.3% of NCS cultured in chondrogenic medium. Scale bar = 200 µm.*

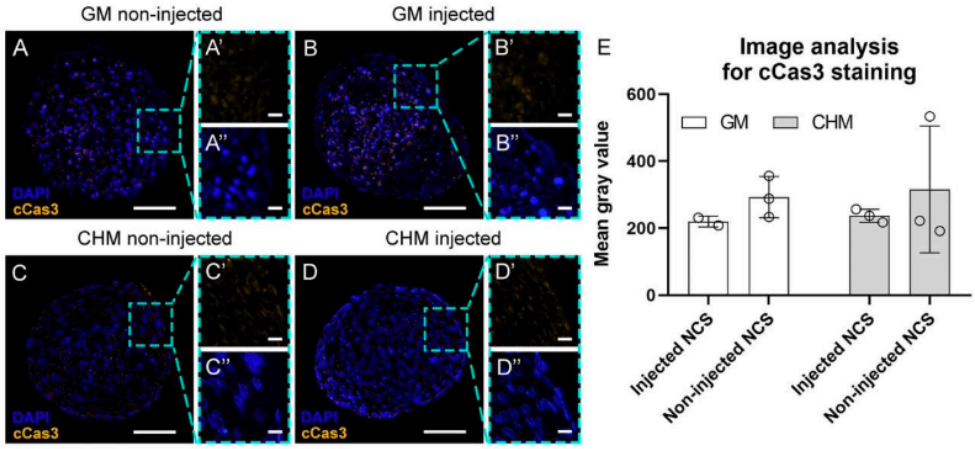

Fig S2. Cleaved caspase 3 (cCas3) staining of nasal chondrospheres (NCS) passed through a spinal needle. *cCas3 staining of NCS generated in growth medium (GM)* ***(A-A'')*** *not-injected and* ***(B-B'')*** *24 h after injection. cCas3 staining of NCS generated in chondrogenic medium (CHM)* ***(C-C'')*** *not-injected and* ***(D-D'')*** *24 h after injection. DAPI (blue) used to stain the nuclei. Scale bars = 200 µm (A-D), 4 µm (rest).* ***(G)*** *Quantification of cCas3 immunofluorescence (n = 2-3 donors, mean ± SD, 2-way ANOVA).*

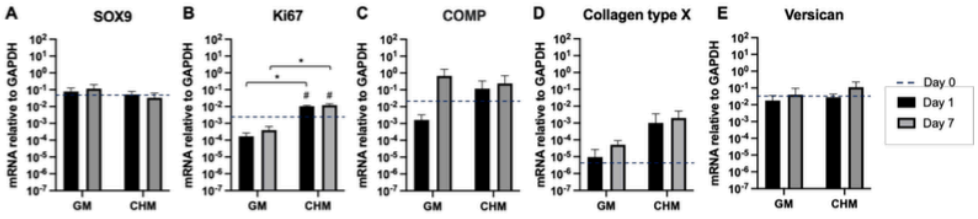

Fig. S3. Changes in gene expression of additional structural/functional cartilage components during 7-day maturation of NCS in growth and chondrogenic medium. *Relative gene expression (2^{-dCt}) of* ***(A)*** *SOX9,* ***(B)*** *Ki67,* ***(C)*** *Thrombospondin 5 (COMP),* ***(D)*** *Collagen type X, and* ***(E)*** *Versican. The expression of IL-6 was under the detection limit (n = 3-5, mean ± SD, *p < 0.05, #p < 0.05 vs. Day 0, ANOVA).*

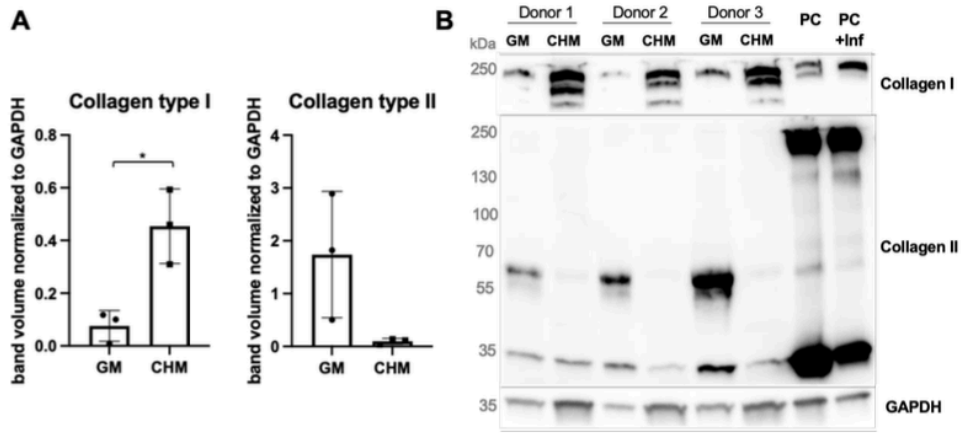

Fig. S4. Protein accumulation of collagen type I and II in NCS cultured for 7 days in growth (GM) and chondrogenic (CHM) medium. ***(A)*** *Quantification of band volume relative to loading control (GAPDH) (n = 3, mean ± SD, *p < 0.05, t-test).* ***(B)*** *Western blot (WB) image. Positive controls (PC) were standard MSC pellets cultured for 17 days in chondrogenic medium without or with low-grade inflammation for the last 10 days. WB indicated significant accumulation of collagen type I by NCS cultured in CHM and trends towards enhanced collagen type II in GM. PC showed mature collagen type II (>130 kDa, triple helix) and lower Mw band, which corresponds to the C-terminal propeptide of collagen type II. An absence of this band in NCS can be related to their immature status when fibrils are not yet well stabilized by intermolecular crosslinks.*

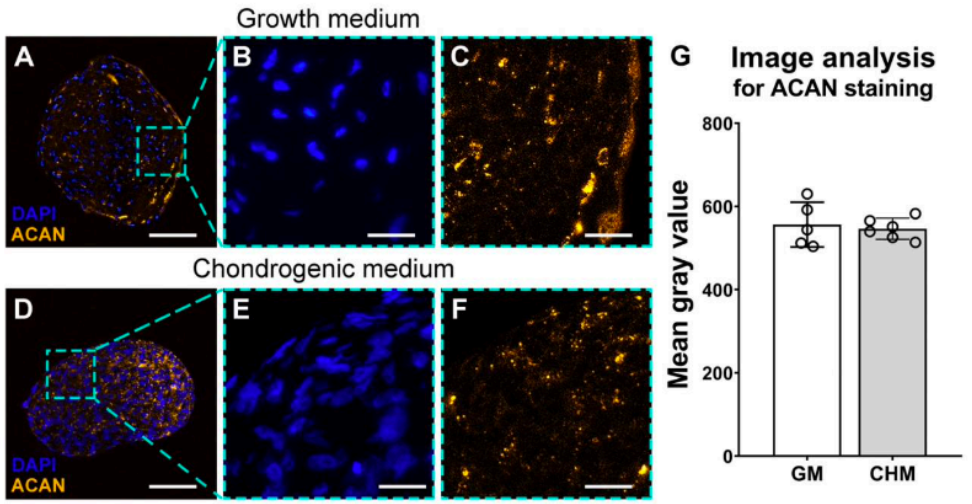

Fig. S5. Aggrecan staining of 7 day-matured nasal chondrospheres (NCS) cultured in growth and chondrogenic medium. *Safranin O staining of NCS cultured in growth medium (**(A, A')** best case, **(B, B')** worst case) and chondrogenic medium (**(E, E')** best case, **(F, F')** worst case). Immunofluorescence staining aggrecan (yellow) in NCS cultured in **(A-C)** growth (GM) and **(D-F)** chondrogenic (CHM) medium. DAPI (blue) used to stain the nuclei. Scale bars = 200 µm (A, D), 4 µm (B, C, E, F'). **(G)** Quantification of aggrecan (ACAN) immunofluorescence (n = 2-3 donors, mean ± SD, 2-way ANOVA).*

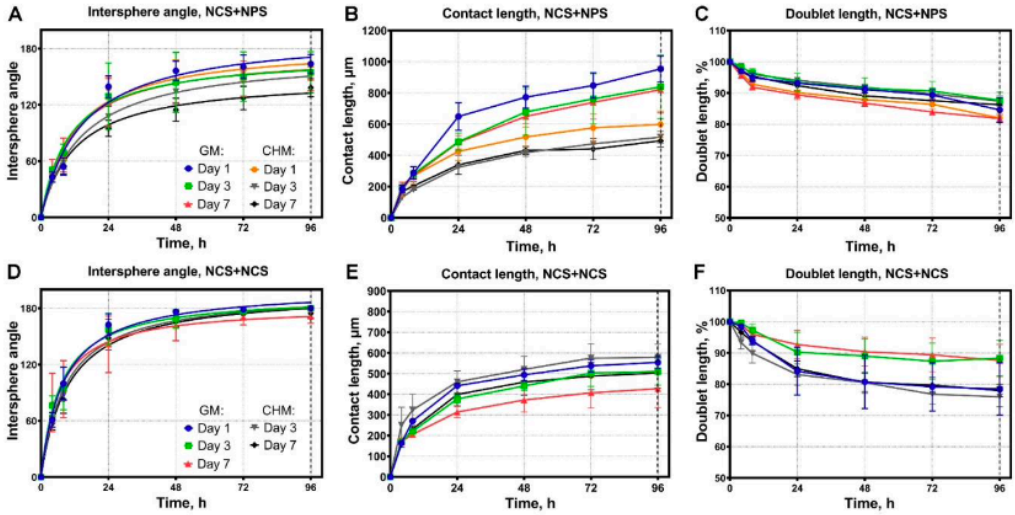

Fig. S6. Fusion kinetics of nasal chondrospheres generated in growth and chondrogenic medium, in DDD-mimicking conditions. (A) *Fusion of NCS with nucleus pulposus spheroids (**A-C**: NCS+NPS) and with each other (**D-F**: NCS+NCS) (n = 3 donors for NCS and 2 donors for NPS, mean ± SD, $p < 0.05$, 2-way ANOVA). GM = growth medium, CHM = chondrogenic medium.*

7. Chapter 4: Human 3D nucleus pulposus micro-tissue model to evaluate the potential of pre-conditioned nasal chondrocytes for the repair of degenerated intervertebral disc”

Results are being prepared for publication in Frontiers in Bioengineering and Biotechnology – 6.1

J. Kasamkattil, Gryadunova, A., M. H. P. Gay, B. Dasen, H. Hilpert, R. Schmid, K. Pelttari, I. Martin, S. Scharen, A. Barbero, O. Krupkova, and A. Mehrkens. Potential title: "Human 3D nucleus pulposus micro-tissue model to evaluate the potential of pre-conditioned nasal chondrocytes for the repair of degenerated intervertebral disc" Envisioned journal for submission: *Frontiers in Bioengineering and Biotechnology*.

Contribution: Data curation, Formal analysis, Investigation, Methodology, Writing – original draft.

Human 3D nucleus pulposus micro-tissue model to evaluate the potential of pre-conditioned nasal chondrocytes for the repair of degenerated intervertebral disc

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1. Abstract

Introduction: An *in vitro* model that appropriately recapitulates the degenerative disc disease (DDD) microenvironment is needed to explore clinically relevant cell-based therapeutic strategies for early-stage DDD. We developed an advanced human 3D nucleus pulposus (NP) micro-tissue (µT) model that includes hypoxia, low glucose, acidity, and low-grade inflammation as well as human NP cells isolated from degenerating NP tissue (Pfirrmann grade: 2-3), to simulate early-stage DDD. We then used this model to test the performance of nasal chondrocytes (NC) suspension or spheroids (NCS) after pre-conditioning with drugs known to exert anti-inflammatory/degrading or pro-anabolic acitivities.

Methods: NPµT model was formed by pooling only spheroids generated with NP cells (NPS) or combining NPS with NC suspension or NCS and were cultured in healthy or DDD condition. Anti-inflammatory and anabolic drugs (amiloride, celecoxib, metformin, IL-1Ra, GDF-5) were used for pre-conditioning of NC/NCS. The effects of pre-conditioning were tested in 2D, 3D, and degenerative NPµT model. Histological, biochemical, and gene expression analysis were performed to assess matrix (glycosaminoglycans, collagen type I&II) content, inflammation/catabolism (IL-6, IL-8, MMP-3, MMP-13) and cell viability (cleaved caspase 3).

Results: The degenerative NPµT contained less glycosaminoglycans, collagens, and released higher levels of IL-8 compared to the healthy NPµT. In the degenerative NPµT, NCS performed superior compared to NC cell suspension but still showed reduced viability. Among the different compounds tested, only IL-1Ra pre-conditioning inhibited the expression of inflammatory/catabolic mediators and promoted proteoglycan accumulation in NC/NCS in DDD microenvironment. In degenerative NPµT model, preconditioning of NCS with IL-1Ra also provided superior anti-inflammatory/catabolic activity compared to non-preconditioned NCS.

Conclusion: In conclusion, degenerative NPµT model allows to study the responses of therapeutic cells to microenvironment mimicking early-stage DDD. We demonstrated that spheroidal organisation of NC is better suited for early-stage IVD repair than NC cell suspension and that IL-1Ra pre-conditioning of NCS could further improve their ability to counteract inflammation/catabolism and support proteoglycan accumulation within harsh DDD microenvironment. Furter *ex vivo* and *in vivo* studies in animal will be required to validate the therapeutic effects of IL-1Ra pre-conditioned NCS in a DDD in vivo environment.

2. Introduction

Low back pain is experienced by 80% of the world population at least once in their life and it is one of the costliest diseases for the healthcare system [1]. 40% of the chronic LBP cases are due to degeneration of the intervertebral disc (IVD) [2, 3]. During IVD degeneration, the extracellular matrix (ECM) of the nucleus pulposus (NP) tissue, especially the proteoglycans (PG), is degraded due to the imbalance of catabolic and anabolic activities [4, 5] and the cell density in the IVD decreases over time [6]. The gradual onset of IVD degeneration is considered as a part of the natural course of ageing [7]. However, with progressing degeneration, the NP tissue could become inflamed and/or herniate through annulus fibrosus (AF) and press against the nerve roots causing pain [8]. The association of IVD degeneration with inflammation and pain is referred to as degenerative disc disease (DDD). Surgical strategies to treat DDD often do not improve patient's quality of life, as they could accelerate degeneration of adjacent IVDs [9, 10]. In order to prevent surgery, minimally invasive biological therapies should be developed and applied at relatively early stage [1]. Ideally, these will restore the structure and function of the mildly affected NP (grade 2-3), allowing the IVD to hydrate and regain the hight, as well as reduce the catabolic shift [11]. However, despite the advancement in biological IVD repair [11], no therapy has been widely adopted clinically yet [12-14].

Numerous *in vitro*, *ex vivo*, and *in vivo* studies have investigated the effects of anti-catabolic or anti-inflammatory factors on IVD repair [15-17]. The aim was to either directly suppress the expression of catabolic enzymes (i.e., MMPs and ADAMTS) [18, 19] or by downregulating pro-inflammatory mediators (i.e., TNF-α, IL-1) [20, 21]. Although these anti-catabolic and anti-inflammatory therapeutic strategies are promising, they are not sufficient enough to regenerate the IVD function since the resident cells often fail to restore their ability to synthesize ECM [1]. Pro-anabolic strategies using growth factors (i.e., TGFβ or GDF-5) have been also investigated to induce anabolic activities within the NP tissue with encouraging *in vitro*, *ex vivo* and *in vivo* results [22-26]. As an example, clinical studies evaluated the safety, tolerability and efficacy of GDF-5 injection into degenerating IVD, with no major adverse events directly related to GDF-5 injection as well as moderate improvement of pain and disability (https://clinicaltrials.gov; NCT01158924, NCT00813813, NCT01182337, and NCT01124006). However, these pro-anabolic approaches are hampered by the limited amounts of healthy/metabolically active cells in the degenerated NP. Therefore, a single intradiscal injection of biological factors with anti-catabolic, anti-inflammatory and pro-anabolic effects combined with healthy therapeutic cells, which survive and produce ECM within the DDD microenvironment, could be a better approach to repair the NP tissue. Nevertheless, translation of cell-based approaches for IVD repair still faces several critical challenges, mainly related to (i) selection of a therapeutic cell source with good performance within the DDD microenvironment and (ii) a lack of an *in vitro* model that appropriately mimics course of the disease and at the same time allows for clinically relevant incorporation of therapeutic cells [27-29].

To repopulate the NP tissue with cells, endogenous stem/progenitor cell recruitment by injecting chemokine ligands (i.e., CCL5 or CXCL12) [30-32] or exogenous cell injection strategies have been explored over the last two decades [1]. For exogenous cell injection, differentiated cell sources (NP, AF, notochordal cells, articular chondrocytes (AC)) as well as stem/stromal cells (derived e.g. from bone marrow or adipose tissue) in combination with or without scaffolds were investigated [33, 34]. However, for both endogenous and exogenous cell supplementation, healthy cells are either not available in sufficient number, donor site morbidity arises, and/or cell survival within the harsh DDD microenvironment is limited [7, 35-38]. These limitations can be overcome by using nasal chondrocytes (NC), isolated from autologous nasal cartilage with minimal donor site morbidity [39-41]. NC showed superior viability (over AC and mesenchymal stromal cells (MSCs)) in simulated DDD microenvironment, thus represent a robust cell population with a likelihood of survival post injection [42]. We have demonstrated that spheroids formed with NC (hereafter referred to as nasal chondrocyte spheroids, NCS) generate own matrix, and survive and fuse with NP microtissues in DDD microenvironment [43]. Notably, NCS are injectable into the IVD using a spinal needle, without losing their structural integrity [43]. Therefore, NCS represent promising alternative for a single-injection-based IVD repair strategy.

The first step towards developing a functional cell-based strategy for IVD repair includes testing in *in vitro* models. At this stage, general proof of principle, intercellular communications, cell functions, and cell behavior are investigated. For the appropriate design of the *in vitro* models, the selection of ideal cell source, culture system and culture condition are of key importance. The right choice of species

from which resident/therapeutic cells are being isolated for *in vitro* culture has to be considered because it is known that species-specific responses could lead to different outcomes [44, 45].

Interaction between IVD and therapeutic cells is commonly investigated in 2D and 3D co-culture models, tissue explants as well as organ culture models [29]. 3D culture models (i.e. insert system, alginate bead system, pellet system) restore the IVD cell phenotype and allow to reproduce *in vivo* spatial distribution of the IVD cells, which makes them more physiologically relevant and predictive than 2D monolayer cultures [46]. Different 3D *in vitro* co-culture models have been used to study the interaction between therapeutic and IVD cells [47-49]. As an example, direct co-culture of human bone marrow stromal cells (BMSCs) with bovine NP cells (ratio: 1:1) encapsulated in 3D alginate beads revealed that hypoxia and co-culture could lead to BMSCs differentiation into NP-like phenotype [50]. In order to overcome several disadvantages of the alginate bead system, such as lack of reproducibility and uniformity of quality and size of the microspheres [51], the 3D pellet culture system could be used. In this model, it has been demonstrated that co-culture with NP cells could modulate the gene expression of therapeutic MSCs towards chondrogenesis and matrix deposition [52, 53]. Furthermore, in a serum free pellet model containing high concentration of TGFβ the co-culture of synovium-derived MSCs with NP cells (ratio: 1:1) leads to similar differentiation state and matrix production as NP pellets alone [54]. The pellet model could be considered superior for chondrogenic induction (in regard to COL2B gene expression and matrix accumulation) compared to alginate culture model, with one disadvantage, namely very large number of cells are needed for the generation of the pellets [55]. However, even the direct -coculture pellet system is not simulating the *in vivo* situation properly since therapeutic cells are not supposed to differentiate with differentiating NP cells but should rather be introduced to an already differentiated NPμT. Tissue explants and organ culture models are excellent to test local tissue responses, integration and delivery of therapeutic cells into IVD tissue and also in regard to biological and cellular functions [56-59]. However, due to the handling and complexity of *in vitro* tissue/organ culture, they are not well suited for fundamental cellular mechanistic studies.

Selecting the ideal culture condition to mimic the DDD microenvironment is essential to study the potential of therapeutic cell for IVD regeneration. The harsh NP microenvironment is characterized by avascularity, hypoxia, low glucose level, acidity, inflammation, high osmolality and restricted biomechanics [60]. Several studies have assessed the performance of the therapeutic cells within *in vitro* models simulating some of the parameters present in the DDD microenvironment [38]. Nevertheless, it has been shown that less than 15% of the *in vitro* studies include either one of these parameters thus not mimic the harsh IVD microenvironment properly [29].

Since available 3D models are still not satisfactory to study the responses of therapeutic cells to the harsh NP microenvironment, we aim to develop a convenient yet sufficiently complex 3D NP micro-tissue (μT) model. Within NPμT model we investigate the responses of therapeutic cell suspensions as well as cell spheroids (NC vs. NCS) to the DDD microenvironment. We test also the efficacy of different clinically relevant compounds to improve NC function exposed to DDD microenvironment. We demonstrate that the model is suitable to test pre-conditioning strategies that enhance the NP repair potential of therapeutic cells.

3. Material and Methods

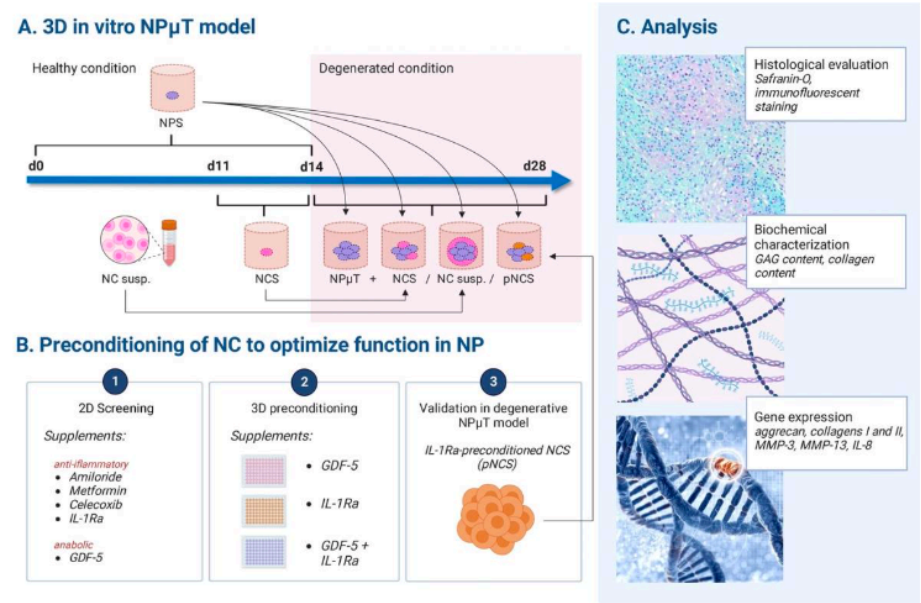

Fig. 1: Experimental design. *(A) Single spheroids were generated by culturing NC (NCS) and NP cells (NPS) for three days or two weeks repectively, in healthy condition. Afterwards, NPS were pooled with NCS/NC to form NPµT (+ NCS / NC susp). (B) NC were preconditioned with anti-inflammatory or pro-anabolic compounds in (B1) 2D culture and (B2) 3D culture. (B3) IL-1Ra-preconditioned NCS were implemented in the NPµT model. (C) Histological, biochemical and gene expression analysis were performed.*

3.1 Cells and cell sources

3.1.1 Tissue harvest and cell isolation

Tissues were collected following local ethical committee approval (EKNZ-2015305, University Hospital Basel). Following informed consent from all donors, human nasal septal cartilage tissue was harvested from the patients undergoing rhinoplasty (total n=6, Suppl. Table S1). NP tissue was acquired from donors undergoing surgery for DDD. Harvested NP tissues were graded using Pfirrmann scale [61]. NP tissues with Pfirrmann grade 2-3 (mild/moderate degeneration) were used in this study (total n=6, Suppl. Table S2). NC and NP cells were isolated after digestion in collagenase type II (0.15% NC; 0.05% NP cells for 22h), and expanded in NC and NP expansion medium respectively (composition in Suppl. Material) up to passage 3.

3.1.2 Lentiviral transduction of nasal chondrocytes

In order to distinguish NC from NP cells, NC were labelled with mEmerald fluorescent protein using lentiviral transduction. Lentiviral expression vector was generated by subcloning mEmerald coding sequence by PCR from pmEmerald-LifeAct-7 vector (Addgene, 54148) into pLVX lentiviral vector (Clontech, USA). For lentivirus production, Lenti-X 293T cells (Clontech, USA) were transfected with lentiviral expression vector and 3rd generation packaging plasmids prMDLg/pREE, pRSV-Rec, and pMD2.G (Addgene, 12251, 12253, and 12259, respectively) using Lipofectamine2000 (Lifetech, 11668019). After 72 hours, the supernatant containing lentiviral particles was collected, and lentiviral titer was assessed by ELISA using Quick Titer Lentivirus titer kit (Cell Biolabs, VPK-1070). Lentiviral transduction of NC was performed according to previously established protocol, reported affecting neither proliferation nor the differentiation capacity of chondrocytes [62]. Briefly, NC were seeded in 6-

well plates as $2.5x10^6$ cells/well and transduced with mEmerald-lentivirus at MOI 5 in the presence of 8 µg/ml polybrene, which yields ≥95% transduction efficiency (TEf). TEf was monitored by assessing the percentage of green-positive cells by flow cytometry (Aria III, BD) 3 days post-transduction.

3.2 *Drug screening in 2D culture of nasal chondrocytes*

NC (n=3) were cultured in 6-well plates (0.1M cells/well) for 24h in NC expansion medium (composition in Suppl. Material) and pre-treated with FDA-approved drugs interleukin 1 receptor antagonist (IL-1Ra), growth and differentiation factor 5 (GDF-5), Amiloride, Metformin, and Celecoxib at different concentrations (Table 1) for the last 3h (21h+3h). Afterwards, the drugs were removed and DDD mimicking condition (composition in Suppl. Material) was supplemented to the cells for further 24h. Then, the cells were harvested and analyzed by reverse transcription quantitative PCR (RTqPCR) (chapter 4.3.1).

***Table 1**: FDA-approved drugs screened in nasal chondrocytes*

Drug	*Desired Effect*	*Concentrations*
Amiloride	Inhibitor of acid sensing ion channel 1/3 [63] which could prevent acid-induced decrease in cell proliferation and ECM gene expression [64].	10, 100µM
Metformin	Inducer of inflammation resistant phenotype [65] and known for its chondroprotective properties [66].	10, 100, 1000µM
Celecoxib	Intradiscal delivery of celecoxib-loaded microspheres restored IVD integrity in preclinical canine model [67].	10, 100, 1000µM
IL-1Ra	Inflammation inhibitor [68] with matrix protective properties in intact human degenerate IVD explants [21].	10, 100, 500ng/mL
GDF-5	Inducing NP-specific ECM forming effects [69] and driving differentiation of therapeutic cells towards NP-like cells [70].	1, 10, 100ng/mL

3.3 *Generation of spheroids and nucleus pulposus micro-tissue*

3.3.1 Fabrication of nucleus pulposus and nasal chondrocytes spheroids

Nucleus pulposus spheroids (NPS) formation: 25'000 NP cells/well were seeded in 2% PolyHEMA (Sigma, P3932) coated 96-well plates and the formed spheroids were cultured for 14 days in NP differentiation medium (composition in Suppl. Material) in Thermo Scientific™ Heracell™ 150i CO_2 incubator (37°C; 5% CO_2, 20% O_2). Media was changed twice a week. Nasal chondrocyte spheroids (NCS) formation: 12'500 NC cells/well were seeded in 2% PolyHEMA (Sigma, P3932) coated 96-well plates and the formed spheroids were cultured for 3 days in NC differentiation medium (composition in Suppl. Material) in Thermo Scientific™ Heracell™ 150i CO_2 incubator (37°C; 5% CO_2, 20% O_2) without medium change.

3.3.2 Preconditioning of nasal chondrocyte spheroids

NCS were formed in 96 well-plates in NC differentiation medium (composition in Suppl. Material) for 3 days [43] with/without GDF-5 (100ng/mL), IL-1Ra (500ng/mL) or the combination of both drugs. Then, the drugs were removed. Preconditioned NCS were either placed directly in DDD-mimicking condition (composition in Suppl. Material) for further 7 days and analyzed (chapter 4.3.2), or introduced into the NP micro-tissue (NPµT) model (see 4.3.3).

3.3.3 Nucleus pulposus micro-tissue model

NPµT was cultured either alone or in combination with NCS or NC cell suspension. 16 NPS were pooled in a polypropylene conical tube to form the NPµT. 8 NPS and 16 NCS were pooled to form the NP µT + NCS. 8 NPS and 0.2M NC cells were pooled to form the NPµT + NC cell suspension. The total number of the cells in each formed micro-tissue was 0.4M. The aggregates were cultured for 14 days in 0.5 mL of either healthy (normoxia, high glucose, NHG) or DDD-mimicking medium (composition in Suppl. Material). The medium was changed twice per week.

3.4 Histologican, biochemical and molecular characterisation

3.4.1 Gene expression analysis

ECM genes (aggrecan, collagen type II) are downregulated in IVD degenerative condition [71] and inflammatory/catabolic genes (IL-6, IL-8, MMP3, MMP13) are upregulated [71-74] thus these targets were analysed, to verify whether pre-conditioning of NC (either in monolayer or 3D spheroidal organisation) could modulate ECM degradation and inflammation. Total RNA from 0.2M cells was extracted using the RNeasy Mini Kit (Quiagen, 74106), according to the manufacturer's protocol. The RNA yield and purity were measured on a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, USA). SuperScriptTM III Reverse Transcriptase kit (Invitrogen, 18080093) was used to reverse-transcribe 0.5 µg of RNA into cDNA in a 30 µL volume. 10 ng of cDNA/well was mixed with TaqManTM Universal PCR Master Mix (Applied Biosystems, 4304437), RNase-free water, and TaqMan primers (ACAN: Hs00153936; COL2A1: Hs00264051; COL1A1: Hs00164004; MMP3: Hs00968305_m1; MMP13: Hs00233992_m1; IL-8: Hs00174103_m1; IL-6: Hs00985639_m1) in a total volume of 10 µL and used for quantitative real-time polymerase chain reaction (RTqPCR) performed on a 7300 Real-time PCR System (Applied Biosystems, USA). For each sample, Ct values of the target were subtracted from the Ct values of a housekeeping gene (human GAPDH, Hs02758991, Applied Biosystems) to derive the ΔCt. Gene expression was quantified relative GAPDH ($2^{-\Delta Ct}$) and relative to control ($2^{-\Delta\Delta Ct}$).

3.4.2 Biochemical content quantification

NPµT (+NCS / NC suspension) were digested for 16 h at 56 °C in 1 mg/mL proteinase K solution [1 mg/ml proteinase K (Sigma-Aldrich, P2308) in 50 mM Tris (Sigma-Aldrich, A5456-3) with 1 mM EDTA (Fluka, 03680), 1 mM iodoacetamide (Sigma-Aldrich, I-1149) and 10 mg/mL pepstatin A (Sigma-Aldrich, P5318)]. Glycosaminoglycan (GAG) content was determined spectrophotometrically using Blyscan GAG Assay (Biocolor, B1000). DNA content was measured using the CyQuant Cell Proliferation Assay Kit (Invitrogen, C7026), with bacteriophage λ DNA as a standard, according to the manufacturer's protocol. Total collagen content was quantified using the Hydroxyproline (HYP) Assay Kit (Sigma-Aldrich, MAK008) according to manufacturer's protocol. Both GAG and HYP contents were normalized either to single NCS or to DNA content.

3.4.3 Enzyme-Linked Immunosorbent Assay (ELISA) and Luminex

The amounts of MMP13, IL-8, and IL-1Ra in the cell culture media was quantified using ELISA. MMP13 and IL-8 are known to be released in IVD tissues experiencing catabolic shift [75, 76]. SensoLyte Plus™ 520 MMP13 Assay Kit (Catalog #: 72019) was used for fluorometric detection of total MMP13, performed according to the manufacturer's protocol. Human IL-8 ELISA Set (555244, BD) with ELISA reagent set B (550534, BD) were used to detect IL-8 according to the manufacturer's instructions. IL-1Ra was quantified using the Human Luminex Discovery Assay (Magnetic Luminex Assay 2 Plex, biotechne, LXSAHM-02) according to the manifacturer's instructions.

3.4.4 Histology and immunohistochemistry

Samples were fixed in 4 % paraformaldehyde (01-1000, formafix), washed in PBS, embedded in Richard-Allan Scientific HistoGel™ (HG-4000-012, ThermoFisher) and processed using Tissue Processing Center TPC 15 Duo (Medite, Germany). 4 µm-thick sections were cut (Microm HM 430 or Microm HM 340E) and collected on poly-L-lysine coated glass slides (J2800AMNZ, Fishersci). After dehydration, safranin-O/fast green (SafO/FG; SafO: 84120, Sigma; FG: F-7252, Sigma) stain with hematoxylin (J.T. Baker, MFCD00078111) nuclear counterstaining was performed to visualize the PG within the sections. Widefield microscopy (Nikon Ti2, Japan; acquisition software: Nikon NIS; Camera: Nikon DS-Ri2; Objective: 20x or 40x; NA: 0.95) was applied for imaging. Images were processed using Fiji/ImageJ software (NIH, Bethesda, MD). For immunohistochemistry, sections were subjected to enzymatic epitope retrieval and blocked with 1 % BSA (A9647, Sigma) supplemented with triton X-100 (1:1000, 93418, Sigma), followed by application of primary antibodies anti-cleaved caspase 3 (1:300, polyclonal, 9661, Cell Signalling) and anti-GFP (1:1500, GFP-1020, Aves). Respective matching secondary antibodies Alexa Fluor 647- or 488-conjugated (1:500, polyclonal, A21245, Invitrogen and 103-605-155, Jackson) were used, with DAPI as a nuclear counterstain. Widefield fluorescence microscopy (Nikon Ti2, Japan; acquisition software: Nikon NIS; Camera: Photometrics Prime 95B;

Objective: 20 or 40x; NA: 0.95) was applied for imaging. Images were processed using Fiji/ImageJ software (NIH, Bethesda, MD).

3.4.5 Image quantification

Multiplexed fluorescence images from tissue sections were analyzed with QuPath version 0.3.0, an open source software for whole-slide images [77] Immunopositive areas containing NCS were used as regions of interest (ROIs). StarDist extension, a deep-learning-based model for nuclei detection, was applied to the DAPI channel of fluorescent images to calculate the amount of cells in ROI. For each image, the mean intensity value representing cCas9 was obtained. The mean fluorescence intensity was normalized to either number of cell detections or the area. The percentage of cCas9-positive areas was assesed by adjusting software built-in pixel classifier. In total, from 6 to 29 images were analyzed for every experimental group.

3.4.6 Cell viability assay

The cell viability of NCS on day 0, 3, and 7 after culturing in DDD mimicking conditions was assessed using the CellTiter-Glo Luminescent Cell Viability Assay (Progmega, G7570) according to the manufacturer's protocol. Briefly, the microplate with NCS was left at room temperature (RT) for 30 minutes prior to examination. Four empty wells were filled with 100µl of the corresponding medium to obtain a value of background control. CellTiter-Flo Reagent and medium (ratio 1:1) was added to each well and mixed to induce cell lysis. Afterwards, the plate was incubation for one hour at RT to stabilize the luminescent signal. Luminescence was recorded using SPARK Multimode-Microplate Reader (Tecan, Switzerland). Luminescence signal, proportional to cellular ATP generation, was expressed in relative light units (RLU) and normalized to control (DDD ctr) (chapter 4.3.2)

3.5 Statistical analysis

All data were analyzed using GraphPad Prism software ver. 8.0.1 (GraphPad Software, Inc., La Jolla, Ca) and reported as mean ± SD. The following tests were used to assess the statistical significance: for normally distributed data, analysis of variance (ANOVA) followed by Tukey's post-hoc test (column analysis) or Sidak's post-hoc test (group analysis); for non-normally distributed data, Kruskal-Wallis test with Dunn's post-hoc test (column analysis) or the Mixed-effects model with Sidak's post-hoc test (group analysis); for data obtained from semi-quantitave image analysis, Mann-Whitney U test was performed. Numerical values of probability smaller than 0.05 were considered as statistically significant.

4. Results

4.1 Development of an 3D in vitro degenerative nucleus pulposus micro-tissue model

To recapitulate the conditions during early stage IVD degeneration such as pro-inflammatory/catabolic shift, onset of ECM degradation, and mild apoptosis [60], NPuT model was designed as follows. Spheroids consisting of NP cells (NPS) were formed for two weeks in healthy (NHG) condition consisting of Normoxia (20% O_2) and High Glucose level (4.5mg/ml) and then pooled to form the NPµT for further 14 days in either NHG (control group) or DDD (early stage IVD degeneration group) condition consisting of hypoxia (2% O_2), Low Glucose (1mg/ml), acidity (pH 6.8), as well as in the presence of inflammation (100pg/ml TNF α, IL-1β, IL-6) (Fig. 2A). 2-stage NPµT formation allows for generation of larger microtissues with low risk of necrotic core formation. This configuration also permits straightforward incorporation of therapeutic cells into 3D NP microenvironment with cell-produced ECM and factors playing a key role in NP degeneration such as low nutrition, acidity, hypoxia and pro-inflammatory cytokines. Histological and quantitative analysis revealed that NPµT formed in DDD microenvironment accumulated significantly less GAG and collagens compared to healthy NPµT (Fig. 2B&D). NP cells also experienced catabolic shift, which confirmed the presence of the harsh DDD micromilieu (Fig. 2C). Even though apoptotic cells were detected in subsections of the NPµT cultured in DDD microenvironment (Fig. 2D'''), semi-quantification of the cleaved caspase 3 staining on the whole section revealed no significant upregulation of apoptotic cell profile in NPµT cultured in DDD compared to control (Fig. 2E).

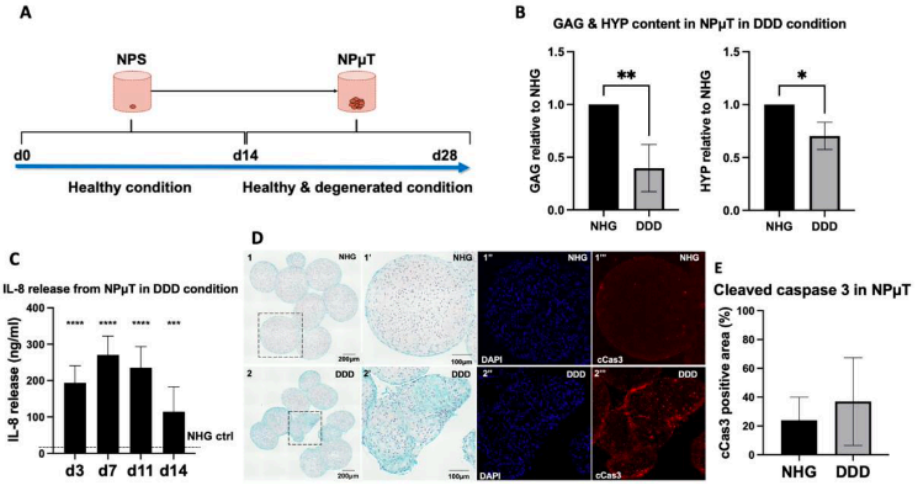

Fig. 2: Development of 3D degenerative NP micro-tissue (µT) model. (A) *NPµT was formed for 14d in NHG or DDD condition using NP spheroids (NPS) pre-cultured in healthy (NHG) condition for 14d. After 14d in NHG or DDD condition, NPµT was analyzed* ***(B)*** *biochemically by quantifying glycosaminoglycans (GAG) and collagens (HYP) (n=6, mean ± SD, *p < 0.05, ANOVA).* ***(C)*** *Catabolic shift in degenerative NPµT was assessed by measuring IL-8 release in culture medium on day 3, 7, 11 and 14. Dashed line represents IL-8 release in NHG control (no IL-8 detected) (n=5, mean ± SD, *p<0.05 vs. NHG ctrl., ANOVA).* ***(D)*** *NPµT formed in* ***(D1)*** *NHG or* ***(D2)*** *DDD condition was stained with Safranin O* ***((D1'')*** *zoomed D1 showing healthy cells producing proteoglycans,* ***(D2'')*** *zoomed D2 showing cells with moderate proteoglycan production) and immunofluorescence* ***((D1''-2'')*** *DAPI and* ***(D1'''-D2''')*** *cleaved caspase 3 (cCas3) visualising nuclei (in blue) and apoptotic cells (in red), respectively) (n =6).* ***(E)*** *quantification of cCas3 staining in NPµT (n =6, mean ± SD, *p < 0.05, ANOVA).*

4.2 *Assessing the performance of nasal chondrocytes in NPμT model*

The NPμT model was designed to study the long-term effects of DDD microenvironment on potential therapeutic cells. As promising cell type for NP repair, NC were implemented in the model [42, 43, 78]. To evaluate possible differences in responses to DDD microenvironment, NC were incorporated in the model either as cell suspension (NPμT+NC cell suspension) or spheroids (NPμT+NCS). To generate the model, NPS were pooled with NCS or NC suspension and cultured for two weeks either in healthy (NHG) control condition, consisting of Normoxia (20% O_2) and High Glucose level (4.5mg/ml), or degenerative (DDD) condition (Fig. 3A). Accumulation of ECM components within degenerative NPμT co-cultures was compared to NHG control and between NCS and NC suspension groups (Fig.3B). GAG and collagen content in NPμT+NCS group did not significantly differ from healthy control, while GAG and collagen in NPμT+NC suspension group was significantly reduced. No significant differences in GAG and collagen between NPμT+NCS and NPμT+NC cell suspension were detected, although trends towards higher ECM content in NPμT+NCS were observed. Addition of NCS to degenerative NPμT tended to increase GAG content, compared to degenerative NPμT only. Catabolic shift was measured by the release of IL-8, typical for DDD [75]. On day 3 a trend towards reduced release of IL-8 by the NPμT+NCS (9+/-5 ng/mL) and NC cell suspension (29+/-17 ng/mL) was detected compared to NPμT (194+/-47 ng/mL) (Fig. 3C). On day 7 the IL-8 released by NPμT+NCS was significantly lower (280+/-117 ng/mL) compared to NPμT+NC suspension (816+/-488 ng/mL) but no difference could be stated on day 14. In NHG control, no IL-8 release was detected at any time point. As the incorporation of NCS into degenerative NPμT tended to increase GAG content, further (immuno)histological analysis of NPμT+NCS group was performed. The staining indicated that NCS within the DDD microenvironment could accumulate proteoglycans (Fig. 3D) but not consistently (Fig. 3D2). GFP-transduction of NCS revealed that apparently non-functional NCS are apoptotic (cCas3-positive), as a trend of higher intensity of cCas3 positive area was observed in the region with less proteoglycan accumulation (Fig. 3D'''&Fig. 3E).

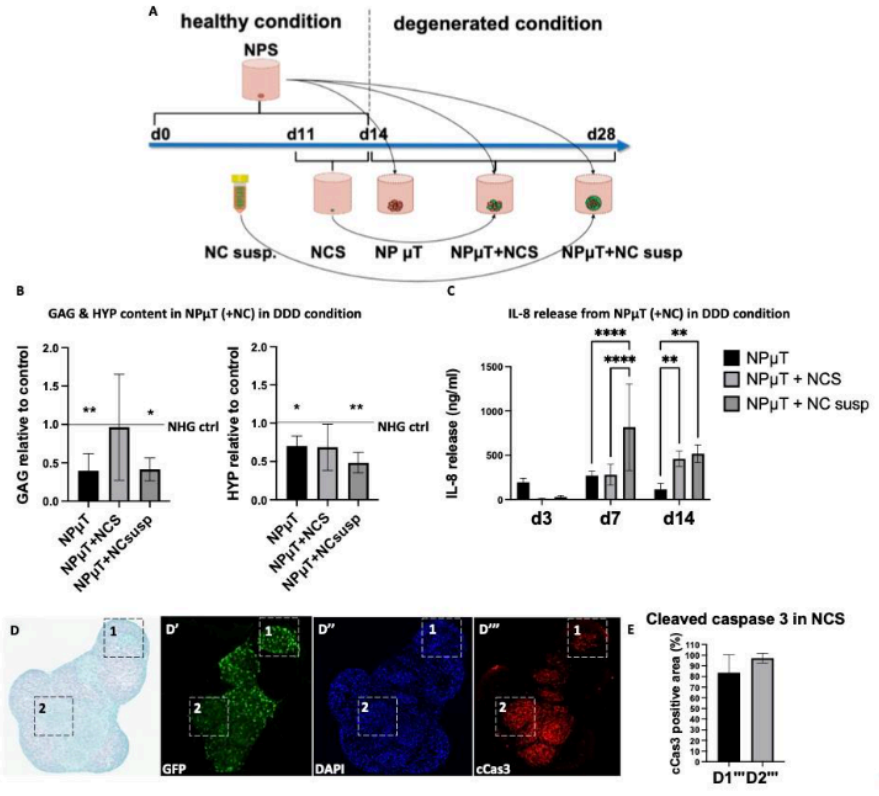

Fig. 3: Assessing the responses of NCs in 3D in vitro degenerative NPμT model. (A) *NP spheroids (NPS) were formed for 2 weeks and NC spheroids (NCS) were formed for 3 days in healthy condition. After pooling NPS with either NCS or NC cell suspension (NC susp.), the aggregates were cultured for two weeks in either healthy or degenerated condition. Afterwards,* ***(B)*** *Glycosaminoglycans (GAG) and hydroxyproline (HYP) were quantified. Dashed lines represent value of healthy control. Asteriks indicate significance compared to healthy control (NP: n=6, NC: n=3, mean ± SD, *p<0.05 vs. NHG ctrl., ANOVA).* ***(C)*** *Catabolic shift was assessed by measuring IL-8 release on day 3, 7, and 14. No IL-8 release was detected in healthy condition (NP: n = 6, NC: n=3, mean ± SD, *p < 0.05, ANOVA).* ***(D)*** *Safranin O staining of NPμT containing NCS accumulating proteoglycans (D1) and (D2) less/no proteoglycans. Immunofluorescence staining visualising (D') GFP transduced NC, (D'') nuclei (DAPI), and (D''') apoptotic cells (cleaved caspase 3 (cCas3)). Black and white squares depict zoomed-in subsections of (D1''') less apoptotic and (D2''') more apoptotic NCS (NP: n = 1, NC: n=3).* ***(E)*** *semi-quantification of cCas3 staining in D1''' and D2''' (NP: n=1, NC: n=3, mean ± SD, *p<0.05, Mann-Whitney U test)*

The 3D *in vitro* degenerative NPμT model was developed and validated using promising therapeutic cell type (NC). NC could be distinguished from NP cells within the model, allowing to explore the responses of both cell types to DDD microenvironment as well as the fate of the therapeutic NC. Furthermore, the model could be used to assess two cell configurations (cell suspension and spheroids). Our data suggested that even if a large amount of NCS in the degenerated NPμT aquired apopotic traits, their overall performance in the DDD microenvironment was superior thatn the one of NC suspension. To optimize NCS function within the DDD microenvironment, the drugs should equip NC with anti-inflammatory and anti-catabolic resistance and/or enhance their anabolic activity.

4.3 Preconditioning of nasal chondrocytes to optimize their function in the nucleus pulposus

Cell preconditioning using inflammatory cytokines or mediators, hypoxia, pharmacological drugs and chemical agents have been investigated to improve IVD cell function, survival, and therapeutic efficacy [79]. In order to facilitate clinical translation, we selected pre-conditioning of therapeutic NC using FDA approved drugs with anti-inflammatory, anti-catabolic, and/or anabolic activities, namely GDF-5, IL-1Ra, metformin, amiloride, and celecoxib. GDF-5 injection was shown to increase ECM accumulation in the IVD in clinical settings (https://clinicaltrials.gov; NCT01158924, NCT00813813, NCT01182337, and NCT01124006) and IL-1Ra was reported to reduce anti-inflammatory and anti-catabolic factor release of NP cells *in vitro, ex vivo,* and *in vivo*, partly by inhibiting the p38 MAPK activity [21, 80]. Intradiscal delivery of celexoxib-loaded microspheres was shown to restore intervertebral disc integrity in a preclinical canine model [67]. Amiloride inhibits acid sensing ion channel 1/3 [63] which was stated to prevent acid-induced decrease in cell proliferation and ECM gene expression [64]. Metformin treatment induce inflammation resistant phenotype [65] and is known for its chondroprotetive properties [66]. A 3-stage experiment was designed (i) to identify compounds/concentrations active in NC (2D screening), (ii) to study the effects of pre-conditioning on NCS cultured in DDD microenvironment (3D pre-conditioning), and finally (iii) to evaluate the function of pre-conditioned NCS using the compounds sleceted in i&ii within the degenerative NPμT model.

4.3.1 Effects of compounds on nasal chondrocytes (2D drug screening)

NC were pre-treated with increasing concentrations of GDF-5, IL-1Ra, metformin, celecoxib, and amiloride for 3h. Afterwards the drugs were removed, DDD mimicking condition was introduced to the cells for further 24h, and the expression of anabolic (aggrecan: ACAN; collagen type II A1: COL2A1) and catabolic genes (matrix metallopeptidase 13: MMP-3; interleukin 6: IL-6) were analyzed. Metformin, amiloride and celecoxib pre-treated NC showed no significant modulation of tested genes (Supplementary figure 2). GDF-5 (100ng/mL) pre-treatment significantly upregulated ACAN and showed trend towards increased COL2A1 expression in the NC cultured in DDD mimicking condition (Fig. 3A). In IL-1Ra (500ng/mL) pre-treatment NCS a trend towards downregulated IL-6 and MMP-3 gene expression was detected (Fig. 3B). Therefore, both GDF-5 (100ng/mL) and IL-1Ra (500ng/mL) were considered suitable for pre-conditioning of NCS.

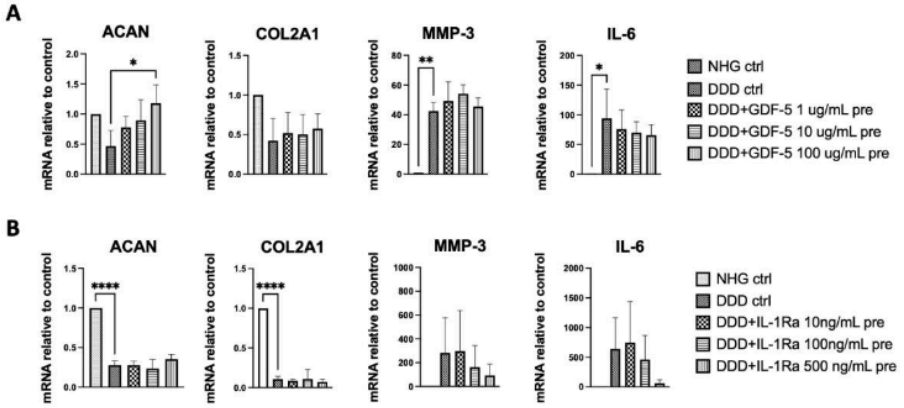

Fig. 3: 2D drug screening: effects of drug pre-treatment on NC in DDD-mimicking conditions. *Gene expression (relative to contro) of anabolic genes (ACAN, COL2A1) and catabolic genes (MMP-3, IL-6) in NC pre-treated with* ***(A)*** *GDF-5 or* ***(B)*** *IL-1Ra. NHG: healthy condition, DDD: DDD mimicking condition (n = 3, mean ± SD, *p < 0.05, ANOVA).*

4.3.2 Preconditioning of nasal chondrospheres with IL-1Ra and GDF-5

NCS were pre-conditioned during their formation with IL-1Ra (500ng/mL, putative anti-inflammatory activity), GDF-5 (100ng/mL, putative anabolic activity), or the combination of both, and introduced to DDD mimicking conditions for 7 days. Pro-inflammatory/catabolic (IL-8, MMP-3) and anabolic (COL2A1, COL1A1, ACAN) gene expression, IL-8 release, GAG and total collagen content in the NCS were assessed on day 0 (before implementing in DDD condition), day 3 and 7. Furthermore, NCS viability was determined, with no significant difference between pre-treated NCS and DDD control.

IL-1RA pre-conditioning. IL-1Ra preconditioning of NCS did not influence the expression of tested genes on day 0 (Fig. 4A, C). In DDD condition, IL-1Ra pre-conditoned NCS significantly downregulated IL-8 and MMP-3 on day 3 and tended to reduce it on day 7 (Fig. 4A). Pre-conditioning of NCS with IL-1Ra significantly downregulated the release of IL-8 protein on day 3 and on day 7 (trend), confirming gene expression data (Fig. 4B). Although no significant effects on anabolic genes were observed (Fig. 4C), IL-1Ra pre-conditioned NCS contained significantly more GAG (but not collagen) on day 7 compared to days 0 and 3 (Fig. 4D), indicating that these NCS could accumulate ECM in DDD condition possibly via anti-catabolic action of IL-1Ra.

GDF-5 pre-conditioning. Significant upregulation of ACAN expression (Fig. 4C) and a trend towards downregulated IL-8 and MMP-3 expression (Fig. 4A) were observed on day 0, before the GDF-5 pre-treated NCS were implemented in DDD condition. In DDD condition, GDF-5 pre-conditioning of NCS had no effect on the expression of tested genes (Fig 4A, C), nor IL-8 release (Fig. 4B) or ECM accumulation (Fig. 4D).

IL-1Ra+GDF-5 pre-conditioning. At day 0, significant upregulation of COL1A1 and ACAN expression (Fig 4C) and reduced IL-8 and MMP-3 expression (not significant) were detected, likely due to the effects of GDF-5. In DDD condition, IL-1Ra+GDF-5 pre-conditioning significantly downdergulated IL-8 and MMP3 genes in day 3 NCS (likely due to IL-1Ra) (Fig 4A) and tended to upregulate COL2A1 on days 3 and 7, possibly as a result of combination treatment (Fig. 4C). The significant downregulation of IL-8 resease from IL-1Ra+GDF-5 NCS on day 3 and on day 7 (trend) confirmed gene expression data, as expected effects of IL-1Ra (Fig. 4B). Despite expectations, ECM accumulation in IL-1Ra+GDF-5 pre-conditioned NCS during 7 days was non-significantly different from the corresponding controls (Fig. 4E).

Altogether, GDF-5 pre-conditioing exerted some anabolic responses (i.e., increased ACAN expression). However, they were lost during NCS culture in DDD condtion. IL-1Ra pre-conditioning downregulated pro-inflammatory responses (the expression of IL-8 on gene and protein level) and catabolism (reduced MMP-3 gene expression), which could allow for GAG accumulation in DDD condition [81]. Since it was feasible to achieve anabolic effects in NCS without GDF-5 pre-conditioning we proceeded to test the performance of IL-1Ra pre-conditioned NCS within the degenerative 3D *in vitro* NPµT model.

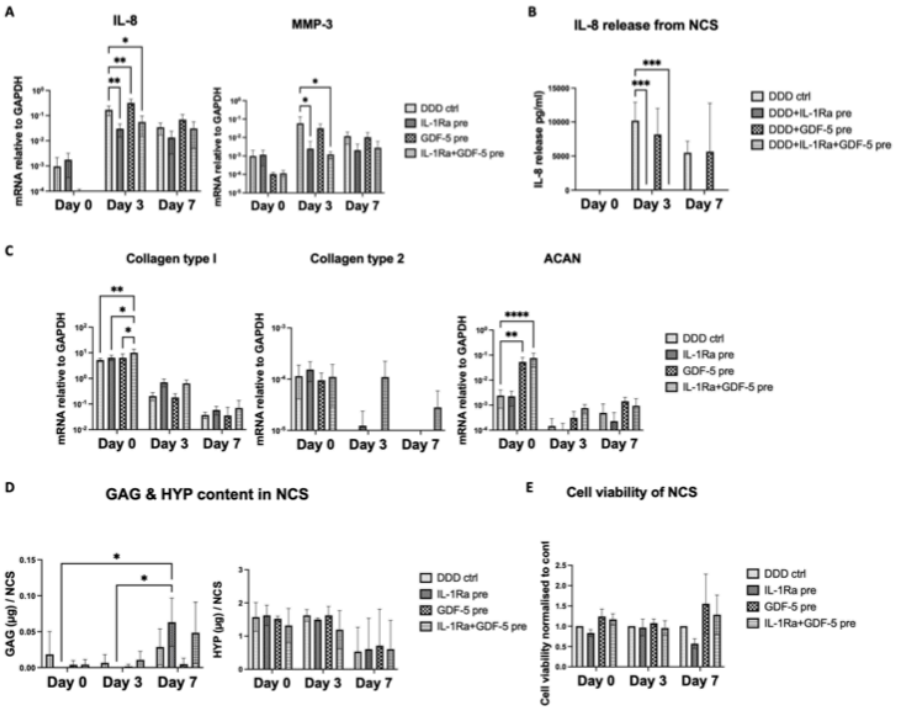

Fig. 4: The effects of NCS pre-conditioning. *Pre-conditioned NCS with IL-1Ra (500ng/mL), GDF-5 (100ng/mL) or combined were cultured for 0, 3, 7 days in DDD mimicking conditions and analysed. (A) Relative gene expression (2^{-dCt}) of catabolic markers (MMP-3, IL-8). (B) Catabolic shift in NCS was assessed my measuring IL-8 release. (C) Relative gene expression ($2^{-\Delta Ct}$) of anabolic markers (aggrecan, collagen type I, collagen type II). (D) Glycosaminoglycan (GAG) and hydroxyproline (HYP) content in NCS were quantified. The (E) The NCS viability was assessed (n = 3, mean ± SD, *p < 0.05, ANOVA).*

4.3.3 Performance of NCS pre-conditioned with IL-1Ra within degenerative NPµT model

NCS or IL-1Ra pre-conditioned NCS (pNCS) were implemented in the NPµT model and cultured for two weeks in DDD or NHG (control) microenvironment. Before implementation into the microtissue model, ECM content in NCS and pNCS was comparable (Supplementary figure 3). Interestingly, throughout their co-culture in the NPµT model, pNCS released IL-1Ra up to day 7 possibly as a result of its entrapment in newly generated ECM of pNCS [82, 83] (Fig. 5C). During co-culture in DDD condition, the NPµT+pNCS released significantly less IL-8 on day 3 and 7 compared to NPµT+NCS without pre-conditioning (Fig. 5A). Similar trend was observed for MMP-13 release (Fig. 5B). However, on day 14, the amount of released IL-8 became comparable between NPµT+pNCS and NPµT+NCS, which could be explained by significant decrease of IL-1Ra release from pNCS at this timepoint (Fig. 5C).

No significant difference in GAG and total collagen content was detected between NPµT+pNCS and NPµT+NCS (Fig.5D). Consistently, no difference in GAG content could be observed histologically (Fig. 5 E&F). Regarding NCS viability, no difference in cCas3 staining intensity could be visualised (Fig. E'''&F'''). However a trend towards less percentage of apoptotic cells within NPµT containing pNCS could be seen when the images were semi-quantified (Fig.5G).

Although the biological half-life of IL-1Ra protein has been reported to be 4-6 hours [84], NPµT co-cultured with IL-1Ra pre-conditioned NCS exhibited superior anti-inflammatory/anti-catabolic properties, compared to NPµT with non-preconditioned NCS at least up to day 7. However, IL-1Ra pre-conditioning was not sufficient to significantly increase the ECM content in NPµT, which could have been the consequence of reduced IL-1Ra release and/or activity in time.

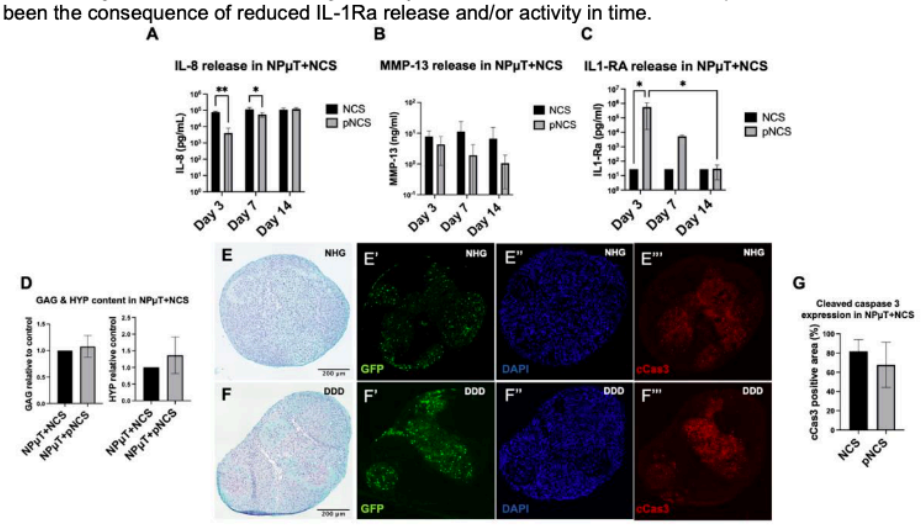

Fig. 5: Implementation of pre-conditioned NCS (pNCS) in NPµT model. *(**A**) IL-1Ra, (B) MMP-13, (C) IL-1Ra release from NPµT+NCS/pNCS was assessed on day 3, 7, and 14 (NP: n=1, NC: n = 3, mean ± SD, *p < 0.05, ANOVA). (**D**) Glycosaminoglycan (GAG) and hydroxyproline (HYP) quantification, normalised to NPµT+NCS (NP: n=1, NC: n = 3, mean ± SD, *p < 0.05, ANOVA). Safranin O staining of NPµT containing (**E**) NCS and (**F**) pNCS, visualising proteoglycans. Immunofluorescence staining visualising (**E'&F'**) GFP transduced NC, (**E''&F''**) nuclei (DAPI), and (**E'''&F'''**) apoptotic cells (cleaved caspase 3, cCas3) within NPµT+NCS/pNCS. (**G**) Quantification of cCas3 staining (NP: n = 1, NC: n=3, mean ± SD, *p < 0.05, ANOVA).*

5. Discussion

In this study, we aimed to develop a new 3D *in vitro* degenerative NPμT model allowing to investigate the responses of therapeutic cells (in this case NC) to DDD microenvironment. Our NPμT model contains hypoxia, acidity, low-grade inflammation as well as 3D degenerated human NP cells with cell-made matrix, pre-stimulated with DDD microenvironment. The model exhibits important features of early-stage IVD degeneration including degrading ECM and catabolic shift [28, 85]. Therapeutic cells can be incorporated into the NPμT model either as cell suspensions or spheroids, acquiring/maintaining 3D organization of target NP tissue. In this model, NC cell suspension showed inferior GAG and collagen accumulation and increased catabolic shift compared to NCS, supporting the use of NCS for clinical IVD repair. As spheroidal organization did not completely recover early-stage DDD signs, we attempted to improve the performane of NCS by drug pre-conditiong during their formation. From five tested candidates (amilorde, celecoxib, IL-1Ra, metformin, GDF-5), a clinically avaiable anti-inflamatory drug IL-1Ra was evaluated as the most promising. Pre-conditioning of NCS with IL-1Ra further downregulated pro-inflammatory and catabolic responses, which could allow for GAG accumulation in DDD-mimicking conidtion. Moreover, pre-conditioned NCS exhibited long-term IL-1Ra release associated with reduced IL-8 and MMP13, which underlines the importance of anti-inflammatory pre-conditioning for IVD repair.

Early stage DDD could still be restored using cell therapy [28]. An ideal *in vitro* model of early-stage DDD should accurately simulate the target tissue, by using NP cells from a patient with an appropriate Pfirrmann grade (2-3), as well as degenerative microenvironmental cues such as low glucose and oxygen levels, pH, and low-grade inflammation [28]. 3D alginate beads and pellet cultures are commonly used in IVD reaseach to study the interaction of NP cells with a therapeutic cell source of interest *in vitro* [86, 87]. In alginate bead co-cultures, expanded NP and cells of interest are mixed and then differentiated together within the beads, while the alginate represents an exogenous ECM [48, 88]. Alternatively, a transwell system is used for indirect co-cultures, to study paracrine interactions between both cell types encapsulated within alginate beads separately [50, 89]. The pellet culture model overcomes several disadvantages of alginate beads such as lack of reproducibility and uniformity of quality/size of the microspheres [51]. The pellet culture system was extensively used for direct co-culture studies, where the therapeutic cells were mixed together with the expanded NP cells and centrifuged in a tube to form a pellet, thus differentiated together thereafter [53, 54, 90]. However, the simple pellet culture model does not accurately mimick the early stage *in vivo* DDD, thus is not ideal for investigating/testing cell therapies.

In clinical settings, the therapeutic cells are introduced to an already mature degenerative NP tissue containing differentiated NP cells and NP cell-produced matrix. Furthermore, the therapeutic cells create initial contacts with the ECM of the NP tissue rather than directly with the NP cells. Therefore, in order to accurately mimic this situation *in vitro*, we designed new 3D degenerative NPμT model. To simulate clinical early-stage DDD, we first re-differentiated NP cells in spheroidal organization and allowed them to accumulate cell-produced ECM, to which the therapeutic cells could be introduced at later stage. We also pre-stimulated the resulting NPS with hypoxia, low glucose, acidity, and low-grade inflammation, to recapitulate the chemical properties of degenerated NP [28]. Pooling the NPS with the therapeutic cells allows to create NP niches where the cells of interest could integrate into. Furthermore, the NPμT model also contains low nutrition, acidity, hypoxia and low-grade inflammation, thus can be used to study the long-term effect of degenerative NP microenvironment on therapeutic cells. As such, this straightforward yet sufficiently complex NPμT model overcomes limitations of currently used models.

For early stage IVD repair, therapeutic cells have to reside, survive, resist inflammation and produce ECM within the degenerative NP tissue. In previous studies, NC showed superior viability in simulated DDD microenvironment over commonly used MSCs and AC, thus represent a promising cell source for IVD repair [42, 43, 78, 91]. In degenerative IVD condition, NC were reported to produce a ratio of low collagen to high GAG content whereas AC produce less favorable high collagen ratios [78]. We have demonstrated the potential of NC as spheroids (NCS) for IVD repair [43]. However, these studies were performed partially in absence of the NP cells, which could modulate the behaviour of NC, or only the short-term DDD mimicking conditions were applied [42, 43]. In the current study we implemented NC either as NCS or NC cell suspension in the denerative NPμT model.

NPμT+NCS cultured in DDD condition possessed similar amount of proteoglycans and collagens compared NPμT+NCS cultured in healthy condition which is already promising for future application of NCS for early stage IVD repair.

We have also shown that pre-conditioning of NCS is necessary to improve their ability to counteract inflammation, increase cell survival, and/or accumulate ECM. FDA approved drugs were used for preconditinong of NCS, to reduce regulatory burden during clinical translation. 2D screening revealed two promising candidates, IL-1Ra (anti-inflammatory) and GDF-5 (anabolic), with GDF-5 being eliminated at next stage (3D) due to its inferior effects in NCS pre-conditioning tests. We expected anabolic effect of GDF-5 on NCS, as benefits of GDF-5 in cartilage and IVD repair are well described [22-24, 92-94].
IL-1Ra preconditioned NCS were further implemented into the NPμT model. Overall IL-1Ra preconditioning inhibited pro-inflammatory and catabolic responses of NCS (IL-8, MMP-3, MMP-13). While IL-1Ra promoted NCS GAG accumulation in DDD mimicking condition, it failed to produce similar signifincant effects in NPμT model, which indicated that degenerated NP microtissue indeed infuences the performance of therapeutic cells, thus it should be present during preclinical therapeutic *in vitro* testing.

The biological half-life of IL1-Ra protein has been reported to be 4-6 hours [84].Several approaches have been taken to prolong the half-life of IL-1Ra such as fusing IL-1Ra with proteins (elastin-like polypeptides, human serum albumin, albumin domain antibodies) or by combining it with biodegradable polymers (poly(D,L-lactidide-co-glycolide), PLGA, polyethylene glycol (PEG), thermo-reversible gel) to prolong its steady-state sustained release at the site of administration [84]. However, another approach could be to entrap IL-1Ra within the ECM of the spheroids by pre-conditioning NCS during their formation time, as in our study. The entrapped IL-1Ra could be slowly released [95], supporting cells to counteract inflammation for longer time periods. In our NPμT model, the anti-inflammatory protection by IL-1Ra lasted up to 14 days and appeared to inversely correlate with the release of IL-8, suggesting that the IL-1Ra entrapped within the NCS was consumed. Even though some modest increase in ECM content within the NPμT with pNCS (vs. NCS) could be observed, IL1-Ra pre-conditioning of NCS alone will likely not be sufficient to sustain ECM-forming activity *in vivo*. Therefore, another approach to directly increase anabolic activity of NCS might be needed.

At the moment our NPμT model has several limitations that could be addressed in the future. We used 1:1 ratio of NP/NC cells based on literature [86, 96-98]. However, it was also reported that a ratio of 75:25 NP/MSC cells leads to optimized MSC differentiation towards NP phenotype [99], thus it still has to be determined which ratio is optimal for co-culture studies of NP cells with therapeutic cells in NPμT model. Another limination is that our micro-tissues were cultured in static conditions. Applying compressive loading to micro-tissues might better mimick early stage IVD degeneration and increase cell survival due to enhanced nutrient diffusion/waste removal within the NPμT model.

The 3D *in vitro* degenerative NPμT model aims to substitute the use of current alginate and pellet culture systems for preclinical *in vitro* investigations of IVD cell therapies. It should allow to study the survival and performance of (primed) therapeutic cells within the NP microenvironment mimicking early stage

6. References

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7. Supplementary Material

7.1 Media composition

7.1.1 Expansion Medium

7.1.1.1 NC Expansion Medium

Complete Medium (10% FBS): Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, 10938-025) supplemented with 10% fetal bovine serum (FBS) (Invitrogen, 10270-106), 1mM sodium pyruvate (SP) (Invitrogen, 11360-039), 10mM 4-(2-hydroxylethyl)-1-piperazineethanesulfonic acid (HEPES) (Invitrogen, 16630-056), 100 units/ml penicillin, 100µg/ml streptomycin, 0.29 mg/ml L-glutamine (PSG) (Invitrogen, 10378-016), 10ng/ml transforming growth factor (TGF-β1) (R&D, 240-BO-10) and 5 ng/ml fibroblast growth factor (FGF2) (R&D, 233-FB-025).

7.1.1.2 NP Expansion Medium

Complete Medium F12 (10% FBS): Dulbecco's Modified Eagle Medium Nutrient Mixture F-12 (DMEMF/12) (Invitrogen, 10938-025) supplemented with 10% FBS (Invitrogen, 10270-106), 1mM SP (Invitrogen, 11360-039), 10mM HEPES (Invitrogen, 16630-056), 100 units/ml penicillin, 100µg/ml streptomycin, 0.29 mg/ml L-glutamine (PSG) (Invitrogen, 10378-016) and 5 ng/ml FGF2 (R&D, 233-FB-025).

7.1.2 Differentiation Medium

7.1.2.1 NC Differentiation Medium

NC chondrogeic medium: DMEM (31885-023, Gibco), 1 mM SP (Invitrogen, 11360-039), 10mM HEPES (Invitrogen, 15630-056), 100 units/ml penicillin, 100 µg/ml streptomycin, 0.29 mg/ml L-glutamine (PSG) (Invitrogen, 10378-016), 10 µg/ml Insulin-Transferrin-Selenium (Gibco, 51300-044), 0.56 mg Linoleic acid (Sigma, L9530-5), 1.25 ml/l human serum albumin (CSL Behring, 43075), 10ng/ml TGF-β3 (100-36E, Peprotech), 0.1µM dexamethasone (Sigma, D-2915) and 0.1mM ascorbic acid (Sigma, A-8960).

7.1.2.2 NP Differentiation Medium

NP chondrogenic medium: DMEMF/12 (Invitrogen, 10938-025) supplemented with 1 mM SP (Invitrogen, 11360-039), 10mM HEPES (Invitrogen, 15630-056), 100 units/ml penicillin, 100 µg/ml streptomycin, 0.29 mg/ml L-glutamine (PSG) (Invitrogen, 10378-016), 10 µg/ml Insulin-Transferrin-Selenium (Gibco, 51300-044), 0.56 mg Linoleic acid (Sigma, L9530-5), 1.25 ml/l human serum albumin (CSL Behring, 43075), 10ng/ml TGF-β3 (100-36E, Peprotech), 0.1µM dexamethasone (Sigma, D-2915) and 0.1mM ascorbic acid (Sigma, A-8960).

7.1.2.3 Normoxic High Glucose medium (NHG)

Dulbecco's Modified Eagle Medium (Invitrogen, 10938-025) supplemented with 1mM SP, 10mM HEPES, 100 units/ml penicillin, 100 µg/ml streptomycin, 0.29 mg/ml L-glutamine (PSG) (Invitrogen, 10378-016), 10 µg/ml Insulin-Transferin-Selenium (Gibco, 51300-044), 0.56 mg/5.6µl Linoleic acid (Sigma, L9530-5), 1.25 ml/l human serum albumin (CSL Behring, 43075), 10ng/ml TGF-β3, 0.1µM dexamethasone (Sigma, D-2915) and 0.1mM ascorbic acid (Sigma, A-8960).

7.1.2.4 DDD mimicking medium (DDD)

For first 3 days: DMEM low glucose (Gibco, 11885-084) supplemented with 1mM SP, 10mM HEPES, 100 units/ml penicillin, 100µg/ml streptomycin, 0.29 mg/ml L-glutamine (PSG) (Invitrogen, 10378-016),

10 µg/ml Insulin-Transferin-Selenium (Gibco, 51300-044), 5.6µg/mL Linoleic acid (Sigma, L9530-5), 1.25 ml/l human serum albumin (CSL Behring, 43075), 0.45 mg/ml lactic acid (MP Biomedicals, ICN19022805) and 100 pg/ml of TNFα, IL1β, IL6 (All Sigma-Aldrich, SRP3177, GF331, SRP3096),10ng/ml TGF-β3, 0.1µM dexamethasone (Sigma, D-2915) and 0.1mM ascorbic acid (Sigma, A-8960).

For further 11 days: DMEM low glucose (Gibco, 11885-084) supplemented with 1mM SP, 10mM HEPES, 100 units/ml penicillin, 100µg/ml streptomycin, 0.29 mg/ml L-glutamine (PSG) (Invitrogen, 10378-016), 10 µg/ml Insulin-Transferin-Selenium (Gibco, 51300-044), 5.6µg/mL Linoleic acid (Sigma, L9530-5), 1.25 ml/l human serum albumin (CSL Behring, 43075), 0.45 mg/ml lactic acid (MP Biomedicals, ICN19022805) and 100 pg/ml of TNFα, IL1β, IL6 (All Sigma-Aldrich, SRP3177, GF331, SRP3096),

7.2 Supplementary tables

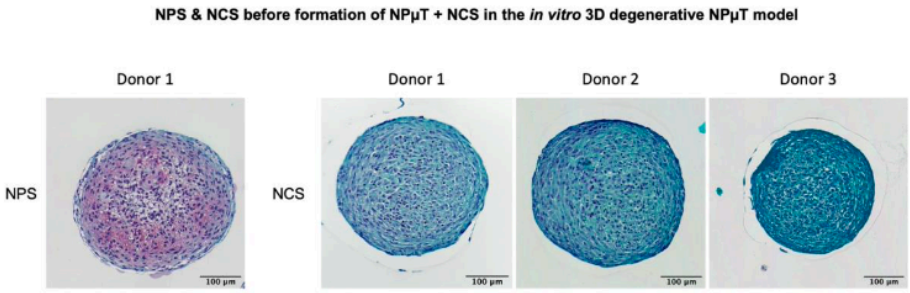
Table S1: *Donor demographics and characteristics of NC source tissues.*

	NC1	**NC2**	**NC3**
Age	52	25	53
Sex	male	female	female
Source	Rhinoplasty	Rhinoplasty	Rhinoplasty
	NC4	**NC5**	**NC6**
Age	53	26	34
Sex	male	male	male
Source	Septoplasty	Rhinoplasty	Rhinoplasty

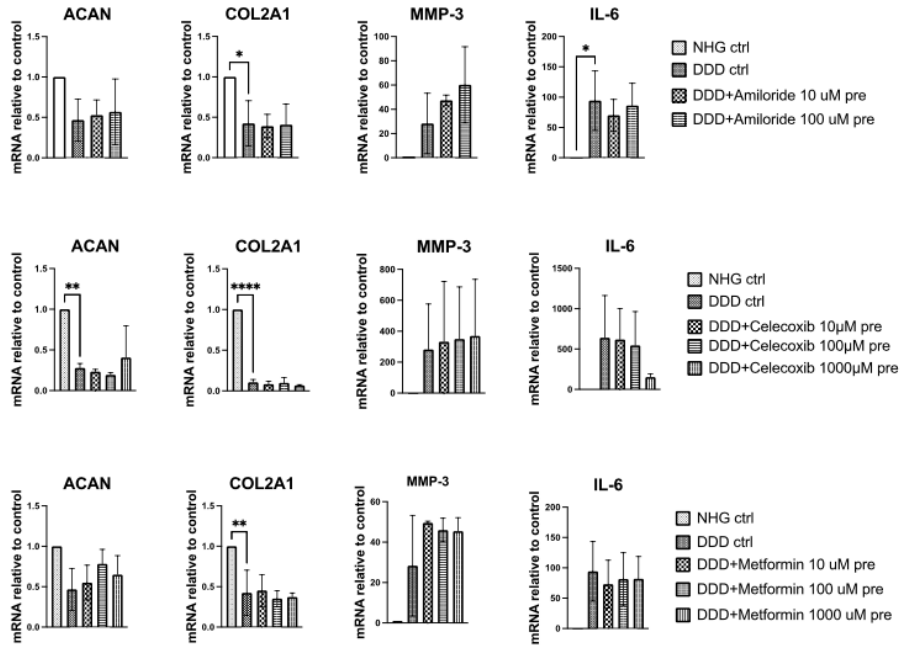
Table S2: *Donor demographics and characteristics of NP source tissues.*

	NP1	**NP2**	**NP3**
Age	72	53	46
Sex	male	male	male
Source	L3/4 grade 3	L4/5 grade 3	L5-S1 grade 3
	NP4	**NP5**	**NP6**
Age	47	43	79
Sex	female	male	female
Source	L5-S1 grade 3	L4/5 grade 2.5	L3/4 grade 2.5

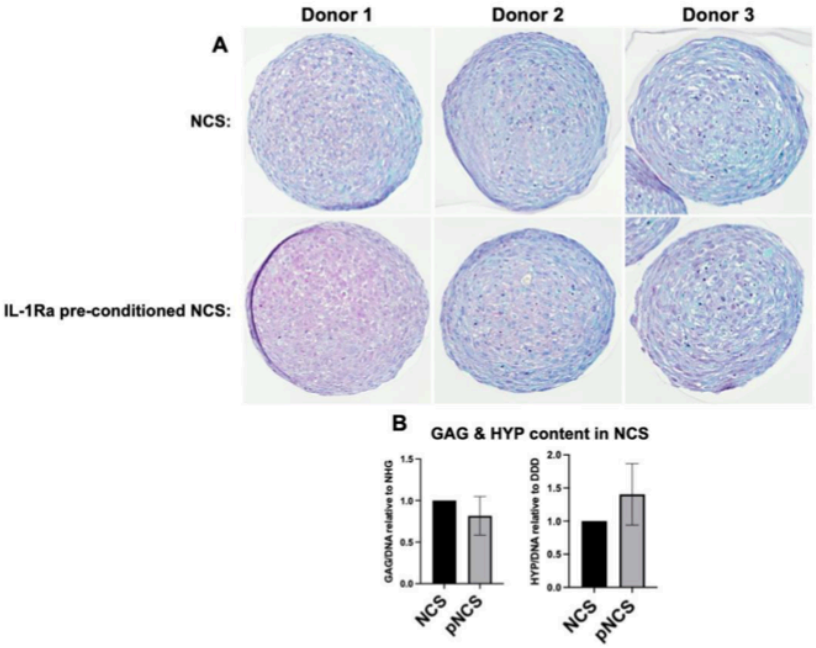
7.3 Supplementary figure



Supplementary figure 1: *Safranin O staining of NPS & NCS before formation of NPµT + NCS in the in vitro 3D degenerative NPµT model.*



Supplementary figure 2: Effects of drug pre-treatment on catabolic and anabolic marker expression in nasal chondrocytes (NC). *Relative gene expression (2^{-dCt}) of anabolic markers (aggrecan, collagen type II) and catabolic markers (MMP3, IL-6) in amiloride, celecoxib, and metformin pre-treated NC. NHG: healthy condition, DDD: DDD mimicking condition. (n = 3, mean ± SD, *p < 0.05, ANOVA).*



Supplementary figure 3: NCS and IL-1Ra pre-conditioned NCS (pNCS) before implementation in the 3D in vitro NPμT model. *(A) Safranin o staining of NCS and pNCS visualising proteoglycan content. (B) Proteoglycan and total collagen quantification in NCS and pNCS (n = 3, mean ± SD, *p < 0.05, ANOVA).*

8. Chapter 5: Discussion and Outlook

8.1 Discussion

In this thesis, I first present an overview of spheroid-based treatments for nucleus pulposus (NP) and annulus fibrosus repair. I also discuss cell sources and methods for spheroid fabrication and characterization, mechanisms related to spheroid fusion, as well as enhancement of spheroid performance in the context of the IVD microenvironment. Furthermore, my studies assess whether spheroids formed by nasal chondrocytes (NCS) represent a suitable graft for cell-based scaffold-free regeneration of the NP and investigate their performance within a novel 3D *in vitro* human degenerative NP micro-tissue (NPμT) model.

Our envisioned therapeutic strategy is scaffold-free. While injectable cell-carrier systems have improved cell delivery into the NP in preclinical studies, the biomaterial itself poses a challenge for clinical translation [1]. Carrier degradation products might accumulate to levels beyond those found in other (vascular) tissues, possibly causing NP-specific toxicity [213, 214]. On the other hand, intradiscal delivery of cell suspensions in a saline solution can lead to cell leakage out of the IVD, hindering the regulatory approval process [147]. For clinical application, spheroid-based strategies represent an ideal approach for IVD repair using minimal invasive treatment of early stage DDD in patients. My suggested NCS-based therapy may benefit patients with mild or moderate levels of degeneration with average Pfirrmann Grade 2-3 [215]. I provided *in vitro* evidence of the applicability of NCS for the augmentation of NP. Non-adhesive technology allowed reproducible fabrication of NCS both with and without additional growth factors. I detected no significant effects of shear stress on gene expression markers for inflammation, catabolism, or apoptosis markers while passing the NCS through a spinal needle. While growth medium ensured stable elastic modulus (E), chondrogenic medium increased E of NCS in correlation with their gene/protein expression of collagen type I in a time-dependant manner. The fusion kinetics of NCS with spheroids formed out of NP cells was not impaired in DDD-mimicking conditions, supporting their integration potential within harsh microenvironments. I also demonstrated that NCS can be delivered into the IVD without damage using spinal needles and rapidly fuse with NPS.

Application of NCS might circumvent regulatory issues related to biomaterials as well as increase cellular retention in the NP space. Previously reported clinical use of spheroid-based technology for articular cartilage repair supports the feasibility and translational potential of this strategy [153]. Optimal NCS dosage (and composition) will likely depend on the stage of degeneration, determined by MRI [95]. The ultimate goal is to alleviate patient's pain (short-term pain relief) and stabilize NP function by reconstituting native tissue structures, to improve long-term quality of life [1]. The proposed mechanism for NCS-based regeneration is both structural (directly supporting NP with their own ECM-forming capacity and stiffness) and trophic (delivering signals to protect or nurture resident NP cells). Indeed, it has been shown that the secretome of nasal chondrocyte-based tissue-engineered cartilage (N-TEC) for the repair of osteoarthritic knee cartilage defect reduced the inflammatory profile of osteoarthritic cells [150]. Downregulation of Wnt signalling pathway mediated by secreted frizzled related protein 1 (sFRP1) was suggested as a possible mechanism leading to superior performance of NC (compared to articular chondrocytes) in pro-

inflammatory milieu. Since IVD degeneration and osteoarthritis are characterized by similar immuno-metabolic alterations [57], the NC might display a comparable anti-inflammatory effect via modulation of Wnt signalling within the DDD microenvironment.

The reaction of therapeutic cells to a degenerative NP microenvironment is critical for the success of cell therapy. Therapeutic cells were reported to suffer from limited nutrient supply and waste removal *in vivo* due to endplate calcification and/or an excess number of introduced cells [95]. Implanted cells are exposed to the avascular niche with low oxygen and glucose, high concentration of lactic acid, and complex non-physiological loading [216]. I developed a novel 3D *in vitro* degenerative NPμT model which allowed to investigate the reaction of nasal chondrocytes, a potential therapeutic cell source, to DDD microenvironment. NPμT exposed to the DDD conditions produced less proteoglycans and collagens while expressing more inflammatory/degrading markers as compared to the healthy control, thus acquiring features of an early stage degenerated IVD tissue [95, 217]. Furthermore, I observed superior performance of NCS compared to NC suspension within the model, supporting further the use of spheroidal organisation of NC for clinical applications. Previous studies demonstrated that NC were superior at surviving and accumulating ECM in an inflamed IVD milieu to AC and MSC. However, their anabolic activity was drastically impaired in such harsh environments [17]. Using our NPμT model, I showed that NC, even within spheroids produced limited amounts of proteoglycans and acquired apoptotic traits. These results thus prompted us to investigate strategies to increase the anti-inflammatory/pro-anabolic properties of NC spheroids.

Among the different anti-inflammatory compounds tested, only IL-1Ra significantly reduced the expression of inflammatory markers in NC and subsequently in NCS, when cultured in DDD microenvironment. We implemented IL-1Ra pre-conditioned NCS (pNCS) within our model to counteract inflammation-driven catabolic activity by NC. Interestingly, NPμT containing pNCS released significantly less IL-8 and also a trend in downregulation of MMP-13 expression was detected till day 7. This observation is even more impressive since the biological half-life of IL1-Ra protein has been reported to be 4-6 hours [218]. Promoting tissue repair via localised delivery of therapeutic bioactive factors in low doses limits side effects caused by diffusion into neighbouring tissues. Local sequential release of therapeutics is often accomplished using biomaterial systems. For example, IL-1Ra was encapsulated in drug delivering biodegradable polymers (poly(D,L-lactidide-co-glycolide), PLGA, polyethylene glycol (PEG), thermo-reversible gel) for steady-state sustained release at the site of administration [218]. In regard to IVD repair, it was reported that bioactive IL-1Ra encapsuled in PLGA microspheres could be successfully delivered via intradiscal injection into rat caudal IVD which prevented IL-1β-induced glycosaminoglycan loss *in vivo* [219]. However, the use of biomaterials for IVD repair can have cytotoxicity and immunogenic effects due to the degradation of the biomaterial itself [220].

Several scaffold-free approaches were investigated in the past to extend the half-life and/or sustained release of IL-1Ra. For example, it has been demonstrated that the binding affinity of IL-1Ra to the ECM is increased by fusing IL-1Ra to an ECM-binding sequence derived from the heparin-binding domain of placenta growth factor (PlGF) [221, 222]. This protein fusion limits the diffusion of low IL-1Ra doses into neighbouring tissues thereby preventing side effects. In my NPμT model I show that the anti-inflammatory and anti-catabolic effect of IL-1Ra diminished on day 14 indicating that

the supplemented IL-1Ra is consumed. It has to be investigated whether NCS entrap the IL-1Ra by binding it to the ECM and if the binding affinity of IL-1Ra should be increased for improved clinical efficacy.

Altogether, this thesis contains an overview of spheroid-based strategies for IVD repair. I generated and characterized NCS which have the potential to be used for clinical treatment of early stage DDD. I tested in a newly developed 3D *in vitro* human degenerative NPμT model performance of NCS to survive, counteract inflammation and accumulate ECM. I could state that pre-conditioning of NCS with IL-1Ra support the NCS to counteract inflammation within harsh DDD microenvironment.

8.2 Outlook

My work can be further developed in different directions, in order to lead to more fundamental biological understanding and to a potential clinical application. The future challenges I have identified are of scientific, technical, translational and regulatory nature.

For generating volume within the degenerating NP tissue, the combination of spheroids with an injectable biomaterial such as alginate/hyaluronic acid (HA) hydrogel should not be excluded from future investigations. In AF tissue engineering, spheroids could serve as building blocks for living AF patches, together with various biomaterials to seal the AF. A biomaterial could regulate the rate of spheroid fusion with AF, define spheroid position in a patch, and/or release factors that regulate spheroid functions (e.g., growth factors). Spheroids capacity for rapid fusion might aid in filling deeper and irregular AF defects. The spheroid functionality could be enhanced by using biomimetic and/or bio-instructive environment, as well as genetic modifications using for example CRISPR technology to increase GDF-5 or IL1-Ra expression in NC. In regard to NP tissue repair, a suitable biomaterial such as hydrogels could be used to encapsulate NCS before intradiscal injection in order to protect them from the harsh DDD microenvironment. However, it has to be investigated whether the NC within NCS could migrate out of the hydrogels to support NP tissue repair. Furthermore, the biomaterial itself could cause regulatory problems due its mechanical properties or cytotoxicity caused by degradation of the biomaterial.

The 3D *in vitro* human NPμT model has several limitations that could be improved. Culture medium was changed twice a week, removing any cell-produced trophic factors and new media containing DDD associated environmental cues were added over two weeks. One way to solve this problem would be to exchange only 50% of the media and new media should only be added once a week. I used 1:1 ratio of NP/NC cells based on literature [223-226]. However, it was also reported that a ratio of 75:25 NP/MSC cells leads to optimized MSC differentiation towards NP phenotype [184]. It still has to be determined which ratio is the optimum for co-culture studies of NP cells with therapeutic cells. The micro-tissues were cultured in static conditions, thus applying compressive loading on them might increase cell survival due to increased nutrient diffusion as well as waste removal within the micro-tissue. With the 3D *in vitro* degenerative NPμT model I aim to substitute the current alginate and pellet culture systems. It should allow to study the survival, anti-inflammatory/catabolic and pro-anabolic activities of (primed) therapeutic cells within the harsh NP microenvironment created during early stage of DDD. However, to obtain more clinically relevant information of NCS such as survival, retention, integration and ECM accumulation within harsh IVD microenvironment, an *ex vivo* disc culture model where loading could be applied [227] should be implemented and further pre-clinical studies using large animals should be performed before clinical application.

As aforementioned, ideal *ex vivo* culture model to study the performance of NCS in DDD microenvironment could be bovine disc culture model using dynamic loading [227]. The bovine disc could be isolated from a bovine tail and IVD degeneration could be induced by chemonucleolysis via intradiscal delivery of an ECM-degrading enzymes such as chondroitinase ABC which recapitulates the loss of proteoglycans in IVD degeneration. Subsequently, NCS (generated with labelled NC) could be

injected into the bovine discs to test their survival, retention, integration, and ECM accumulation properties during long-term culture under (hyper)-physiological loading. The model will also allow researchers to study whether the NCS fuse with the NP tissue or if NC migrate out of the NCS to the damaged area for either accumulating ECM themselves or supporting the NP cells to restore their cell function and ECM production capabilities. Within the IVD research field, it is well known that intradiscal delivered cells could leak causing osteophyte formation [147]. Therefore, it is crucial to study the retention of the NCS within the IVD. For example, by injecting NCS, which were incubated in a contrast solution, into the degenerated bovine IVD the retention could be quantified using MRI analysis.

Animal models of disc degeneration range in scale from small species (mouse, rat, rabbit) to large (sheep, goat, pig, dog) species [228-232] However, there are several reasons why a large animal model should be considered to study IVD degeneration. Large animal models possess similarities in morphology to human lumbar IVD as well as sufficient disc height to mimic the nutrient deprived microenvironment in human IVD compared to small animal models [233]. Furthermore, in sheep and goat the proteoglycan content and the IVD mechanics are similar to human [234, 235]. The canine model is also promising since it could reflect natural IVD degeneration [236]. However, in case of animal models without natural occurring IVD degeneration, chemonucleolysis could be performed to induce IVD degeneration [229]. The animal model enables to assess the survival of the injected cells and to study through which mechanisms the implanted cells induce IVD repair. Moreover, only the animal model will allow to monitor pain which represents the major burden for the patient.

For clinical application of NCS, production must be carried out according to standard operating procedures (SOPs), following good manufacturing practice (GMP) guidelines and applying in-process controls (IPCs) and defined release criteria to ensure high quality, sterility and safety of the product. NCS is a scaffold-free tissue engineered product. Advanced therapy medical products (ATMP) can only be produced following legal framework since it has been standardized in Europe since 2007 [237]. Therefore, main criteria for release include viability, identity, potency and purity. This requires demonstration of the functionality and performance through validated assays. For example, release criteria for NCS generated considering GMP compliance could be to score a roundness above 0.8 and diameter >600µm to prevent blockage of the spinal needle which will be used for intradiscal injection. Cell viability/morphology should be tested for each batch produced. Ideally, the concentration of secreted factors should also be considered to understand the mode of action of the NCS. Biomechanical properties of the NCS could be also added as release criteria to ensure elastic modulus of the NCS similar to the NP tissue (~5kPa in healthy NP tissue). In addition, sterility, endotoxin levels and absence of mycoplasma have to be demonstrated.

To conclude, the progression of IVD degeneration is complex and modulated by multiple biological processes. For the IVD research field, a standardised 3D *in vitro* model simulating early stage IVD degeneration could help to optimize and compare cell-based therapeutic strategies. Several approaches based on biological factors or cell and gene therapy, also in combination with biomaterials, have shown promising results. However, a more advanced strategy is needed to simultaneously counteract inflammation and catabolic activities, repopulate the degenerating IVD and finally to

promote ECM accumulation within the IVD. Addressing all these aspects, spheroid-based strategies are promising and could change the field of IVD repair.

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10. About the author

10.1 Publications

First-authorship publication *(Review, Part of PhD project)**:***
Kasamkattil, J., A. Gryadunova, I. Martin, A. Barbero, S. Scharen, O. Krupkova, and A. Mehrkens. "Spheroid-Based Tissue Engineering Strategies for Regeneration of the Intervertebral Disc." *Int J Mol Sci* 23, no. 5 (Feb 25 2022). https://dx.doi.org/10.3390/ijms23052530.

Co-first-authorship publication *(Part of PhD project)**:***
Gryadunova, A., **J. Kasamkattil**, M. H. P. Gay, B. Dasen, K. Pelttari, V. Mironov, I. Martin, S. Scharen, A. Barbero, O. Krupkova, and A. Mehrkens. "Nose to Spine: Spheroids Generated by Human Nasal Chondrocytes for Scaffold-Free Nucleus Pulposus Augmentation." *Acta Biomater* 134 (Oct 15 2021): 240-51. https://dx.doi.org/10.1016/j.actbio.2021.07.064.

Planned first-authorship publication *(Part of PhD project)**:***
J. Kasamkattil, Gryadunova, A., M. H. P. Gay, B. Dasen, H. Hilpert, R. Schmid, K. Pelttari, I. Martin, S. Scharen, A. Barbero, O. Krupkova, and A. Mehrkens. Potential title: "Human 3D nucleus pulposus micro-tissue model to evaluate the potential of pre-conditioned nasal chondrocytes for the repair of degenerated intervertebral disc" *Envisioned journal for submission: Frontiers in Bioengineering and Biotechnology.*

Co-authorship publication *(not related to PhD project)**:***
Filippi, M., F. Garello, O. Yasa, **J. Kasamkattil**, A. Scherberich, and R. K. Katzschmann. "Engineered Magnetic Nanocomposites to Modulate Cellular Function." *Small* 18, no. 9 (Mar 2022): e2104079. https://dx.doi.org/10.1002/smll.202104079.

Associated evaluator in Faculty Opinions *(not related to PhD project)**:***
Siles, L., et al., *ZEB1 protects skeletal muscle from damage and is required for its regeneration.* Nat Commun, 2019. **10**(1): p. 1364.

Potential co-author publication in collaboration with Novartis *(not related to PhD project)**:***
“Inhibition of BMP signaling in hMSC cultures for stable cartilage formation.”

10.2 Posters / Presentations / International Meetings on PhD Topic

10/2021: Podium presentation at "**DBM PhD Retreat 2021**" in Switzerland, Schwarzenburg.
Topic: "Nasal Chondrocytes for Intervertebral Disc Regeneration"

05/2021: Lecture at "**CABMM** (Center for Applied Biotechnology and Molecular Medicine) **2021**" online
Topic: "Nose to back: Spheroids generated by human nasal chondrocytes for scaffold-free nucleus pulposus augmentation"

05/2021: Poster presentation at "**ISSLS** (international society for the study of lumbar spine) **2021**" online
Topic: "Nose to back: spheroids derived from human nasal chondrocytes for nucleus pulposus repair"

05/2021: Oral presentation at "**ISCT** (international society for cell & gene therapy) **2021**". Online
Topic: "Spheroids Derived from Human Nasal Chondrocytes for Nucleus Pulposus Repair"

02/2020: Podium presentation at "**Surgical Research Day 2020**" in Switzerland, Basel
Topic: "The Effects of Nasal Chondrocytes on Formation of Extracellular Matrix"

08/2019: Podium presentation at "**SGS** (swiss society for spinal surgery) **2019**" in Switzerland, St. Gallen
Topic: "Nasal Chondrocytes for Intervertebral Disc Regeneration"

06/2019: Poster presentation at "**DBM PhD Retreat 2019**" in Switzerland, Schwarzenburg.
Topic: "Nasal Chondrocytes for Intervertebral Disc Regeneration"

05/2019: Podium presentation at "**TERMIS EU 2019**" in Greece, Rhodes
Topic: "Nasal Chondrocytes for Intervertebral Disc Regeneration"

04/2019: Poster presentation at "**Biospine 2019"** in Italy, Rome
Topic: "Nasal Chondrocytes for Intervertebral Disc Regeneration"