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

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ABSTRACT

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

Statement of Significance

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

1. Introduction

Intervertebral disc (IVD) degeneration is one of the most common causes of chronic low back pain [1], which is a major problem worldwide [2]. Treatment strategies currently under development aim at regeneration of the nucleus pulposus (NP), the soft, central core of the IVD, where the first signs of degeneration become apparent [3]. A common regenerative strategy is the use of biomaterials [4,5], and specifically hydrogels, as cell carriers. Cells can be the endogenously present NP cells, but also NP cells isolated from the affected disc and retransplanted together with the gel. Widely used carriers include hydrogels of alginate, agarose, and collagen, with variable effects on NP-mediated regeneration, but their application is mainly trial- and error-based. Insight into hydrogel characteristics determining the NP cell response would greatly enhance future development and application of hydrogels for NP regeneration. Adequate mechanical properties of the hydrogel, an appropri-

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ate degradation rate to accommodate new tissue formation, and accessibility to nutrients and oxygen all provide for an environment which induces cell differentiation and matrix deposition. These properties and thus cell behavior are likely to be affected by hydrogel parameters such as the polymer's physicochemical properties (e.g. fixed charge density), polymer concentration and cross-link density (influencing stiffness, porosity, and diffusion of nutrients and oxygen) [4].

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

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

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

2. Materials & methods

2.1. Culture

2.1.1. Cell isolation and expansion

Human IVDs were collected during post-mortem examination from patients deceased at the University Medical Center Utrecht. Redundant IVD tissue was used anonymously according to the regulations of the Local Medical Ethical Committee (METC) (number 12–364), and hence without information on existing spine pathology. From 9 donors (age 44–67, mean age 52), IVDs macroscopically scored as Thompson grade III, NP tissue was visually identified and dissected. Thereafter, cells were isolated using 14 U/ml pronase (Roche, Basel, Switzerland) + 110 U/ml DNAse (Sigma, St. Louis, MO) for 1 h followed by 13.7 U/ml collagenase (Roche) + 110 U/ml DNAse overnight. NP cells were expanded by seeding at 3000 cells/cm^2 [26] using DMEM (31885, Gibco, Life Technologies, Carlsbad, CA) + 10% heat-inactivated fetal bovine serum (HyClone, Life Technologies) + 2% antibiotics mixture (final concentration 200 U/ml penicillin, 20 kU/ml streptomycin, 50 mg/L amphotericin-B and 50 mg/L gentamycin (Lonza, Basel, Switzerland)) and 10 ng/ml recombinant human basic fibroblast growth factor (bFGF) (R&D Systems, Minneapolis, MA). During the second passage, anti-microbial agents were reduced to 1% penicillin and streptomycin (100 U/ml + 100 μg/ml, pen/strep, Gibco). NP cells were expanded for 2 passages until use in hydrogel cultures.

2.1.2. Hydrogel preparation

Three hydrogels with different stiffness and adjustable polymer concentration were prepared to determine whether stiffness affects matrix deposition by NP cells (see Table 1). Alginate (medium viscosity, Sigma) was mixed with the NP cell suspension and the solution was added dropwise into a sterile 102 mM $CaCl_2$ solution to form beads. The beads were allowed to cross-link for 10

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

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

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

2.1.3. Hydrogel culture

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

2.2. Analyses

2.2.1. Dynamic mechanical analysis

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

2.2.2. Biochemistry

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

2.2.3. Vinculin/actin immunohistochemistry

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

2.2.4. Extracellular matrix histology and immunohistochemistry

For a qualitative impression of extracellular matrix production, after 18 days of culture, hydrogels were fixed in 4% buffered formalin, dehydrated and embedded in paraffin. A Safranin-O/Fast Green staining was performed by incubating 5 µm sections with Mayer's hematoxylin (Merck) for 5 min, washing in tap water for 10 min and staining with 0.4% w/v Fast Green (Merck) during 4 min. After washing in 1% acetic acid, the sections were stained for 5 min in 0.125% (w/v) Safranin-O (Merck), followed by dehydration and mounting. For aggrecan immunohistochemistry, antigen retrieval was performed by incubating in citrate buffer at 96 °C for 20 min and cooling to room temperature (RT) for an additional 20 min. For types I and II collagen, antigen retrieval consisted of incubation with pronase (1 mg/ml in PBS, Roche) and hyaluronidase (10 mg/ml in PBS, Sigma) (both at 37 °C for 30 min). Immunostaining protocols were the same after the antigen retrieval. Washing in between steps was performed with PBS-T. Sections were blocked with PBS-BSA for 30 min at RT and incubated overnight at 4 °C with the primary antibody diluted in PBS-BSA (aggrecan (diluted 1:150, 6-B-4 (Novus Biologicals, Littleton, CO, USA), type I collagen (2 µg/ml, EPR7785 (abcam, Cambridge, UK), or type II collagen (0.4 µg/ml, DSHB II-II6B3 (DSHB, Iowa City, IA)). The EnVision kit (α-mouse for aggrecan and type II collagen, α-rabbit for type I collagen, Dako, Glostrup, Denmark) was used for visualization according to the manufacturer's protocol. Microscopy pictures were taken with a BX43 upright microscope and CellSens software (Olympus, Tokyo, Japan).

2.2.5. Chondrogenic, osteogenic and adipogenic gene expression

After 18 days of culture, hydrogels were snap frozen at −80 °C. For RNA isolation hydrogels were cut into small pieces and TRIzol® reagent (Thermo Scientific) was added. The lysate was then homogenized with an Ultra-turrax T10 homogenizer (IKA, Staufen, Germany) and purified with Direct-zol™ columns (Zymo, Irvine, CA, USA). 100 % ethanol was added to the lysate and centrifuged over the Zymo-Spin columns and treated with DNAse I for 15 min. The columns were then washed with pre-wash and wash buffer and RNA was eluted with RNAse-free water. Quality and quantity were checked using a Nanodrop (Thermo Scientific). A High-capacity

cDNA kit (Life Technologies) was used to synthesize complementary DNA from RNA on an iCycler Thermal Cycler (Bio-Rad, Hercules, CA, USA). In order to determine towards which lineage NP cells redifferentiated after expansion, a panel of genes was determined: aggrecan (ACAN), type I, type II and type X collagen (Col1A1, Col2A1, ColX), matrix metalloproteinase 13 (MMP13, a matrix degrading enzyme), osteopontin (SPP1, an osteogenic marker), and peroxisome proliferator-activated receptor gamma (PPAR-γ, an adipogenic marker). All genes of interest were normalized to the geometric mean of the housekeeping genes 18S and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Sequences and details of all genes are shown in Table 2. RT-qPCR was performed on a LightCycler 96 (Roche) using SYBR Green (Bio-Rad).

2.3. Statistical analyses

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

3. Results

3.1. Mechanical analysis

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

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

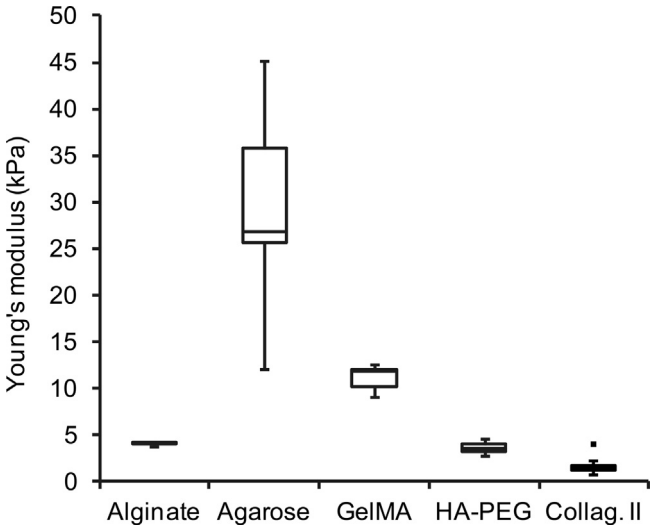

Fig. 1. Compression testing shows the highest Young's modulus for agarose hydrogels, followed by gelMA, and alginate and HA-PEG, and the lowest was measured for type II collagen hydrogels.

3.2. Focal adhesion formation

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

3.3. Inhibition of focal adhesion kinase

3.3.1. Cytotoxicity

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

Table 2
List of employed primers for RT-qPCR.

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

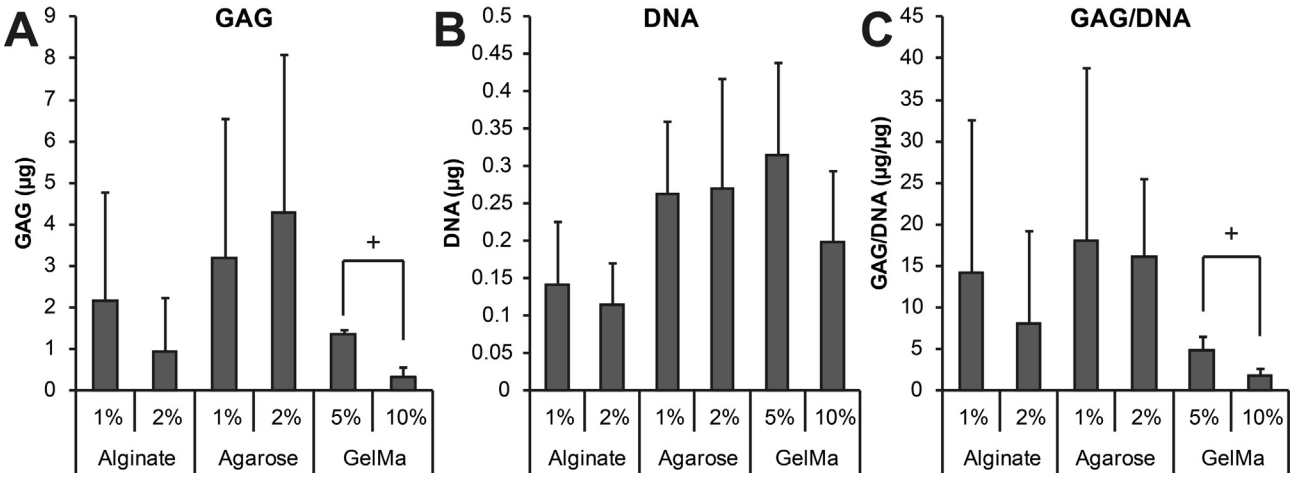

Fig. 2. Nucleus pulposus cells from human degenerated discs from 4 donors (n = 5 gels per condition per donor) were cultured in hydrogels of different concentrations. A: GelMA hydrogels of 5% (w/v) contained more GAGs than those of 10% (w/v). B: No differences in levels of DNA were found. C: GAG corrected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

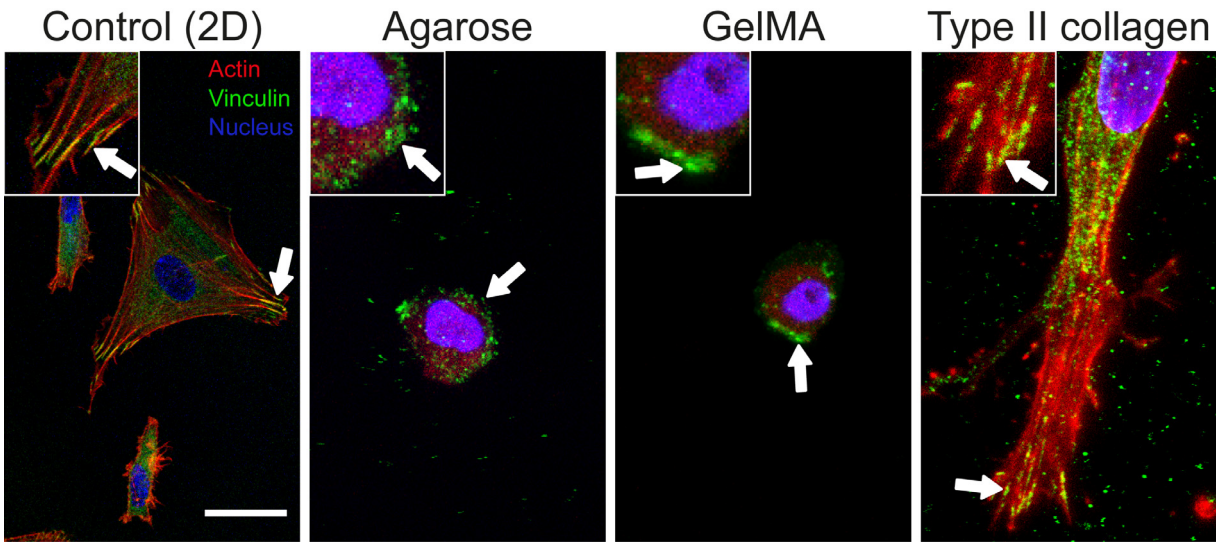

Fig. 3. Confocal microscopy of human degenerated nucleus pulposus cells cultured on type I collagen-coated glass coverslips and in several hydrogels. Red staining is actin, green staining is vinculin, and blue staining is the cell nucleus. Arrows indicate focal adhesions. Z-stack images are represented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

3.3.2. DNA and GAG content

Serum was omitted from the medium to prevent interference with focal adhesion. Consequently, GAG levels were lower in serum-free medium than when serum was present (Fig. 4 compared to Fig. 2). The GAG content of alginate hydrogels cultured with 5 µM FAK-i-14 was higher than with 10 µM FAK-i-14 (p = .004, Fig. 4A). No differences were measured for cells in agarose hydrogels. In gelMA hydrogels, the GAG content increased with increasing concentration of FAK-i-14, as measured by a weak but significant correlation (Spearman coefficient 0.286, p = .011). No effects of FAK inhibition were detected for cells cultured in HA-PEG hydrogels. In type II collagen hydrogels, a weak but significant negative correlation was found between GAG content with FAK-i-14 concentration (Spearman coefficient −0.265, p = .024). No differences in DNA content were detected (Fig. 4B). GAG/DNA was higher in alginate hydrogels cultured with 5 µM FAK-i-14 than with 10 µM FAK-i-14 (p = .004), but no correlation with inhibitor concentration was found (Fig. 4C). No differences in GAG/DNA were detected for NP cells in agarose, gelMA, and HA-PEG. GAG corrected for DNA was higher for NP cells in type II collagen hydrogels cultured without FAK-i-14 than with 10 µM, and a moderate negative correlation was found with increasing FAK-i-14 concentration and GAG/DNA in type II collagen hydrogels (Spearman coefficient -0.354, p = .002).

3.3.3. Vinculin and actin localization with FAK inhibition

NP cells were cultured in agarose, gelMA and type II collagen hydrogels in the presence or absence of the FAK inhibitor. In agarose, inhibition of FAK did not seem to influence the pattern of vinculin localization or actin polymerization (Fig. 5). In the presence of FAK-i-14, all NP cells in gelMA hydrogels were round and there was a reduced diffuse cytosolic non-polymerized actin staining. Some focal vinculin staining was present at the cell membrane, but at a clearly lower intensity. Cells in type II collagen changed

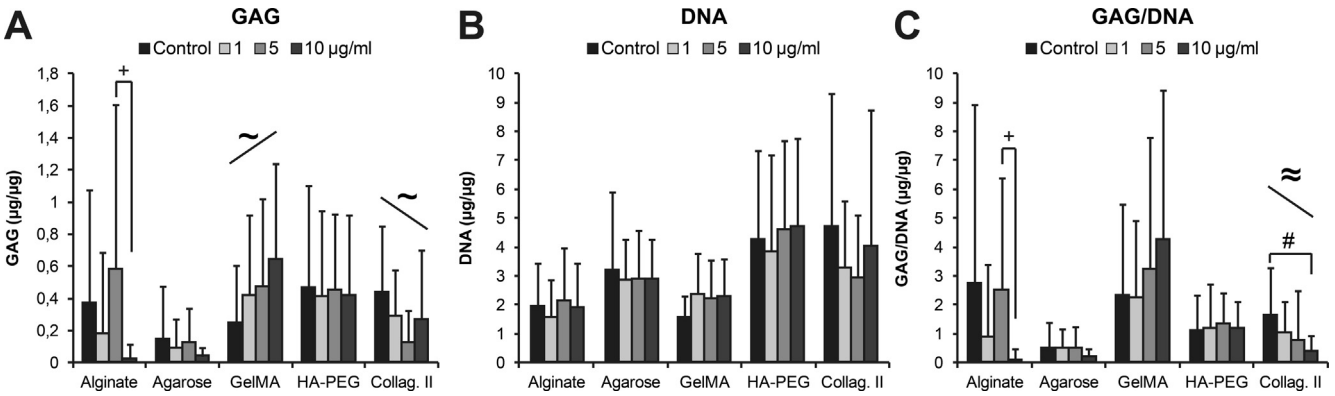

Fig. 4. Human degenerated nucleus pulposus cells from 5 donors cultured in several hydrogels in the presence of focal adhesion kinase inhibitor 14 (FAK-i-14) at different concentrations (1, 5 or 10 µM)(n = 5 gels per condition per donor). Control contains the carrier (DMSO)). A: A positive correlation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

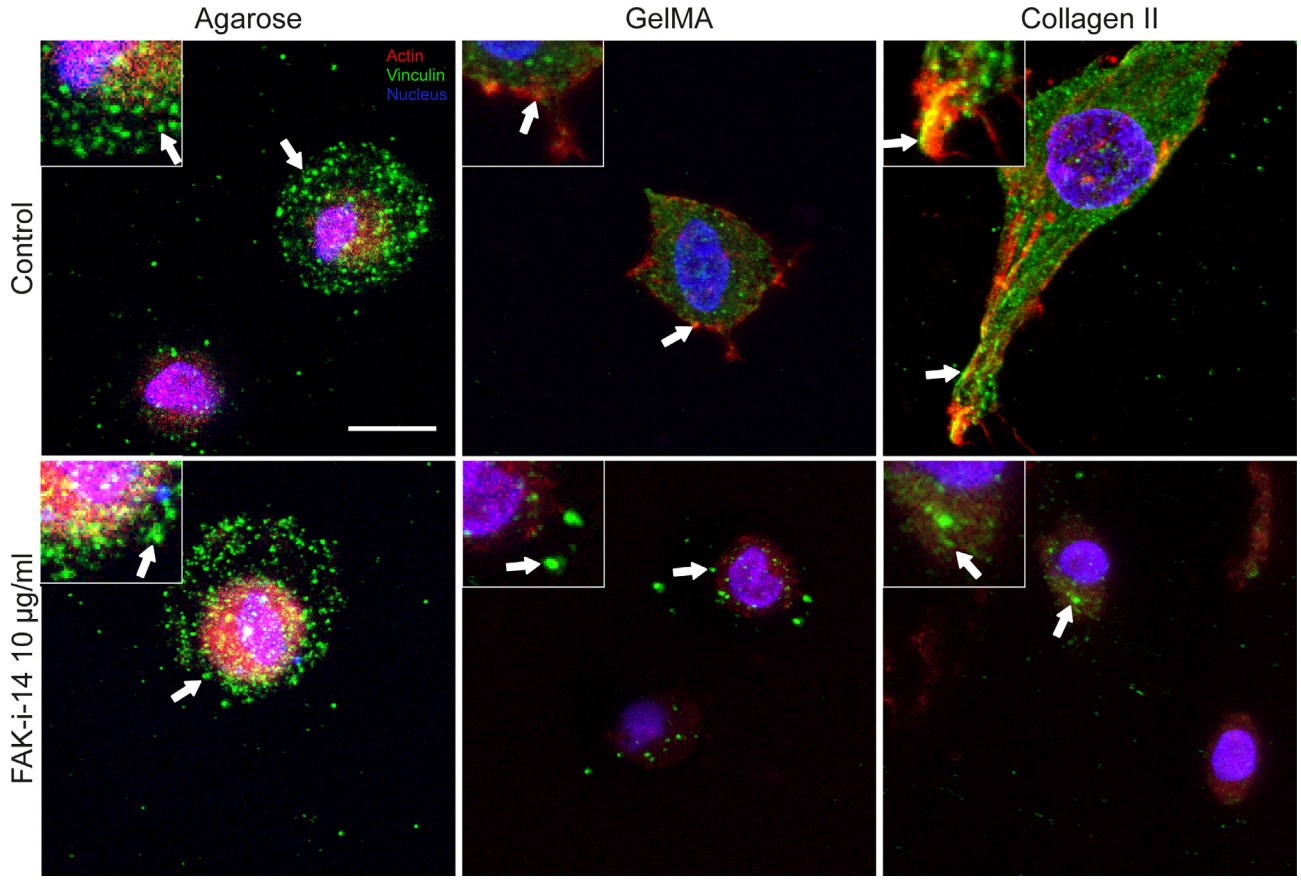

Fig. 5. Confocal images of cells in agarose, gelMA, and type II collagen hydrogels that were cultured in the presence of 10 µM FAK-i-14, and compared to controls. Cells were stained for vinculin (green), actin (red), and the cell nucleus (blue). White arrows indicate focal adhesions. Z-stack images are represented here as maximum intensity projections. No actin or vinculin was present in the nuclei; any apparent staining comes from adjacent structures that were superimposed. Scale bar indicates 10 µm. Images are representative for two donors. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

from being elongated and stretched to rounded cells in the presence of FAK-i-14 and diffuse low-intensity staining for actin without stress fibers was present in the cytoplasm. Also in these hydrogels vinculin staining was greatly reduced.

3.3.4. No visible staining for collagens or proteoglycans

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

3.3.5. Gene expression in response to FAK inhibition

In order to explain the differential effect of FAK inhibition on NP cell fate in gelMA versus type II collagen hydrogels, gene expression was measured after 18 days of culture in gelMA or type II collagen hydrogels in the presence or absence of 10 µM FAK-i-14 (Fig. 6A). Type X collagen expression could not be detected in

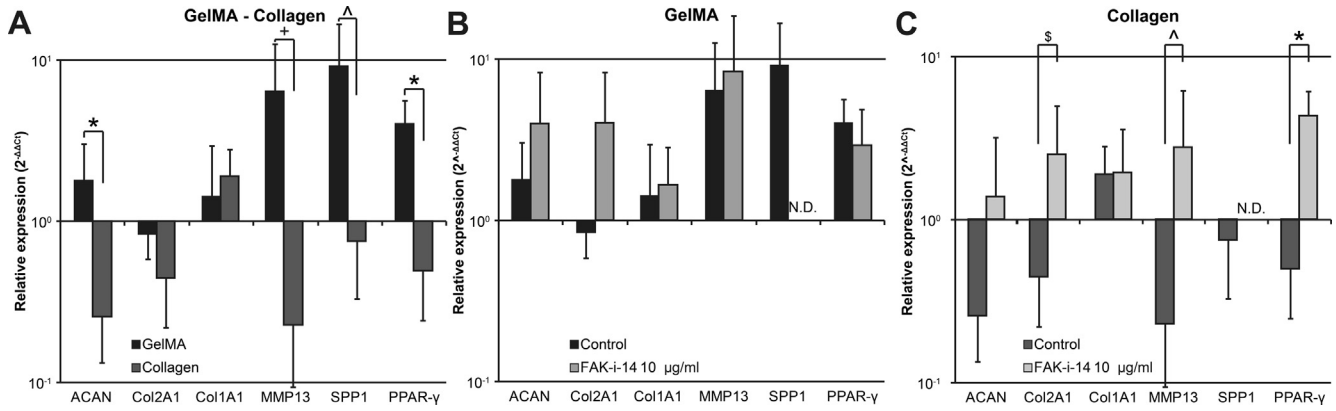

Fig. 6. Gene expression of human degenerated nucleus pulposus cells (4 different donors) cultured in gelMA or type II collagen hydrogels for 18 days in the absence or presence of FAK-i-14, relative to housekeeping genes, are displayed on a logarithmic y-axis. Gene expression of type X collagen could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

either hydrogel, while gene expression of the osteogenic marker osteopontin (SPP1) was completely inhibited in cells cultured in the presence of FAK-i-14. In controls without FAK inhibition, expression of chondrogenic marker aggrecan, hypertrophic marker MMP13, osteogenic marker osteocalcin (SPP1), and adipogenic marker PPAR-γ was 15-fold, 11-fold, 9-fold, and 32-fold higher in gelMA hydrogels compared to type II collagen hydrogels (p = .001, p = .003, p = .016, and p = .001, respectively, see Fig. 6A). Gene expression profiles of cells cultured in gelMA remained unchanged in the presence of FAK-i-14 (Fig. 6B). However, in cells in type II collagen hydrogels, FAK-i-14 increased the gene expression of type II collagen (4.6-fold, p = .05), MMP13 (6-fold, p = .023) and PPAR-γ (30-fold, p < .001), whereas gene expression of aggrecan and type I collagen remained unchanged (Fig. 6C).

4. Discussion

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

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

Focal adhesion signaling appeared to be involved in the response of NP cells to polymer concentration in hydrogels that contain integrin binding sites. MSCs have been shown adapt their differentiation pathway in response to matrix stiffness, with low stiffness leading to adipogenic (~2 kPa), higher to chondrogenic (~20 kPa), and even higher stiffness leading to osteogenic differentiation (~30–40 kPa) [49]. Stem cell fate appears to be mediated, among others, by a cascade involving adhesion signaling, subsequent FAK activation, actin filament bundling and thereby translocation of YAP/TAZ to the nucleus, which induces osteogenesis and inhibits chondrogenesis and adipogenesis [50] and as such can only differentiate between an osteogenic versus non-osteogenic state. In the current study, despite the fact that NP cells are already in the chondrogenic lineage, they appeared to respond in a similar manner to hydrogels of different stiffness. Previously, Arg-Gly-Asp (RGD)-binding integrins were shown to be involved in mechanosensing in healthy, but not degenerated NP cells [23]. Here, also in degenerated NP cells stiffness sensing is suggested to be mediated by collagen-binding integrins which can be modu-

lated by FAK inhibition. However, the extensive actin stress fibers formed in collagen II gels were in contrast with the low stiffness of the hydrogel and concomitant low expression of the osteogenic marker osteopontin, compared to gelMA. This may be related to the observation that after focal adhesion formation, F-actin networks can play other roles than cytoskeleton tensioning [51]. Osteopontin was completely inhibited in the presence of a FAK inhibitor in both gelMA and type II collagen. However, in the much softer type II collagen hydrogel FAK inhibition resulted in a reduction of chondrogenesis and enhanced adipogenic gene expression, which is in accordance to its stiffness already predisposing more towards the adipogenic lineage [49]. Assuming that FAK was inhibited fully, this suggests that also other mechanisms were at play in the response of the NP cells to their surroundings in the gelMA versus type II collagen hydrogels. In addition to differences in matrix stiffness, different collagens are known to bind to different (combinations of) integrins [52], possibly explaining why the response of NP cells in the two hydrogels was different. In line with this concept, differentiation of MSCs was shown to also be influenced by specific integrin combinations [53]. Adipose-derived MSCs respond differently to type I and type II collagen and show more NP gene expression in response to type II collagen. However, chondrocytes in gelMA hydrogels of lower stiffness (around 2–3 kPa) showed a more fibroblastic morphology and high cell proliferation, suggesting dedifferentiation [54]. Here it should be noted that gene expression data should be interpreted with caution and may only be used to provide an explanation rather than proof for a particular phenomenon. First of all, gene expression does not always coincide with protein production, as we have shown before for the response of NP cells to osmolarity [26]. This is in fact also shown here by an absence of a change in aggrecan mRNA levels in GelMA gels upon FAK inhibition, while at the protein level aggrecan production was clearly enhanced in the GelMA gels. Moreover, the co-expression of transcription factors for different lineages have been shown before for undifferentiated human degenerate NP-derived cells, where exposure to differentiation media was required for final differentiation [55]. Most likely, the final balance with other pathways in cells will determine differentiation at the protein level. This may also explain why in the current study PPARγ mRNA was detected in GelMA, even at a similar level to those in collagen with FAK inhibitors, despite the higher proteoglycan production in the former, further cautioning against overinterpretation of mRNA expression data.

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

Surprisingly, vinculin patches were also observed with confocal microscopy in NP cells in agarose hydrogels, while agarose by itself does not contain any cell binding motifs. The fact that no apparent differences were seen between the absence and presence of a FAK inhibitor and no actin polymerization was evident, suggests that these adhesions in agarose have formed in the absence of FAK activity. Indeed, it was shown that FAK activity is not required for focal adhesion formation [58], nor does it affect the molecular composition of the adhesion complexes [59]. Nevertheless, its activity is required for focal adhesion formation-mediated cell behavior such as migration [59]. FAK activity mediates focal adhesion protein phosphorylation and thereby activation of actin linkage to focal adhesions and subsequent signalling. Hence the activation stage and conformation of focal adhesion complex components rather than their quantity determines signalling [60,61]. The absence of actin polymerization in agarose further supports the absence of FAK activity in these gels. The lack of effect of changes in agarose concentration, and hence hydrogel stiffness, may be related to the absence of serum in our cultures. Serum is known to contain various integrin binding molecules, including laminin and fibronectin, which may mediate tethering to the surrounding hydrogel [62]. In addition, it enhances cell-based production of ECM [30,63]. This also explains the observation that in the current study only limited matrix production was observed, as serum nor TGFβ was added to enhance this [64]. As such, stiffness sensing in alginate, agarose, and HA-PEG hydrogels may occur only when cells have produced integrin-binding matrix [65]. In line with this thought, bovine chondrocytes cultured in agarose hydrogels respond to mechanical loading with increased matrix synthesis rates only after the initial deposition of extracellular matrix [66,67]. However, to what extent this is relevant for clinical application, is unclear, as the presence of serum proteins will be very limited in the largely avascular IVD [68].

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

5. Conclusion

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

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Disclosures

The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.actbio.2017.11.029.

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