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DISSERTATION

In vitro differentiation of chondrogenic cells in three-dimensional scaffold-assisted culture for cartilage repair and characterization of cartilage sources

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ABKÜRZUNGSVERZEICHNIS

3D	three-dimensional	AB	alcian blue
ACI	autologous chondrocyte implantation	AF	annulus fibrosus
Alg	alginate	(h)BM-MSC	human bone-marrow derived MSC
BSA	bovine serum albumin	CSC	cross-linked chondroitin sulfate
CTCF	corrected total cell fluorescence	CS	chondroitin sulfate
DAPI	6-diamidino-2-phenylindole	DMMB	1,9-dimethylmethylene blue dye binding assay
ECM	extracellular matrix	EDTA	ethylene diamine tetraacetic acid
ELISA	enzyme-linked immunosorbent assay	EM6F	expansion medium with 6 factors
EtBr	ethidium bromide	FBS	fetal bovine serum
FDA	fluorescein diacetate	FNF	femoral neck fracture
HE	hematoxylin eosin	IVD	intervertebral disc
MSC	mesenchymal stromal cells	NP	nucleus pulposus
OA	osteoarthritis	OHP	orthohydroxyproline
PBS	phosphate buffered saline	PFA	paraformaldehyde
RT	room temperature	SEM	scanning electron microscopy
sGAG	sulphated glycosaminoglycans	TBS	tris buffered saline
TGF-β	transforming growth factor beta		

Abstrakt (Deutsch)

Weder der hyaline Gelenkknorpel noch der Faserknorpel der Bandscheibe verfügen über ein ausreichendes Heilungspotential. Langfristig führen Knorpelverletzungen daher zu Arthrose oder Bandscheibendegeneration. Über das Knorpel-Tissue Engineering, bei dem Knorpelzellen oder zu Knorpelzellen differenzierbare Vorläuferzellen wie mesenchymale Stromazellen (MSCs) mit einer Trägermatrix (Scaffold) kombiniert werden, könnten Knorpelzellimplantate für den Knorpelersatz zur Verbesserung der Heilung gewonnen werden.

Um den Einfluss von hochporösen Alginat-basierten Scaffolds, die mit Chondroitinsulfat (CS) funktionalisiert wurden, auf die Knorpelbildung (Chondrogenese) mesenchymaler Stromazellen (MSCs) zu beurteilen wurde die Zelladhärenz, das Zellüberleben und die Expression von Knorpel-Matrixproteinen von MSCs und Chondrozyten im Alginatscaffold analysiert. In der zweiten Teilstudie wurde zur Herstellung eines Scaffolds aus natürlicher extrazellulärer Matrix (ECM) eine Zellbefreiung (Dezellularisierung) von Bandscheiben-gewebe etabliert und optimiert. Anschließend wurde die Rekolonisation der dezellularisierten ECM mit MSCs im Vergleich zu Bandscheibenzellen vorgenommen. Die dritte Teilstudie beschäftigte sich mit der vergleichenden Charakterisierung von Hüftkopf-knorpel und aus diesem gewonnenen Knorpelzellen von Patienten mit Arthrose oder Schenkelhalsfraktur. Dieser findetsehr oft für die Zellisolierung für experimentelle Zwecke Verwendung. Zur Beurteilung der Arthrose- assoziierten Veränderungen wurde ein makroskopisches *in-house* Scoring-System etabliert und validiert.

Die Alginatscaffolds erlaubten das Wachstum der MSCs und die Synthese einer ECM, die knorpelspezifisches Kollagen Typ II enthielt. Die Chondroitinsulfat- Funktionalisierung führte zu einer erhöhten Proteoglykansynthese im Vergleich zu den CS-freien Scaffolds. Es konnte eine zellfreie Bandscheiben-ECM hergestellt und nach Vorbehandlung mit Serumalbumin, erfolgreich mit MSCs und IVD-Zellen rekolonisiert werden, wobei der Gesamtkollagengehalt in den mit MSCs besiedelten Konstrukten höher war. Das *in-house* Score System war zur Erfassung arthrotischer Veränderungen geeignet, unterschied zwischen beiden Knorpelquellen (Arthrose und Schenkelhalsfraktur) und korrelierte mit dem histologischen Mankin-Score. Aus dem Knorpel von Coxarthrose- Patienten isolierte Zellen wiesen gegenüber den nach Schenkelhalsfraktur isolierten Zellen Unterschiede im Phänotyp auf.

Die Ergebnisse dieser Arbeit zeigen zwei grundsätzlich verschiedene Ansätze für die Gewinnung tissue-engineerter Knorpelimplantate auf und helfen bei der Beurteilung von Knorpelzellen für experimentelle Zwecke, die aus dem nach Implantation einer Endoprothese anfallendem Hüftkopfknorpel isoliert werden.

Abstract (Englisch)

Neither hyaline articular cartilage nor the fibrocartilage of the intervertebral disc have a sufficient self-healing potential. In the long term, cartilage injuries lead to osteoarthritis (OA) or degenerative intervertebral disc disease. With the help of cartilage tissue engineering techniques, combining chondrocytes or chondrogenic progenitor cells such as mesenchymal stromal cells (MSCs) with a supportive matrix (scaffold), cartilage cell implants could be produced to improve cartilage defect healing.

To evaluate the influence of highly porous alginate-based scaffolds functionalized with chondroitin sulfate (CS) on the cartilage formation (chondrogenesis) by MSCs, the cell adherence, survival and expression of cartilage matrix proteins of MSCs and chondrocytes were analyzed. In the second subpart of the study, a decellularization protocol was established to prepare and optimize a cell-free scaffold made of a natural intervertebral disc-derived extracellular matrix (ECM) from disc tissue. Subsequently, the acellular IVD-derived ECM was repopulated either with allogenic MSCs or IVD cells to compare the reseeding efficacy, cell viability as well as to quantify the collagen and sGAG. The third part of the study focused on the comparative characterization of hip cartilage and chondrocytes derived from patients suffering from OA or femoral neck fracture (FNF). A macroscopic *in-house* scoring system was established and validated to grade OA associated cartilage alterations.

The alginate scaffolds allowed the growth of MSCs and the synthesis of an ECM that contained cartilage-specific type II collagen. The CS functionalization resulted in an increased sGAG synthesis compared with the CS-free scaffolds. The cell-free IVD-ECM was established and successfully re-colonized with MSCs and IVD cells after pretreatment with serum albumin. The total collagen content in the MSCs populated constructs was higher than in those containing IVD cells. The *in-house* scoring system was suitable for detection of OA changes, differed between the two cartilage sources and correlated with histological Mankin score. Cells isolated from OA cartilage showed differences in phenotype compared with cells isolated from the FNF.

The results of this study demonstrated two fundamentally different approaches for obtaining tissue-engineering cartilage implants and help with the assessment of chondrocytes for experimental purposes, which are isolated from the femoral head cartilage discarded after implantation of an endoprosthesis.

EINFÜHRUNG (INTRODUCTION)

Cartilage is characterized by its avascular and aneural conditions as well as its low supply of progenitor cells and the lack of lymphatic drainage, which result in a very limited intrinsic regenerative capacity of chondrocytes, the only cell type in mature cartilage [1]. The results of traumatic cartilage lesions usually are progressive chondral deterioration and eventually osteoarthritis (OA) [2, 3]. OA is characterized by an imbalance between anabolic and catabolic processes in synovial joints [4]. Current operative treatment strategies widely used for well-defined traumatic cartilage defects include osteochondral transplantation (OATS), subchondral bone microfracture, and autologous chondrocyte implantation (ACI) [5-9].

Cartilaginous repair tissue can be produced by chondrocytes, mesenchymal stem cells, and pluripotent stem cells [10]. Given the limitations of differentiated chondrocytes, human bone marrow-derived mesenchymal stem cells (hBM-MSCs) represent a promising cell alternative to support cartilage repair, which have been widely used for tissue repair or regeneration purposes due to their potential for multi-lineage differentiation and self-renewal [11]. To achieve mechanical stability of the implant, 3D organization of the implanted cells supporting chondrogenesis of the cells within the cartilage defect and an appropriate scaffold are needed.

Alginate-based scaffolds, a natural polysaccharide-based polymer, have been widely used as a matrix to support chondrogenesis of BM-MSCs and for articular cartilage tissue engineering [12]. In term of manufacturing stable hydrogels, alginate can be easily polymerized via interaction with cations (such as Ca^{2+}, Cu^{2+} and Sr^{2+}), forming a gel-like and only slowly degradable structure [13, 14]. It can be easily depolymerized using chelators to release the cells. In addition, the alginate gels can be transformed to a foam- or sponge-like structures with highly interconnected pores by using the application of a lyophilisation method, which leads to a structure of higher mechanical strength [15-17]. Alginate is expected to be supportive of chondrogenic differentiation due to the absence of adhesive domains that may inhibit chondrogenesis [18]. Chondroitin sulphate (CS) is the major sulphated glycosaminoglycan (sGAG) component of native cartilage ECM and

its benefit is reported for OA therapy due to having a high negative charge density as well as its ability to stimulate the production of ECM by chondrocytes and its capacity to induce the chondrogenic differentiation of multipotent MSCs [19].

Tissue engineering is also an emerging approach for the treatment of intervertebral disc (IVD) degeneration since it might restore the functionality of native tissues. Although numerous studies have focused on the *nucleus pulposus* (NP) tissue engineering and achieved successes in laboratory settings, IVD tissue engineering without including the *annulus fibrosus* (AF) is deemed to fail for the end stage of disc degeneration [20].

The complex architecture of the IVD remains an obstacle for the development of regenerative approaches to reconstruct IVD and to develop a mechanocompetent scaffold, which supports a differentiated IVD-like phenotype of cells seeded on it. Therefore, an IVD-derived cell-free ECM was prepared in the present study. Decellularized ECMs have brought some efforts in many areas of tissue engineering. The objective of any decellularization process is to maintain as many as possible of the ECM components within a tissue without any loss, damage or disruption. Removing the cellular component in its entirety [21] allows to abolish immunogenicity, which is cell-associated. This aim is implicitly impossible, as any process that disrupts cells and transports the contents from a tissue will necessarily alter the ECM. Practically, the goal is maximizing the removal of cellular material, while minimizing ECM loss and damage [22]. Decellularization of dense cartilage ECM is challenging also due to the demand to create interconnectivity for cell seeding. Decellularization is necessary to remove cellular antigens, which are the target of immunologic attack. Up to now, there exist only few approaches utilizing IVD decellularized ECM [22]. Whether chondrogenically differentiated MSCs can achieve an IVD cell-like phenotype within a natural decellularized IVD ECM scaffold, that presents chondrogenic stimuli by its specific nanotopology and released growth factors bound in the ECM, remains completely unclear and was another topic of this study. For this reason both MSCs and IVD cells were compared as cell sources for scaffold colonization.

Insufficient cartilage repair leads to OA. Within OA there is a shift of the tightly regulated anabolic and catabolic process toward the catabolic state resulting in up-regulation of

inflammatory markers, most notably Interleukin-1β (IL-1β) and Tumor Necrosis Factor-α (TNF-α) [23] which results in cartilage loss. Since OA-cartilage derived chondrocytes might change their metabolic and morphological phenotype, cells should be characterized before using them for experimental purposes such as tissue engineering. Experimentally used human articular chondrocytes derive very often from femoral heads of OA or FNF patients discarded during endoprosthesis implantation. Hence, the aim of the third subproject was to characterize carefully these samples applying a self-designed *in-house* macroscopical score system, a well-established histological score (Mankin score) and in addition, assessing properties of isolated and cultured chondrocytes.

ZIELSTELLUNG (OBJECTIVE)

The first substudy was undertaken to investigate the influence of highly porous alginate-based scaffolds supplemented with CS on the attachment, survival and chondrogenesis of BM-MSCs and articular chondrocytes. The physico-mechanical properties of the scaffolds and morphology, survival, chondrogenesis as well as ECM formation of the cells cultured in the scaffolds was evaluated.

The aim of the second substudy was to establish a decellularized scaffold for IVD tissue engineering. For this purpose firstly the decellularization protocol had to be optimized to maximally remove the cell components, whereby retaining the ECM components. Secondly, the acellular IVD-derived ECM had to be repopulated with either allogenic MSCs or IVD cells using a rotatory culture system to compare the reseeding efficacy and viability of both cell sources. At last, the main cartilage ECM components (collagen and sGAG) of the IVD-ECM scaffolds reseeded with either MSCs (either chondrogenically induced or not), or with IVDCs was quantified.

The third substudy, was undertaken to compare joint cartilage derived from hip OA and FNF patients using both microscopic and, a novel *in-house*, macroscopic scoring system, while further analyzing chondrocyte culture characteristics. In addition, the expression of specific cell surface located complement pathway associated glycoproteins and complement anaphylatoxin receptors was assessed.

METHODIK (MATERIALS AND METHODS)

Fabrication of alginate/chondroitin sulfate (Alg/CS) foams

The scaffolds were prepared and provided for this study by Patcharakamon Nooeaid (Institute of Polymer Materials, Dep. Material Science and Engineering, University of Erlangen-Nuremberg). Sodium alginate and chondroitin-4-sulfate A sodium salt were prepared in deionized water with the ratio of 85/15 wt% of Alg/CS and total concentration of 3 wt/v%. The mixture was stirred at room temperature (RT) for 2 hours. 1 mL of the Alg/CS solution was added into a 48 well-plate, while 100 µL of 0.1 M $CaCl_2 \cdot 2H_2O$ solution was added per well. The gelation reaction was finished after 30 minutes at RT. After this, the gel was placed into the freezer at -20℃ and it was frozen for 24 hours. The frozen samples were lyophilized by using the freeze-drying technique for 24 hours at -50 ℃ under vacuum conditions. Cylindrical three-dimensional (3D) porous Alg/CS-foams were obtained, having dimensions of 8 mm in diameter and 8 mm in height. Then the foams were immersed in 0.5 M $CaCl_2 \cdot 2H_2O$ solution (pH～2) for 4 hours, in order to achieve ionic crosslinking. The crosslinked foams were dried at RT for 24 hours.

Human bone marrow-derived MSCs isolation

BM-MSCs were harvested from human femoral head spongiosa using Biocol gradient. Subsequently, BM-MSCs were resuspended in stem cell expansion medium with 6 factors (EM6F), seeded in T175 culture flasks and incubated at 37°C, 90% air humidity and 5% CO_2. After 3 days, non-adherent cells were discarded, and adherent BM-MSCs were cultured with EM6F medium containing 15% FBS changed every 2-3 days. The cells were grown to sufficient numbers for the experiments by regular passaging at least until passage 4-5, following standard protocols using 0.05% trypsin/0.02% EDTA and re-plated when they reached 80% confluence.

Human chondrocyte isolation

Macroscopically normal human femoral head articular cartilage chips or IVD tissue

fragments discarded during hip joint endoprothesis or anterior lumbar vertebral interbody fusion (lumbar and cervical IVDs) were cut into small slices followed by digestion with pronase (20 mg/mL) in Ham's F-12/Dulbecco's modified Eagle's medium for 30 minutes at 37°C and subsequently with collagenase NB5 at 1 mg/mL in chondrocyte growth medium for 16 hours. Isolated chondrocytes were resuspended in chondrocyte growth medium containing 10% FBS and supplements (1%L-glutamine, 1% essential amino acids, 1% penicillin/streptomycin, 1% partricin, 1% ascorbic acid) and seeded in culture flasks.

Chondrogenic induction of BM-MSCs

Subsequently to the cell expansion process, azacytidine was added to the growth medium (EM6F) for 24 hours. To induce chondrogenic differentiation, BM-MSCs were then cultured for 7 and 14 days in serum-free chondrogenic medium consisting of high-glucose DMEM supplemented with sodium pyruvate, ascorbic acid-2-phosphate, dexamethasone, 25 mg/mL insulin, 25 mg/mL transferrin, and 25 ng/mL sodium selenite), and recombinant human TGF-β1. To determine the chondrogenic differentiation alcian blue (AB, to detect sGAG) staining and type II collagen immunolabellings were performed.

Chondrocytes and BM-MSCs vitality assay

Cell viability was determined by fluorescein diacetate (FDA) /ethidium bromide (EtBr) staining of the cells after 7 and 14 days of cultivation. The seeded alginate or IVD scaffolds were incubated for 2 minutes in a FDA and EtBr solution dissolved in PBS in the dark. The green (living cells, FDA-positive) or red (dead cells, EtBr-positive) fluorescence was monitored by fluorescence microscopy with a digital camera or by confocal laser scanning microscopy.

Decalcification and embedding of cartilage bone cylinders of femoral heads

Cartilage-subchondral bone cylinders (1x1 cm) of twenty-two samples were fixed using 4% paraformaldehyde (PFA) solution. The formic acid solution was changed every 24 hours

while a chemical endpoint test was also carried out on the supernatant to access the rate of decalcification. When chemical testing exhibited no significant residual calcium the samples were rinsed in water and incubated in ammonia solution to ensure neutralization. Samples were then rinsed under running tap water to remove any residual acid. Dehydration involved a series of ascending ethanol solutions. Following this, each sample was embedded in paraffin.

Histological staining

After 14 days of culturing, the seeded alginate and IVD scaffolds were fixed in 4% PFA solution and dehydrated through an ascending ethanol series before they were embedded in paraffin wax. Paraffin tissue blocs were sectioned with a thickness of 5 µm. The general histological examination was performed by a hematoxylin and eosin Y (HE) and alcian blue (AB) staining. All slices were analyzed by light microscopy (Zeiss, Axioskop 40 microscope with camera XC30).

SGAG and DNA quantification

DNA content (per construct) was determined using the CyQuant NF Cell Proliferation Assay Kit with calf thymus DNA as a standard. The absorption shift from λ = 485 nm/ 530 nm was measured immediately. SGAG content (total and percent wet weight) was determined using the DMMB dye binding assay. The results were normalized to the cell number according to the DNA content of the respective lysed scaffold piece.

Collagen quantification

The orthohydroxyproline (OHP) content was quantified via colorimetric reaction with chloramine T and diaminobenzaldehyde, against an OHP standard curve. All supernatants and scaffolds seeded with BM-MSCs or IVD cells were collected at day 7 and 14.

Immunofluorescence labelling

Paraffin sections of seeded alginate scaffolds or cartilage bone cylinders were

deparaffinized. Then, they were immunolabeled for type II, type I collagen or complement receptors (C5aR, C3aR) and complement regulatory proteins (CRPs [CD46, CD55, CD59]) using specific antibodies combined with a counterstaining of the cell nuclei using 4',6-diamidino-2-phenylindole (DAPI). Negative controls included omitting the primary antibody or using mouse IgG_1 as primary antibody during the staining procedure. The primary antibodies (rabbit-anti-human type II, type I collagen, rabbit-anti-human C3aR, mouse-anti-human C5aR, mouse-anti-human CD46, CD59 and goat-anti-human CD55) were diluted in blocking buffer containing Triton X-100 and incubated overnight at 4°C in a humidified chamber. The labeled secondary antibodies (donkey–anti–rabbit- or donkey-anti-mouse–Alexa488 or -Alexa555) were diluted in blocking buffer and incubated for one hour at RT in a dark room.

Decellularization of IVD ECM

IVD fragments were prepared, the transition and NP areas were punched into cylindrical 3D segments using a 6 mm diameter biopsy puncher. Then, the cylindrical IVDs were trimmed into segments. Segments were subjected to six repetitive snap freeze/thaw cycles. Following washing in PBS, the segments were exposed to decellularization Tris-buffer for 24 hours at RT. To inhibit protease activity, phenylmethanesulfonyl fluoride and one Complete Protease Inhibitor Tablet were added to the buffer. The specimens were treated with 2% SDS (24 hours at RT), 0.25% trypsin/0.04% EDTA (30 minutes, at 37°C) and 3% Triton X-100 (48 hours at RT) under continuous agitation.

Recellularization of IVD ECM

Frozen decellularized IVDs were thawed at RT before being rinsed 3 times intensively with sterile PBS. Subsequently, the decellurized matrices were preconditioned in bovine serum albumin (BSA) dissolved in sterile distilled water upon a rotatory device in the incubator with gentle rotation for 24 hours at 37°C. Then, they were rinsed with PBS three times and afterwards, incubated for 24 hours in FBS on the rotatory device under similar conditions. Finally, the discs were washed in PBS and immersed in EM6F or IVD cell growth media. The hBM-MSCs or hIVD cells were cultured for 14 days, whereby

hBM-MSC cultures were maintained either in chondroinductive or non-inductive (control, EM6F) growth medium at 37^{o}C in 5% CO_2. At the time points specified, seeded ECMs were harvested and analyzed using DMMB assay. Cell viability was monitored using FDA /EtBr staining and confocal laser scanning microscopy (Leica, SPE II).

Statistical analysis

Substudy 1

For each experiment and donor, at least three independent experiments were assessed and the values presented as mean ± standard deviation of measurements. Differences between experimental groups were analyzed using two-tailed Wilcoxon test. The unpaired student`s t test was used to compare the differences between the scaffolds types as well as between differentiated or non-differentiated samples based on the data distribution (parametric or not parametric data). Statistical significance was set at a *p*-value of $\leqslant$ 0.05.

Substudy 2

For each experiment and donor, at least three independent experiments were performed and data is presented as mean values with standard deviation. The Grubbs test identified outliers. Data was tested for normal distribution and variance analysis (homogeneity of variance). The Brown–Forsythe test (n=6, α=0.05) was used to determine the equality of group variances before performing ANOVA. Statistical analyses were made using the ordinary one-way ANOVA and Tukey's multiple comparisons test, with *p*-values of $\leqslant$ 0.05 considered statistically significant.

Substudy 3

Data was expressed as an average value with standard deviation or median and analyzed. The Rout test was performed to identify outliers. The Kolmogorov-Smirnov test was used to analyze the data for the presence of a Gaussian distribution. In the presence of a Gaussian distribution: unpaired t-Test was applied to the interval scaled data to compare respective data with the control. The Mann-Whitney test was used for

ordinally scaled values. Statistical significance was set at a *p*-value of $\leqslant$ 0.05. The Pearson coefficient was determined to assess correlations.

ERGEBNISSE (RESULTS)

Substudy 1

Scaffolds had a high porosity ~93-95% with a mean pore sizes of 237 ± 48 µm and 197 ± 61 µm. Most of the cells survived in the scaffolds, whereby the DNA content did not significantly differ. BM-MSCs, but also articular chondrocytes formed rounded clusters within the scaffold pores. The BM-MSCs, irrespective of whether cultured under non/chondrogenic conditions and chondrocytes produced an ECM containing sGAG, types II and I collagen. Total collagen and sGAG contents were higher in differentiated BM-MSCs cultures supplemented with CS than in CS-free foams after 14 days.

Substudy 2

After decellularization, the structure of the IVD became loosen and porous, nevertheless, the tissue microarchitecture was mainly maintained in response to the procedure as shown by HE staining. The modified protocol was effective to prepare mostly cell-free IVD-derived scaffolds. Histological evaluation of DAPI, HE, and alcian blue (AB) stainings revealed only very few discernible cell nuclei in the decellularized scaffolds compared to the native tissue.

BSA preconditioning of the scaffolds before recellularization could apparently protect cells from death, probably caused by toxicity of remanants of SDS. Reseeding of the scaffolds without BSA-treatment led to more dead cells. DAPI, HE and AB stainings indicated that the recolonized cells spread more evenly on the surface of the scaffolds than in the inner part of the scaffold for both hBM-MSCs and hIVDC cells.

The DNA content of scaffolds reseeded with hIVDC cells was significantly higher than in the other groups (native tissue, decellularized scaffolds or decellularized scaffolds seeded with induced and non-induced MSCs). The DNA content in native IVDs was very low and showed no significant difference compared to the decellularized IVD group. The sGAG content in the native tissue was significantly higher than that in the cell-free ECM

and the scaffolds reseeded with hIVD cells and undifferentiated hBM-MSCs. However, the sGAG of the native tissue did not significantly differ compared to the scaffolds seeded with chondrogenically induced hBM-MSCs. The collagen content of the scaffolds reseeded with chondrogenically induced hBM-MSCs was significantly higher than the amount of total collagen in the groups of native IVDs, decellularized IVDs or the decellularized ECM reseeded with hIVDCs.

Substudy 3

A self-designed score system was used to determine the macroscopical grade of OA-associated cartilage degradation. The macroscopical score was significantly higher in patients with hip OA compared with those suffering from FNF. The median of the macroscopical score for OA was 11 whereas that for FNF was 3.5 (p=0.0002). The histological score [24] did not significantly differ between OA and FNF (p=0.0241). The median of OA samples was 5.5 and that of FNF samples was 3. The correlation between the macroscopical and the Mankin scoring results including all patients was significant (p=0.0001) and also that of the OA samples separately (p=0.0357), but not that of the FNF samples separately (p=0.25).

The average number of isolated cells was lower in FNF cartilage than in OA cartilage. It did not correlate with the donor age or the macroscopical score. However, the histological score correlated significantly with the cell number isolated per gram cartilage, so that a higher score went along with a lower cell number.

C3aR receptor protein revealed only a weak immunoreactivity in the investigated samples. C5aR was especially expressed by the cells of the surface cartilage layer. The CRP CD46 could be found in two of five donors analyzed. CD55 was synthesized in the samples of all investigated donors. Signals were not confined to the cells, but also observable in the whole lacunae. Nevertheless, it could not be found in the interterritorial ECM. Protein expression of CD59 was shown in all investigated donors with divergent distribution.

DISKUSSION (DISCUSSION)

The first substudy characterized the chondrogenesis of hBM-MSCs and human articular chondrocytes in Alg-foam based scaffolds supplemented with CS. MSCs could be an ideal option to improve cartilage repair, because they are readily available with not only minimal tumorigenic risk and they have the capacity for considerable cell proliferation to allow sufficient cell expansion and chondrogenic differentiation. The cells used for the experiments revealed a marker expression typical for BM-MSCs . Alginate was chosen in this study as a scaffold basis because of its well known cytocompatibility and ability to stabilize and maintain the rounded chondrocyte morphology as well as the chondrocytic phenotype [25]. The CS supplementation was performed to improve chondrogenic properties of the scaffold, the cell cluster formation induced by the scaffolds might stimulate chondrogenesis via initial intense cell-cell contacts. It is also known that alginate or agarose hydrogels support hMSCs growth for long periods. In the present study Alg-scaffolds were prepared using a freeze-dry technique, leading to uniform, directional and interconnective pore formation within the scaffolds, which resulted in highly porous and interconnected scaffolds giving way to migrating cells in all sides. This strategy allows manufacturing highly porous scaffolds with pore sizes in excess of 100 µm, which is recommended to be optimal for cells in scaffold culture and cartilage regeneration. In addition, large enough porosity (greater than 90%) is as important as pore size, all of which are indispensable to make transfer of cells and metabolites more effectively [26]. In addition, the structural integrity of the Alg-foams was maintained over 6 weeks of immersion in PBS solution, while the Alg/CS-foams started to decompose in PBS solution after 3 weeks of immersion. This phenomenon is suggested to be the result of higher water absorption capability of Alg/CS-foams, which is caused by the negative charges of CS [27]. Based on the physico-mechanical properties (porous structure, biodegradation and mechanical properties), it can be concluded that the Alg/CS-foams presented in the present work are suitable to be used as a scaffold for cartilage tissue engineering applications.

In our second substudy with the topic to prepare a natural IVD-derived ECM scaffold,

snap freeze-thaw cycles in the beginning of the decellularization procedure could facilitate cell devitalization and increase the efficiency of cell removal. Freezing and thawing helps to open up the ECM and facilitate the out-diffusion of the cellular contents and chemicals. In the meantime, the production of ice crystals may produce gaps between the collagen fibers [28] needed for cell entry. However, the results presented here demonstrate, that cell penetration into the decellularized IVD remained still limited at day 14. Obviously, even after the extensive decellularization procedure, the decellularized scaffolds remained so dense that most cells could only colonize the scaffold surface. The decellularization efficacy of detergents depends on the structure of tissue. The composition of IVD differs from hyaline articular cartilage since it consists of fibro-cartilage. Our pre-experiments (data not shown) indicated that the IVD decellularization with 2% SDS for 8 hours was not as effective like a 24 hours lasting processing of the IVD tissue. Therefore, we used 2% SDS for 24 hours. Our initial experiments indicated that the utilization of SDS in the decellularization procedure could affect cell survival. However, the histological IVD structure was barely altered. The DNA content in native IVD tissue was low and no significant difference compared to the decellularized IVD control group was detected. As histological stainings revealed only very few remaining cell nuclei, we assume that although SDS treatment achieved complete lysis of cell membranes and nuclear membranes, the majority of nuclear debris could not be completely expelled from the compact tissue and still remained in the scaffold. The residual intracellular and intercellular ice crystals during the freeze-thaw process might prevent the cell remnants from exiting the discs [29]. It revealed also that the decellularization protocol applied could preserve a certain extent of sGAG content during the decellularization procedure. We suppose that the divergence between the histological staining and the biochemical quantification could be caused by regional inhomogeneities in the IVD and possibly by interference of reagents of the decellularization solution such as SDS and Triton X-100 captured within the ECM. The collagen content of the chondrogenically induced hBM-MSCs group was significantly higher than others. Therefore, we presume that the decellularized IVD ECM scaffolds here might still provide some stimulating mediators such as residual growth factors,

cytokines or ECM motifs, which facilitate the chondrogenesis of MSCs and induce collagen synthesis. Furthermore, the scaffolds maintained the annular collagen architecture, typical for the IVD, which might induce increased collagen deposition. This substudy showed that most of the reseeded cells survived on decellularized scaffolds after 14 days of cultivation, however, the grade of immunogenic rejection has to be analyzed in future performing *in vivo* experiments.

In the third substudy a simple *in-house* macroscopical score was established and was approved to distinguish FNF- and OA-cartilage samples. It is advisable to categorize the quality of cartilage used for chondrocyte isolation and subsequent experiments since chondrocyte phenotype differed between FNF- or OA-cartilage and cells might therefore, respond differentially to various stimuli. Since it is known that complement plays a role in OA [30], some representative samples were analyzed for complement expression, Expression of complement receptors (C5aR, C3aR) and regulatory proteins (CD55, CD59) were detected in all analyzed samples, while CD46 could only be detected in two of the five investigated donors. Since we found in a previous study no CD35 expression in cartilage, it was not further adressed here [31]. Like CD35, CD46 inhibits the opsonins C3b and C4b, whereby CD46 promotes the proteolysis of these opsonins as a cofactor [32]. Possibly these opsonins play no major role in OA, but rather in rheumatoid cartilage disorders. In contrast, CD55 inhibits the upstream cleavage of C3 and C5 by antagonizing formation of the respective C3 and C5 convertases as prerequisites for ongoing complement cascade activation and terminal MAC formation. Investigation of a higher number of OA and non-OA samples of different grades would indicate, whether down-regulation of cytoprotective CD55 and CD59 might be directly associated with different OA stages (early or late) in humans.

Conclusion and Outlook

This *in vitro* study proposed two different tissue-engineering-based approaches for repair of two selected cartilaginous tissues based on chondrogenic progenitor cells and either a synthesized or a natural scaffold. Both approaches allowed chondrogenesis of MSCs.

The third substudy indicated that the cartilage layer of explanted femoral heads can be graded for OA using a simple in-house score which can also be used by unexperienced lab staff. Chondrocytes derived from OA cartilage show a slightly modified phenotype in culture a fact that has to be considered in future *in vitro* experiments.

LITERATURVERZEICHNIS (REFENRENCES)

1 Steinert AF, Ghivizzani SC, Rethwilm A, Tuan RS, Evans CH, Noth U. Major biological obstacles for persistent cell-based regeneration of articular cartilage. Arthritis research & therapy. 2007;9(3):213. doi:10.1186/ar2195.

2 Logerstedt DS, Snyder-Mackler L, Ritter RC, Axe MJ, Orthopedic Section of the American Physical Therapy A. Knee pain and mobility impairments: meniscal and articular cartilage lesions. The Journal of orthopaedic and sports physical therapy. 2010;40(6):A1-A35. doi:10.2519/jospt.2010.0304.

3 Mandelbaum BR, Browne JE, Fu F, Micheli L, Mosely JB, Jr., Erggelet C et al. Articular cartilage lesions of the knee. The American journal of sports medicine. 1998;26(6):853-61.

4 Loeser RF. Molecular mechanisms of cartilage destruction in osteoarthritis. Journal of musculoskeletal & neuronal interactions. 2008;8(4):303-6.

5 Asik M, Ciftci F, Sen C, Erdil M, Atalar A. The microfracture technique for the treatment of full-thickness articular cartilage lesions of the knee: midterm results. Arthroscopy : the journal of arthroscopic & related surgery : official publication of the Arthroscopy Association of North America and the International Arthroscopy Association. 2008;24(11):1214-20. doi:10.1016/j.arthro.2008.06.015.

6 Bekkers JE, Inklaar M, Saris DB. Treatment selection in articular cartilage lesions of the knee: a systematic review. The American journal of sports medicine. 2009;37 Suppl 1:148S-55S. doi:10.1177/0363546509351143.

7 Bugbee WD, Convery FR. Osteochondral allograft transplantation. Clinics in sports medicine. 1999;18(1):67-75.

8 Chu CR, Convery FR, Akeson WH, Meyers M, Amiel D. Articular cartilage transplantation. Clinical results in the knee. Clinical orthopaedics and related research. 1999(360):159-68.

9 Brittberg M, Lindahl A, Nilsson A, Ohlsson C, Isaksson O, Peterson L. Treatment of deep cartilage defects in the knee with autologous chondrocyte transplantation. The New England journal of medicine. 1994;331(14):889-95. doi:10.1056/NEJM199410063311401.

10 Tuan RS, Chen AF, Klatt BA. Cartilage regeneration. The Journal of the American Academy of Orthopaedic Surgeons. 2013;21(5):303-11. doi:10.5435/jaaos-21-05-303.

11 Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD et al. Multilineage potential of adult human mesenchymal stem cells. Science. 1999;284(5411):143-7. doi:DOI 10.1126/science.284.5411.143.

12 Safran MR, Kim H, Zaffagnini S. The use of scaffolds in the management of articular cartilage injury. The Journal of the American Academy of Orthopaedic Surgeons. 2008;16(6):306-11.

13 Kuo CK, Ma PX. Maintaining dimensions and mechanical properties of ionically crosslinked alginate hydrogel scaffolds in vitro. Journal of biomedical materials research Part A. 2008;84(4):899-907. doi:10.1002/jbm.a.31375.

14 Stevens MM, Qanadilo HF, Langer R, Prasad Shastri V. A rapid-curing alginate gel system:

utility in periosteum-derived cartilage tissue engineering. Biomaterials. 2004;25(5):887-94.

15 Despang F, Börner A, Dittrich R, Tomandl G, Pompe W, Gelinsky M. Alginate/calcium phosphate scaffolds with oriented, tube-like pores. Materialwissenschaft und Werkstofftechnik. 2005;36(12):761-7. doi:10.1002/mawe.200500961.

16 Nooeaid P, Roether JA, Weber E, Schubert DW, Boccaccini AR. Technologies for Multilayered Scaffolds Suitable for Interface Tissue Engineering. Advanced Engineering Materials. 2014;16(3):319-27. doi:10.1002/adem.201300072.

17 Petrenko YA, Ivanov RV, Petrenko AY, Lozinsky VI. Coupling of gelatin to inner surfaces of pore walls in spongy alginate-based scaffolds facilitates the adhesion, growth and differentiation of human bone marrow mesenchymal stromal cells. Journal of materials science Materials in medicine. 2011;22(6):1529-40. doi:10.1007/s10856-011-4323-6.

18 Connelly JT, Garcia AJ, Levenston ME. Interactions between integrin ligand density and cytoskeletal integrity regulate BMSC chondrogenesis. Journal of cellular physiology. 2008;217(1):145-54. doi:Doi 10.1002/Jcp.21484.

19 Kubo M, Ando K, Mimura T, Matsusue Y, Mori K. Chondroitin sulfate for the treatment of hip and knee osteoarthritis: current status and future trends. Life sciences. 2009;85(13-14):477-83. doi:10.1016/j.lfs.2009.08.005.

20 Jin L, Shimmer AL, Li X. The challenge and advancement of annulus fibrosus tissue engineering. European spine journal : official publication of the European Spine Society, the European Spinal Deformity Society, and the European Section of the Cervical Spine Research Society. 2013;22(5):1090-100. doi:10.1007/s00586-013-2663-2.

21 Crapo PM, Gilbert TW, Badylak SF. An overview of tissue and whole organ decellularization processes. Biomaterials. 2011;32(12):3233-43. doi:10.1016/j.biomaterials.2011.01.057.

22. Gilbert TW. Strategies for tissue and organ decellularization. Journal of cellular biochemistry. 2012;113(7):2217-22. doi:10.1002/jcb.24130.

23 Haseeb A, Haqqi TM. Immunopathogenesis of osteoarthritis. Clinical immunology. 2013;146(3):185-96. doi:10.1016/j.clim.2012.12.011.

24 Mankin HJ, Dorfman H, Lippiello L, Zarins A. Biochemical and metabolic abnormalities in articular cartilage from osteo-arthritic human hips. II. Correlation of morphology with biochemical and metabolic data. The Journal of bone and joint surgery American volume. 1971;53(3):523-37.

25 Connelly JT, Wilson CG, Levenston ME. Characterization of proteoglycan production and processing by chondrocytes and BMSCs in tissue engineered constructs. Osteoarthritis and cartilage / OARS, Osteoarthritis Research Society. 2008;16(9):1092-100. doi:10.1016/j.joca.2008.01.004.

26 Harley BA. Cell-matrix interactions: collagen-GAG scaffold fabrication, characterization, and measurement of cell migratory and contractile behavior via confocal microscopy. ScD Thesis, Mechanical Engineering, Massachusetts Institute of Technology. 2006:393.

27 Jerosch J. Effects of Glucosamine and Chondroitin Sulfate on Cartilage Metabolism in OA: Outlook on Other Nutrient Partners Especially Omega-3 Fatty Acids. International journal of rheumatology. 2011;2011:969012. doi:10.1155/2011/969012.

28 Kheir E, Stapleton T, Shaw D, Jin Z, Fisher J, Ingham E. Development and characterization of an acellular porcine cartilage bone matrix for use in tissue engineering. Journal of biomedical materials research Part A. 2011;99(2):283-94. doi:10.1002/jbm.a.33171.

29 Chan LK, Leung VY, Tam V, Lu WW, Sze KY, Cheung KM. Decellularized bovine intervertebral disc as a natural scaffold for xenogenic cell studies. Acta biomaterialia. 2013;9(2):5262-72. doi:10.1016/j.actbio.2012.09.005.

30 Wang Q, Rozelle AL, Lepus CM, Scanzello CR, Song JJ, Larsen DM et al. Identification of a central role for complement in osteoarthritis. Nature medicine. 2011;17(12):1674-9. doi:10.1038/nm.2543.

31 Schulze-Tanzil G, Kohl B, El Sayed K, Arens S, Ertel W, Stolzel K et al. Anaphylatoxin receptors and complement regulatory proteins in human articular and non-articular chondrocytes: interrelation with cytokines. Cell and tissue research. 2012;350(3):465-75. doi:10.1007/ s00441-012-1497-2.

32 Piccoli AK, Alegretti AP, Schneider L, Lora PS, Xavier RM. Expression of complement regulatory proteins CD55, CD59, CD35, and CD46 in rheumatoid arthritis. Revista brasileira de reumatologia. 2011;51(5):503-10.

Eidesstattliche Versicherung

"Ich, Zhao Huang, versichere an Eides statt durch meine eigenhändige Unterschrift, dass ich die vorgelegte Dissertation mit dem Thema: "*In vitro* differentiation of chondrogenic cells in three-dimensional scaffold-assisted culture for cartilage repair and characterization of cartilage sources" selbstständig und ohne nicht offengelegte Hilfe Dritter verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel genutzt habe.

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Datum Unterschrift

ANTEILSERKLÄRUNG

Der Promovend Zhao Huang hatte folgenden Anteil an der vorgelegten Publikation:

Huang Z, Nooeaid P, Kohl B, Roether JA, Schubert DW, Meier C, Boccaccini AR, Godkin O, Ertel W, Arens S, Schulze-Tanzil G. Chondrogenesis of human bone marrow mesenchymal stromal cells in highly porous alginate-foams supplemented with chondroitin sulfate. Mater Sci Eng C Mater Biol Appl. 2015 May; 50:160-72. http://dx.doi.org/10.1016/j.msec.2015.01.082. Epub 2015 Jan 27. First authorship.

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Ausgewählte Publikationen

Publikation 1:

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Huang Z, Kohl B, Kokozidou M, Arens S, Schulze-Tanzil G.

Establishment of a cytocompatible cell-free intervertebral disc matrix for chondrogenesis with human bone marrow mesenchymal stromal cells.

Cells, tissues, organs. 2016; 201:354-365. http://dx.doi.org/10.1159/000444521. First authorship.

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Quellenangabe: http://dx.doi.org/10.1016/j.msec.2015.01.082.

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Macroscopical, Histological, and *In Vitro* Characterization of Nonosteoarthritic Versus Osteoarthritic Hip Joint Cartilage

Jessica Badendick[1,*], Owen Godkin[1,*], Benjamin Kohl[1], Carola Meier[1], Michal Jagielski[1], Zhao Huang[1], Stephan Arens[1], Tobias Schneider[1,2] and Gundula Schulze-Tanzil[1,2]

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ABSTRACT: Osteoarthritis (OA) might affect chondrocyte culture characteristics and complement expression. Therefore, this study addressed the interrelation between macroscopical and microscopical structure, complement expression, and chondrocyte culture characteristics in non-OA and OA cartilage. Femoral head cartilage samples harvested from patients with femoral neck fractures (FNFs) and OA were analyzed for macroscopical alterations using an in-house scoring system, graded histologically (Mankin score), and immunolabeled for complement regulatory proteins (CRPs) and receptors. Morphology of monolayer cultured chondrocytes isolated from a subset of samples was assessed. The macroscopical score distinguished the FNF and OA cartilage samples and correlated significantly with the histological results. Chondrocyte phenotype from FNF or OA cartilage differed. Complement receptor C5aR, CRPs CD55 and CD59, and weakly receptor C3AR were detected in the investigated FNF and OA cartilage, except for CD46, which was detected in only two of the five investigated donors. The in-house score also allows inexperienced observers to distinguish non-OA and OA cartilage for experimental purposes.

KEYWORDS: osteoarthritis, chondrocyte, hip joint cartilage, complement regulatory proteins, complement receptors, Mankin score

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Introduction

Osteoarthritis (OA) is a chronic disease, which affects, depending on age, a large number of patients. More than 60% of the German population suffers from OA by the time of their 65th birthday. In combination with demographic changes, OA has led to an enormous rise of health-care costs over the past two decades.[1] Various nonmodifiable entities such as age, genetic, metabolic, biochemical, biomechanical, and modifiable factors, such as obesity, are all suspected to support this degenerative change.[2,3] It has been observed that OA is initiated by multiple microtraumas to the joint or one single traumatic event.[4] However, the exact etiology remains unclear. Unfortunately, there is currently no effective biological and/or pharmacological therapy available for OA, despite some efforts in OA-related research. Within OA, there is a shift of the tightly regulated anabolic and catabolic process toward the catabolic state resulting in the upregulation of inflammatory markers, most notably interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α).[3] Numerous animal model studies have shown the role of IL-1β in the upregulation of catabolic enzymes matrix metalloproteinases (MMP)-1, -3, and -13, while also identifying its ability to suppress the anabolic process, within the extracellular matrix (ECM), by inhibiting type II collagen and proteoglycan expression in chondrocytes.[5] Furthermore, IL-1β may initiate apoptosis via production of nitric oxide or reactive oxygen species within the chondrocyte.[6] Similarly, TNF-α exhibits enhanced expression not only in traumatic articular cartilage but also in OA synovium, inducing the same MMP enzymes as IL-1β, which suggests a synergistic catabolic effect during OA.[7] This loss of homeostasis between anabolic and catabolic factors might contribute to activation of the complement cascade as recently demonstrated for OA pathogenesis.[8] Complement activation results in the secretion of chemotaxins arising from cleaved complement factors, such as anaphylatoxins C3a and C5a. In addition, it induces opsonization of pathogenic particles, and finally, pathogen or cell lysis by membrane attack complexes (MACs).[8] The complement split fragments C3a and C5a bind to anaphylatoxin receptors, C3aR and C5aR, and induce a multiple inflammatory response, including cytokine release, vasoconstriction and permeability, histamine release,

and leukotaxis, as well as platelet aggregation.[9–11] Complement regulatory proteins (CRPs) protect cells from lysis induced by complement activation. The anaphylatoxin receptors are expressed on various cell types.[12] Interestingly, C5aR expression was observed as normal in OA and rheumatoid cartilage, while its upregulation was noted in normal, rheumatoid arthritis samples, but not in OA cartilage, when chondrocytes were stimulated by IL-1β.[13] In addition to C5aR, we wanted to demonstrate the expression of C3aR and crucial CRPs in OA and non-OA cartilage. We hypothesized in 2007 that complement expression could be involved in OA[14] and demonstrated the complement regulation by cytokines in cultured chondrocytes, in cartilage *in situ* as well as in another joint-associated musculoskeletal connective tissue cell type, tenocytes, and altered/healthy tendon tissue.[15–17] An impaired expression of particular CRPs could contribute to OA[8] and was, therefore, investigated here.

Femoral neck fracture (FNF) is a typical feature of older patients suffering from sarcopenia and osteoporosis.[18,19] An FNF usually cannot be treated by conservative strategies due to loss of femoral head vascularization. Therefore, endoprothetic joint replacement of the hip is usually performed. OA diagnosis is based mainly on clinical mobility and pain scores, eg, Western Ontario and McMaster Universities Arthritis Index, visual analog scale, and radiologic measurements, such as joint space narrowing (eg, Kellgren score). Histology provides more detailed information about OA progression in cartilage, but it cannot be applied in the clinical routine. When human articular cartilage is recruited for *in vitro* studies, latent OA might markedly influence chondrocyte culture characteristics. For this reason, we developed an in-house macroscopic scoring system to categorize cartilage samples in a first look manner with respect to OA-related changes. Other scoring systems such as ICRS, O'Driscoll, MOCART, OAS scores, or scores adapted and proposed by Goebel et al, 2012, are specialized to assess the degree of cartilage repair of local defects and might not be suitable for less experienced persons.[20–23]

Therefore, the aim of this study was to compare joint cartilage derived from FNF and hip OA patients using both microscopic and, a novel in-house, macroscopic scoring system while further analyzing chondrocyte culture characteristics. In addition, the expression of specific cell surface-located complement pathway-associated glycoproteins and complement anaphylatoxin receptors was assessed.

Materials and Methods

Samples. Samples of 37 patients (17 males, 20 females; 18 FNF, 19 hip OA; Table 1) were harvested during joint replacement surgery in accordance with the institutional ethics committee of the Charité-Universitätsmedizin Berlin, Campus Benjamin Franklin. The research was conducted in accordance with the principles of the Declaration of Helsinki, and patients gave their written, informed consent to participate. The average age of all donors included in the study was 68.72 years. The age range was between 30 and 91 years. In a subset of samples (15 donors), primary human chondrocytes were isolated from the femoral heads of patients who underwent joint replacement due to OA (4 donors) and FNF (11 donors).

Table 1. Donor characteristics of samples included.

Total number of donors	**37**			
Gender	Male	Female		
	17 (45.9%)	20 (54.1%)		
Age	≤60	≤75	≤90	≤100
	6 (16.2%)	21 (56.8%)	9 (24.3%)	1 (2.7%)
Diagnosis	OA	FNF		
	19 Donors (51.3%)	18 Donors (48.7%)		

Macroscopical scoring. All femoral heads included in this study were scored macroscopically using the self-designed score before the joint cartilage was scratched off for chondrocyte isolation. Observers (one or two observers per time point, but different observers at different time points) were blinded for the diagnosis (FNF/OA). If two observers were included, the results were averaged. The topics such as color, extent of lesions (quadrants affected), consistency, and cartilage thickness were addressed during the scoring process as summarized in Table 2.

Isolation and culturing of articular chondrocytes. Apart from a 1-cm^2 area reserved for histology, cartilage chips were harvested from macroscopically, mostly unaffected cartilage areas of human femoral head articular cartilage with a macroscopical score of ≤9, since at higher scores, the amount and quality of the remaining cartilage appeared too low for successful cell isolation. The rationale to also include OA-affected cartilage was to study the effect of the OA milieu in the joint on chondrocyte phenotype. The harvested cartilage was weighed and cut into small slices (~1 mm^2) followed by digestion with pronase derived from *Streptomyces griseus* at 20 mg/mL (7 U/mg, Roche Diagnostics) in Ham's F-12/Dulbecco's modified Eagle's medium (50/50, Biochrom-Seromed) for 30 minutes at 37 °C. Subsequently, the samples were treated with collagenase NB5 derived from *Clostridium histolyticum* at 1 mg/mL (Serva) in chondrogenic growth medium and slowly stirred in a spinner flask overnight. Isolated chondrocytes were resuspended in growth medium (Ham's F-12/Dulbecco's modified Eagle's medium) containing 10% fetal bovine serum (Biochrom AG), ascorbic acid (25 µg/mL), 1% essential amino acids, partricin (0.5 µg/mL), and penicillin/streptomycin (both: 50 IU/mL). Cell vitality after cell isolation was determined using trypan blue exclusion assay. Cells were counted and then seeded in culture flasks (Cell plus culture flask; Sarstedt AG) at 42,857.142 cells/cm^2 for cell expansion and subsequent storage in liquid nitrogen at passage 1.

Table 2. Self-designed macroscopical scoring system.

	GRADE
Osteoarthritis macroscopically	
Color	
White, Beige	0
Yellowish	1
Gray-brown/Other discolorations	2
Expansion of osteoarthritic changes	
How many quadrants of the femoral head contain recognizable arthritic tissue?	
No osteoarthritis	0
Up to 25%	1
More than 25% up to 50%	2
Up to 75%	3
More than 75% up to 100%	4
Degree of severity of osteoarthritic changes?	
Healthy	0
Little	1
Moderate	2
Severe	3
Cartilage consistency (when cutting)	
Normal	0
Soft	1
Mushy	2
Cartilage thickness	
Normal (ca. >1 mm)	0
Focally thin (clearly <1 mm)	1
Mostly thin	2
Special	
Cartilage–Bone–Osteophytes (chondro-/osteophytes), defects	
complete cartilage loss (no points)	
Maximal: 13 score points	

Culture morphology. For the measurement of cell morphology and the number of adherent cells, freshly isolated cells (passage 0) of 15 donors were seeded into Petri dishes (35 mm diameter) at 52,083.33 cells/cm^2 and cultured for 72 hours. Measurement was taken using cell^D software at ×100 magnification under a light microscope (Axioskop 40, Zeiss), while cellular morphology, including length (µm), width (µm), and overall cell area (µm^2), was assessed at ×200 magnification. Images were taken at ×100 using a XC30 camera system (Olympus, Europa Holding).

Decalcification and embedding of cartilage bone cylinders. A 1-cm^2 cartilage–subchondral bone sample was fixed using 4% paraformaldehyde (PFA) solution (USB, USA) for three days. Twenty-two samples were recruited for histology (derived from 11 female and 11 male donors, 16 of them suffering from FNF and 6 from OA). The samples were placed in a volume 20 times their own volume of formic acid to ensure satisfactory decalcification. The acid solution was changed every 24 hours, while a chemical end point test was also carried out on the supernatant to access the rate of decalcification. When chemical testing exhibited no significant residual calcium, the samples were rinsed in water and incubated in 5% ammonia solution (Merck KGaA) for 30 minutes to ensure neutralization. Samples were then rinsed under running tap water for 24 hours to remove any residual acid. Dehydration involving a series of ascending ethanol solutions was completed over a 48-hour period. Following this, each sample was embedded in paraffin at 60 °C with subsequent sectioning using a microtome Microm HM 325 (Thermo Fisher Scientific).

Histological staining. The histological staining was conducted on paraffin sections (thickness between 3 and 5 µm). Paraffin was removed using a series of descending alcohol solutions. For hematoxylin and eosin (H&E) staining, the sections were incubated for 4 minutes in Harris hematoxylin solution (Sigma-Aldrich), briefly rinsed in tap water and then counterstained for 1.5 minutes in eosin Y (Sigma-Aldrich). For the safranin-O staining, the slides were incubated for 10 minutes in Weigert's iron hematoxylin (Carl Roth GmbH) and afterward rinsed under running tap water for 10 minutes. Subsequently, each section was stained in 0.001% fast green solution (Sigma-Aldrich) for five minutes. After rinsing the slides briefly in 1% acetic acid, the slices were stained in 0.1% safranin-O solution (Merck KGaA) for up to five minutes. For alcian blue (AB) staining, the sections were incubated for 3 minutes in 1% acetic acid followed by 1% AB (Carl Roth GmbH) for 30 minutes. Then, they were rinsed in 3% acetic acid and washed in deionized water (A. dest.) (two minutes). Additionally, cell nuclei were counterstained in nuclear fast red aluminum sulfate solution (Carl Roth GmbH) for five minutes.

Finally, all the slides were dehydrated using an ascending alcohol series and mounted with Entellan mounting medium (Merck KGaA). Images of the slides were taken using the Axioskop 40 light microscope and the Olympus digital camera XC30.

Microscopical scoring for OA. The stained sections were further analyzed by applying the Mankin score[24,25] to them by one observer. The observers were blinded for the diagnosis (FNF, OA) of the cartilage donors. Both cartilage structure and chondrocyte distribution were scored, while safranin-O and AB staining allowed the grading according to the amounts of glycosaminoglycans detectable in the sample. Finally, tidemark integrity was assessed for blood vessel penetrance.

Immunofluorescence labeling. Paraffin sections of five donors (two with OA, two with FNF, and one with OA and FNF) were immunolabeled for complement receptors (C5aR and C3aR) and CRPs (CD46, CD55, and CD59) using specific antibodies combined with a counterstaining of the cell nuclei by 4′,6-diamidino-2-phenylindole (DAPI). The sections were deparaffinized and fixed in 4% PFA before being rinsed for two minutes in tris-buffered saline (TBS; 0.05M Tris, 0.015M NaCl, pH 7.6). The sections were overlaid for 20 minutes with 0.1% pronase solution at 37 °C for demasking and rinsed two times

with TBS (2 minutes). Then, a 20-minute incubation period at room temperature (RT) with 100 µL protease-free blocking buffer (5% donkey serum [Merck Millipore] diluted in TBS) followed. Negative controls included omitting the primary antibody or using mouse IgG_1 as primary antibody during the staining procedure. The primary antibodies (rabbit anti-human C3aR [1:30, Assay Biotechnology], mouse anti-human C5aR [1:100, GeneTex, Biozol], mouse anti-human CD46, CD59 [1:20 and 1:40, both AbD Serotec], and goat anti-human CD55 [1:40, R&D Systems]) were diluted in blocking buffer containing 0.1% Triton X-100 (Sigma-Aldrich) and incubated overnight at 4 °C in a humidified chamber. After rinsing three times with TBS, the labeled secondary antibody (donkey anti-rabbit or donkey anti-mouse-Alexa488 or -Alexa555, 10 mg/mL; Life Technologies Corporation) was diluted 1:200 in blocking buffer containing 0.1% Triton X-100 and 0.1 µg/µL DAPI and incubated for one hour at RT in a dark room. Labeled sections were rinsed with TBS three times and covered with Fluoromount G (Biozol Diagnostica). The fluorescence images were acquired using a confocal laser scanning microscope (SPEII; Wetzlar Leica).

Statistical analysis. Data were expressed as an average value with standard deviation or median and analyzed using GraphPad Prism 5. The ROUT test was performed to identify outliers. If applicable, the Kolmogorov–Smirnov test was used to analyze the data for the presence of a Gaussian distribution. In the presence of a Gaussian distribution, unpaired *t*-test was applied to the interval scaled data (Fig. 4: femoral head diameter, Fig. 5B: cell numbers) to compare the respective data with the control. The Mann–Whitney test was used for ordinally scaled values, for which a Gaussian cannot be determined (Fig. 3: score values). Statistical significance was set at a *P* value of ≤0.05. The Pearson coefficient was determined to assess correlations.

Results

Patients. Patients were sorted according to their age following the WHO classification as follows: less than 60 or 60 years (aging), between 61 and 75 years (old), between 76 and 90 years (older), and between 91 and 100 years (very old). Most of them were between 61 and 75 years (21, 56.76%) and females (20, 54.05%). The average age of the OA cartilage donors included was 63.68 years, whereas that of the FNF cartilage donors was 74 years. The body mass index (BMI) was significantly higher in OA compared with FNF patients (Table 3). The percentage of each comorbidity (diabetes, cardiovascular diseases, infection, and kidney disease) was generally higher in the FNF patients (Table 3).

Table 3. Additional donor characteristics of FNF and OA samples.

FNF: Donor number	**18**			
Gender	Male 50% (9)	Female 50% (9)		
Age[a]	≤60 5.6% (1)	61–75 50% (9)	76–90 38.8% (7)	91–100 5.6% (1)
BMI (Average)		24 ± 2.84		
Comorbidities	Diabetes 16.7% (3)	Cardiovascular disease 72.2% (13)	Infection 27.8% (5)	Kidney disease 55.6% (10)
OA: Donor number	**19**			
Gender	Male (8) 42.1%	Female (11) 57.9%		
Age	≤60 26.3% (5)	61–75 63.2% (12)	76–90 10.5% (2)	91–100 0% (0)
BMI[b] (Average)		27 ± 5		
Comorbidities	Diabetes 5.3% (1)	Cardiovascular disease 47.4% (9)	Infection 10.5% (2)	Kidney disease 5.3% (1)

Note: [a]The FNF donors were significantly older than the OA donors. [b]The FNF donors had a significantly lower BMI than the OA donors.

Grading of OA using the self-designed macroscopical scoring system and the Mankin score for histological scoring. A self-designed score system was used to determine

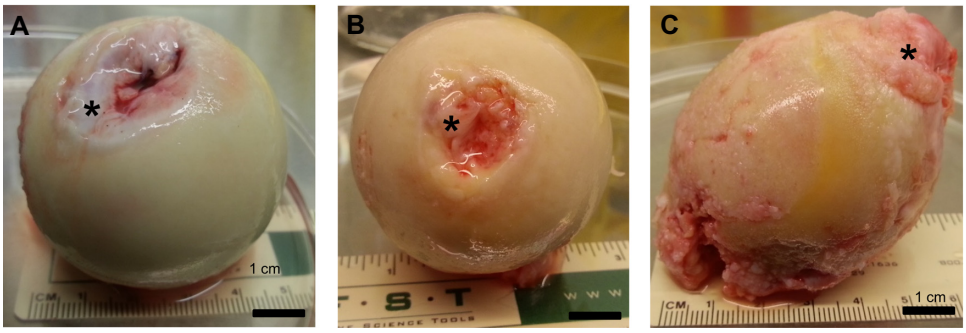

Figure 1. Exemplary macroscopical images of explanted femoral heads and scoring results. Depicted are femoral heads of three representative donors. (**A**) FNF, macroscopical score 1, (**B**) OA, macroscopical score 2, and (**C**) OA, macroscopical score 12.
Notes: *Insertion of the femoral head ligament. Scale bar: 1 cm.
Abbreviation: Macrosc., macroscopical.

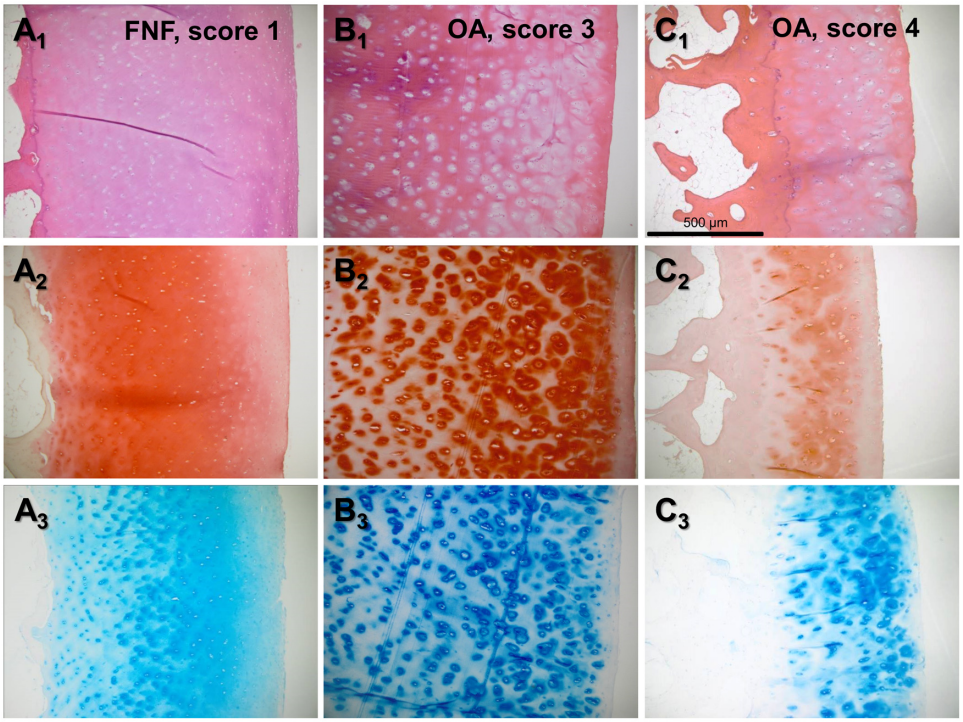

Figure 2. Exemplary histological images of cartilage samples. H&E (A_1–C_1), Safanin O (A_2–C_2), and AB (A_3–C_3) stained paraffin sections of femoral head articular cartilage of three representative donors are shown. (**A**) FNF, Mankin score 1, (**B**) OA, Mankin score 3, and (**C**) OA, Mankin score 4.
Note: Scale bar: 500 µm.

the macroscopical grade of cartilage degradation (Table 2 and Fig. 1A–C). Using this scoring system, the same samples were evaluated by inexperienced versus experienced observers. The differences of the scoring results between both observer groups were not significant (data not shown). For histological evaluation of OA-associated changes (Fig. 2), the Mankin score was used. The macroscopical score was significantly higher in patients with hip OA compared with those suffering from FNF (Fig. 3A). The median of the macroscopical score for OA was 11, whereas that for FNF was 3.5 (P = 0.0002). The histological score did not significantly differ between FNF and OA (P = 0.0241). The median of FNF samples was 3 and that of OA samples was 5.5 (data not shown). The correlation between the macroscopical and the Mankin scoring results including all the patients was significant (P = 0.0001) and also that of the OA samples separately (P = 0.0357), but not that of the FNF samples separately (P = 0.25; Fig. $3B_{1–3}$).

Femoral head diameters. When the femoral head diameters were measured using the X-ray images, the male femoral heads were, as expected, significantly larger compared with the female (P = 0.0001), but no significant differences between FNF- and OA-derived femoral heads could be detected (P = 0.313; Fig. 4A and B).

Chondrocyte morphology, adherence, and vitality. Cells were isolated enzymatically, and the percentage of adherent cells as well as cell morphology (overall cell area, length, and width) was determined after 72 hours of culturing (Fig. $5A_{1–2}$). No significant correlation could be detected between the cell density of cultured chondrocytes and the macroscopical score (P = 0.673; data not shown). The cell density in OA-derived chondrocyte cultures was lower than that in FNF samples (P = 0.0264, Fig. 5A and B and Table 4). The percentage of dead cells (trypan blue positive) after isolation was around 3%. The number of isolated cells showed high variance (between 0.86 and 7.1 mio isolated chondrocytes/g cartilage were counted [data not shown]). The average number of isolated cells was lower in FNF cartilage (2.21 mio isolated chondrocytes/g) than in OA cartilage (3.84 mio isolated chondrocytes/g; Table 4, not significant). It did not correlate with the donor age (P = 0.582; data not shown) or the macroscopical score (P = 0.382, Fig. 5C). However, the histological score correlated significantly with the cell number per gram (P = 0.031, Fig. 5D), so that a higher score went along with a lower cell number. Nevertheless, the number of OA samples (n = 4) versus FNF (n = 11) used for cell isolation was too low to calculate a valid correlation. As determined for FNF and OA samples separately, after 72 hours of culturing, an average 67.18% versus 73.25% of the cells adhered, detectable by an elongated shape with focal adhesion sites formation (Fig. $5A_1$, Table 4). The average cell density of FNF and OA samples together was 366 cells/mm^2 (data not shown). The average cytoplasmic area of all samples (FNF and OA) was 630 µm^2 with a length of 62.27 µm and a width of 17.93 µm (data not shown). FNF samples had a smaller cell area (559.45 versus 824 µm^2, not significant, P = 0.104), and the cells were shorter (59.27 versus 70.5 µm) than the cells of OA samples

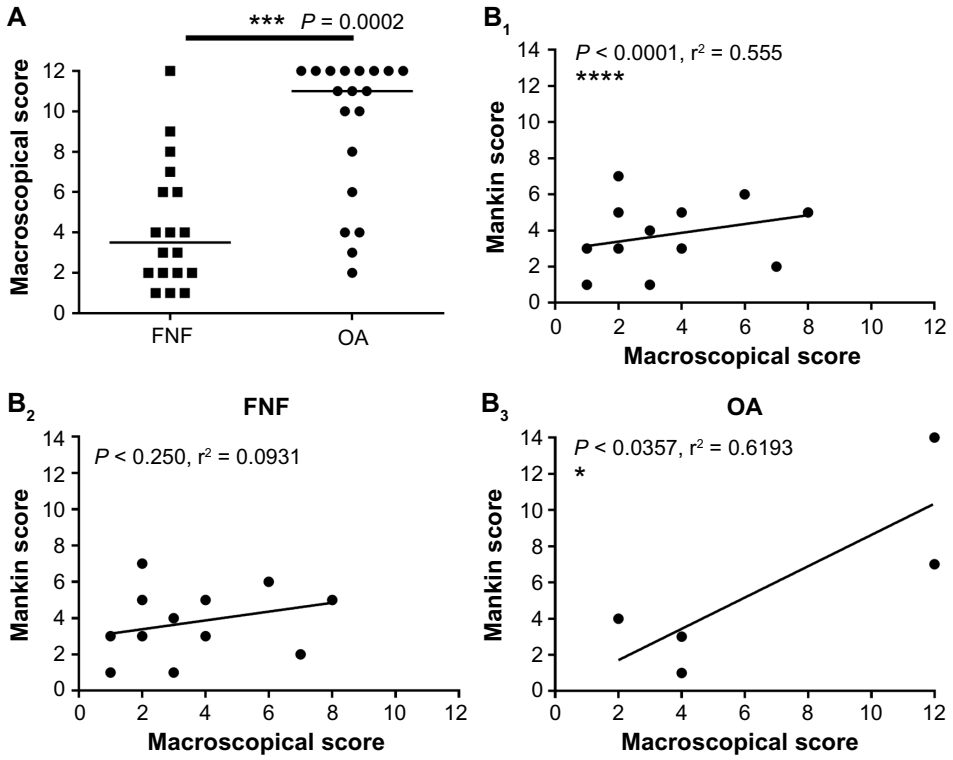

Figure 3. Macroscopical and Mankin scoring results of OA and FNF cartilage samples. (**A**) Macroscopical scoring results of FNF and OA samples. (**B_1**) Correlation between all macroscopical and Mankin scoring data (FNF and OA) and separated for FNF (**B_2**) and OA (**B_3**).
Note: *Represent significant differences.

(not significant, $P = 0.206$). Cells derived from FNF samples grew at a significantly higher cell density in culture (426.36 versus 200 cells/mm, $P = 0.0264$), but presented a lower number of adhering cells (67.18% versus 73.25%, not significant, $P = 0.147$) than OA samples.

Detection of regulatory complement proteins *in situ*. C3aR receptor protein revealed only a weak immunoreactivity in the investigated samples. C5aR was expressed by the cells of the surface cartilage layer. The CRP CD46 could be found in two of the five donors analyzed. CD55 was synthesized in the samples of all investigated donors. Signals were not only confined to the cells but also observable in the whole lacunae. Nevertheless, it could not be found in the interterritorial ECM. Protein expression of CD59 was shown in all investigated donors with divergent distribution (Fig. 6).

Discussion

In this study, a simple in-house macroscopical score was established, and we approved it to distinguish FNF and OA cartilage samples. Macroscopical scoring results correlated significantly with the histological analysis. It is advisable to categorize the quality of cartilage used for chondrocyte isolation since chondrocyte phenotype differed between FNF or OA cartilage. The complement receptor C5aR and the weakly expressed receptor C3aR, as well as CRPs CD55 and CD59, were detected in the investigated FNF and OA cartilage

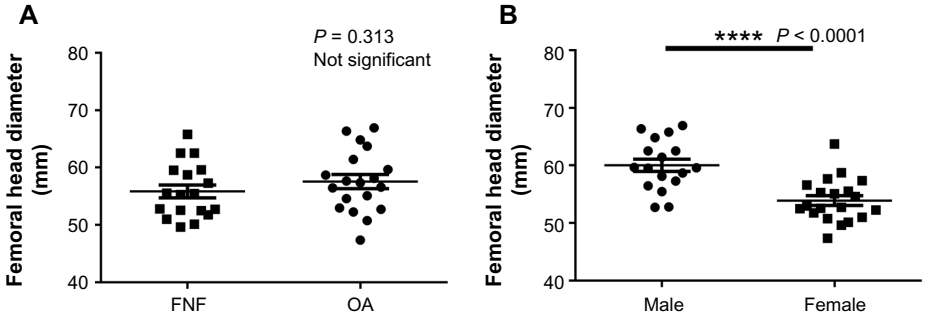

Figure 4. Femoral head diameters with respect to OA and gender. (**A**) Femoral head diameters of FNF and OA donors. (**B**) Femoral head diameters of male and female donors.
Note: *Represent significant differences.

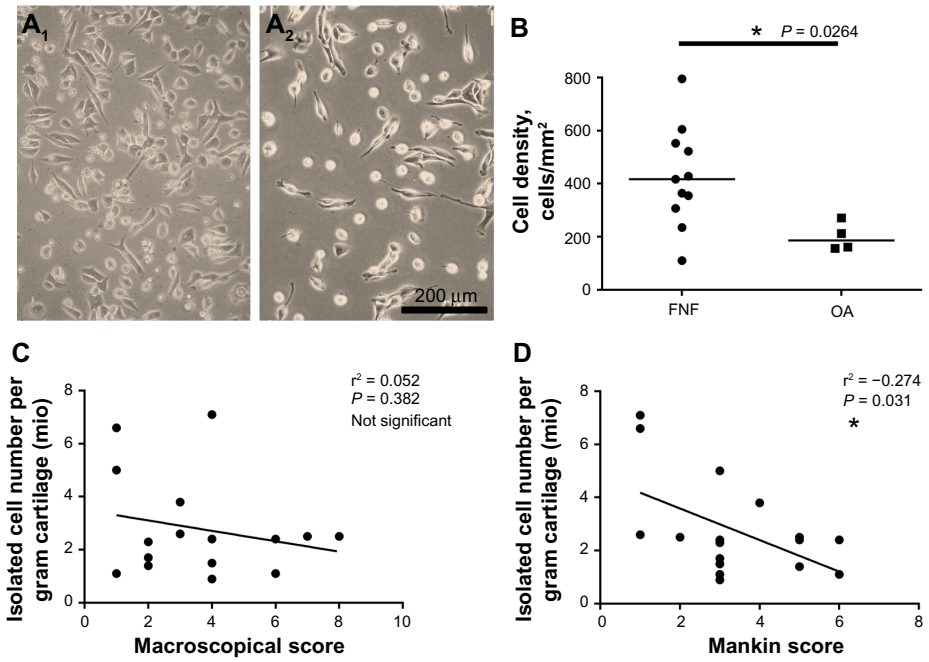

Figure 5. Vitality, adherence, and cytoplasmic area of cultured femoral head chondrocytes isolated from FNF or OA samples. Images of cultured chondrocytes derived from femoral head cartilage prone to FNF (A_1) or OA (A_2). (**B**) Cell density in FNF and OA samples. (**C**) Isolated cell numbers per gram versus macroscopical scoring results. (**D**) Isolated cell numbers per gram versus Mankin scoring results. Images and calculations derived from chondrocytes cultured for 72 hours.

Note: Scale bar: 200 µm.

samples. However, CD46 could only be found in two of the five investigated donors.

The macroscore was established for a rapid cartilage grading before cell isolation as simple as possible to allow even inexperienced laboratory staff to estimate OA in the recruited samples. Indeed, the results deduced from this easy-to-handle macroscopical score did not show major differences between experienced and inexperienced observers. This score could help to spare the time-consuming histology.

The total donor cohort analyzed macroscopically reflected a higher number of female individuals than male individuals as usually reported for OA.[26] On average, hip OA led to total joint replacement with 63.7 years, whereas the FNF (FNF) occurred around 10 years later with 74 years. A higher number of these FNF patients suffered simultaneously from other diseases such as diabetes, infections, kidney disease, and cardiovascular diseases than the OA patients. This fact might be age dependent. However, it was reported that women who have hip OA also attain a higher rate of comorbidities.[27] Furthermore, the BMI in the OA group was significantly higher.

Comparison of the OA and FNF patient-derived cartilage samples indicated a highly significant difference between both groups concerning the macroscopical score (macroscopical scoring values of OA samples varied between 2 and 12 [median: 11]), and that of the FNF group in a similar range (also between 1 and 12), but the median was lower (median: 3.5). However, only samples with a scoring value lower than 9 were used for cell isolation, since samples with higher scores presented insufficient amounts of cartilage. From these samples, only the macroscopically barely changed cartilage was recruited. Also, the FNF group presented only three samples with a low macroscopical score (one score point), indicating macroscopically nearly unaffected cartilage. For this observation, age-associated and early OA changes might be responsible since the average age in the FNF group was around 74 years.

Histological scoring can be done using several scoring systems.[28,29] The Mankin scoring system, which was selected in this study, is well-established one.[30] The Mankin and the recently recommended Osteoarthritis Research Society

Table 4. Characteristics of FNF- and OA-derived chondrocytes cultured for 72 hours.

DONOR	ISOLATED CELL NUMBER PER G CARTILAGE	CELL DENSITY CELLS/mm²	CELL ADHERENCE [%]	CELL AREA [µm²]	CELL LENGTH [µm]	CELL WIDTH [µm]
Average value OA ($n = 4$)	2.6 ± 1.7	200 ± 53.7*	73.3 ± 4.99	824 ± 267.6	70.5 ± 12.8	19.5 ± 3.1
Average value FNF ($n = 11$)	2.2 ± 1.1	426.4 ± 187.8	67.2 ± 10.3	559.5 ± 128.9	59.3 ± 4.8	17.4±3.3

Note: *Significant difference, $P = 0.0264$.

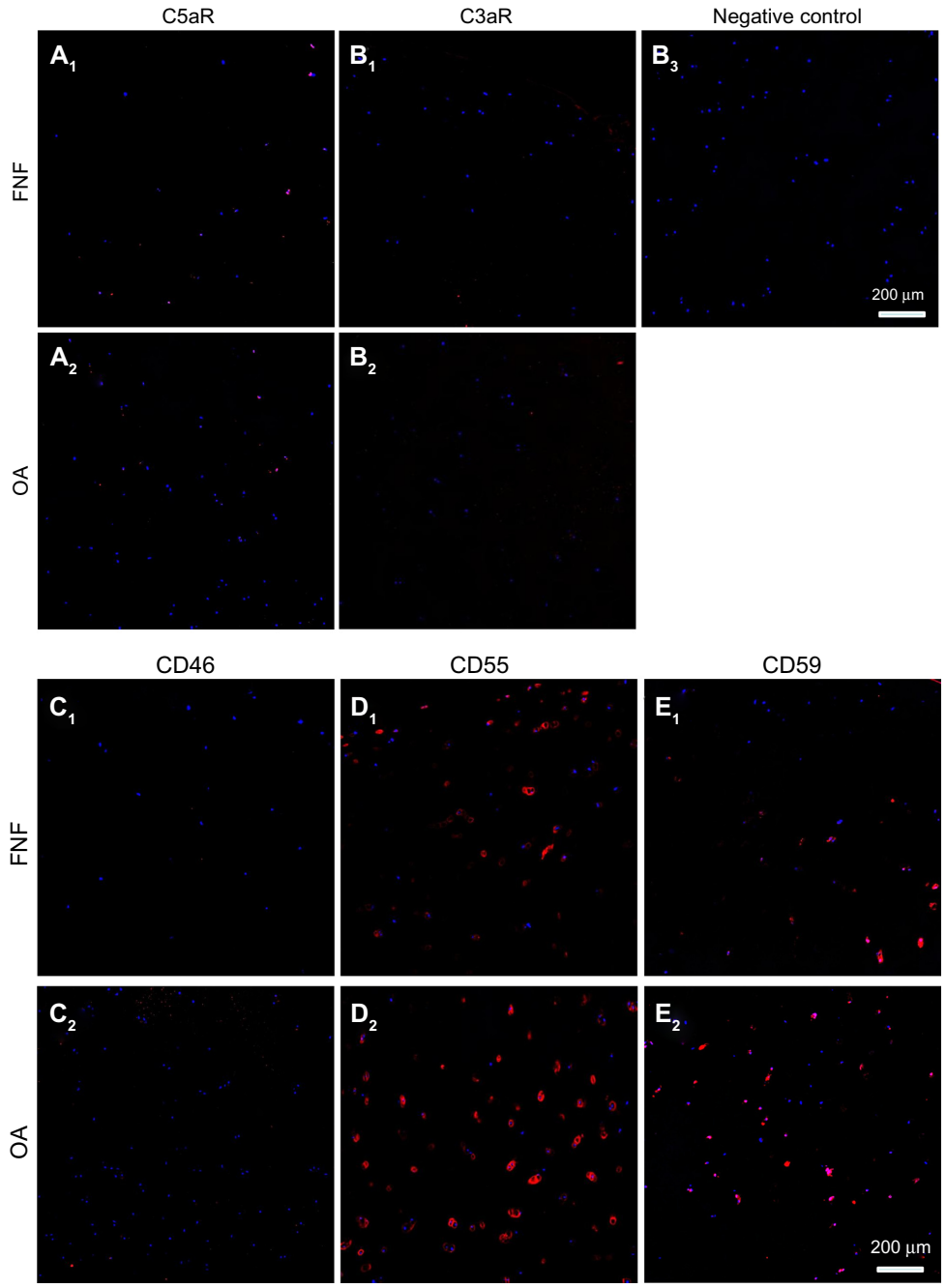

Figure 6. Expression of complement receptors (C5aR and C3aR) and CRPs (CD46, CD55, and CD59) in cartilage in situ. FNF and OA cartilage sections immunolabeled for (**A_{1-2}**) C5aR, (**B_{1-2}**) C3aR, (**C_{1-2}**) CD46, (**D_{1-2}**) CD55, and (**E_{1-2}**) CD59.
Notes: Cell nuclei were counterstained using DAPI (blue). Scale bar: 200 µm. Negative controls (B_3) performed by using only the secondary antibody revealed no unspecific staining.

International (OARSI) cartilage histopathology assessment system revealed a high reliability.[31] The histological score and the macroscopical score correlated significantly. Many samples revealed focal OA due to incongruency of the femoral head and acetabulum. However, the Mankin score revealed differences between OA and FNF group (not significant). One has to consider the fact that the macroscopical score allows an assessment of the whole femoral head, whereas for histology, only one exemplary sample was selected, which represented an area of the best cartilage quality of the whole head. This cartilage area was selected to be representative for the cartilage subsequently harvested for cell isolation. Therefore, the histology was performed to get information concerning the chondrocytes isolated later from the same samples.

It has been reported that chondrocyte morphology changes in hip OA.[32] Holloway et al, detected a subpopulation of chondrocytes with multiple elongated cytoplasmic extensions.[32] We found the tendency of a larger cell area in OA samples compared with FNF cartilage, but the length and width of the cells did not significantly differ. The reason remains unclear. However, we

evaluated only cell culture characteristics, but we did not measure the chondrocyte diameter in the histological sections. This was not necessary, since we wanted to elucidate chondrocyte differences in culture, which might influence future *in vitro* experiments.

OA is known to be associated with a higher rate of cell death *in situ*.[33] It is also known that aging cartilage reveals increased rates of apoptosis.[34] The cell vitality of cultured chondrocytes was slightly lower in OA than in the FNF group, but the difference was not significant. In agreement with the larger cell dimensions, the cell density of cultured chondrocytes was significantly lower in the OA samples compared with the FNF-derived chondrocytes. Against expectation, the cell numbers isolated were higher in OA than in FNF cartilage. This observation could be explained by the higher donor age in the FNF group and the lower ECM stability in the OA cartilage, which allows a more complete and easier digestion by the enzymes used for cell isolation. The cell adherence was lower in the FNF group, which could be explained by remnants of pericellular ECM after digestion. This might impair the rate of adherent cells. Nevertheless, we found no significant correlation between the donor age and the isolated cell numbers (data not shown).

Complement has recently been implicated in OA pathogenesis.[8,14,15] We previously observed gene expression and regulation of complement receptors and cell surface-associated regulatory proteins in cultured chondrocytes.[15] Now, we studied the expression of selected complement receptors and regulatory proteins *in situ*. In agreement with the study by Onuma et al.[13], we detected in all samples, irrespective of either prone to OA or not, an expression of C5aR, but only a focal and faint immunoreactivity for C3aR. C5aR seems to play a superior role in articular cartilage, in addition to its role in traumatic cartilage injury.[35] In addition, we demonstrated the CRPs CD55 and CD59 in all the analyzed samples, but CD46 could be detected in only two of the five investigated donors. Both CD46 positive donors had a history of hip OA, but the third investigated OA donor and the FNF donors were CD46 negative. Since we found in a previous study that there was no CD35 expression in cartilage, it was not further addressed here.[15] Like CD35, CD46 also inhibits the opsonins C3b and C4b, whereby CD46 promotes the proteolysis of these opsonins as a cofactor.[36] Possibly these opsonins play no major role in OA, but rather in rheumatoid cartilage disorders. In contrast, CD55 inhibits the upstream cleavage of C3 and C5 by antagonizing formation of the respective C3 and C5 convertases as prerequisites for ongoing complement cascade activation and terminal MAC formation. CD55 is also found in a soluble form,[36] possibly an explanation for the observed immunostaining of chondrocyte lacunae. CD59 interferes directly with the formation of the MAC complex.[36] Its deficiency has been shown to promote the OA phenotype in a mouse model.[8] Investigation of a higher number of OA and non-OA samples of different grades would indicate whether downregulation of cytoprotective CD55 and CD59 might be directly associated with different OA stages (early or late) in human beings.

Limitations of this Study

This study is limited by the heterogenous donor collective and their high age, particularly in the FNF subgroup. So, age-related influences could not be excluded. For the determination of the cell parameters in culture, the OA group was rather small ($n = 4$). The classification "OA" and "FNF" was based on clinical diagnosis; therefore, some FNF samples might also have OA, which leads to the high macroscopical and histological scores observed in several FNF patients. There is also the limitation that the FNF samples, which represent the controls, are derived from hip fracture patients. These samples are not entirely healthy due to the fact that the most common reason for this event is osteoporosis.[19]

For histology, only one sample could be included from each donor, since the majority of cartilage was needed to be harvested for cell isolation. So, the best cartilage area of each femoral head was selected. This could be the reason why the Mankin scoring provided generally lower scoring numbers, suggesting higher cartilage quality, and hence, superior results than the macroscopical score. In this study, the macroscopical score is more predictive than the Mankin score, concerning cartilage quality. In this respect, sampling and time-consuming processing for histology could be omitted in future.

Conclusion

The self-designed scoring system is applicable for an easy grading of cartilage samples by inexperienced observers for experimental purposes. We are not expecting that our macroscopical scoring system will also find clinical application in arthroscopy of the hip joint. For our coworkers in the laboratory setting, who did not have XRs, etc., available, this score was accepted as helpful and practical. Experiments with human-derived chondrocytes are superior to using animal-derived samples due to interspecies differences.[37,38] Therefore, the facts reported here for FNF- versus OA-derived chondrocytes, which are the main chondrocyte sources of human origin for research, help to assess their peculiarities and its potential influence on experiments. With regard to further characterize the extent of complement activation during OA progression, other complement factors such as C3, C4, and C5 and the split fragments such as C3a and C5a should also be included in future studies.

Acknowledgments

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Author Contribution

Conceived and designed the experiments: JB, OG, GS-T, CM, MJ. Analyzed the data: JB, BK, TS, GS-T. Wrote the first draft of the manuscript: OG, GS-T. Contributed to the writing of the manuscript: ZH, SA. Agree with manuscript results and

conclusions: JB, OG, BK, CM, MJ, ZH, SA,TS, GS-T. Jointly developed the structure and arguments for the paper: GS-T, BK, MJ. Made critical revisions and approved final version: JB, OG, MJ, ZH, SA, GS-T. All authors reviewed and approved the final manuscript.

REFERENCES

1. Roach H, Tilley S. The pathogenesis of osteoarthritis. In: Bronner F, Farach-Carson M, eds. *Bone and Osteoarthritis*. Vol 4. London: Springer; 2007:1–18.
2. Creamer P. Intra-articular corticosteroid injections in osteoarthritis: do they work and if so, how? *Ann Rheum Dis*. 1997;56:634–6.
3. Haseeb A, Haqqi TM. Immunopathogenesis of osteoarthritis. *Clin Immunol*. 2013;146:185–96.
4. Brandt KD, Dieppe P, Radin E. Etiopathogenesis of osteoarthritis. *Med Clin North Am*. 2009;93:1–24, xv.
5. Wheaton AJ, Borthakur A, Shapiro EM, et al. Proteoglycan loss in human knee cartilage: quantitation with sodium MR imaging – feasibility study. *Radiology*. 2004;231:900–5.
6. Wu L, Liu H, Li L, et al. Mitochondrial pathology in osteoarthritic chondrocytes. *Curr Drug Targets*. 2014;15:710–9.
7. Wojdasiewicz P, Poniatowski LA, Szukiewicz D. The role of inflammatory and anti-inflammatory cytokines in the pathogenesis of osteoarthritis. *Mediators Inflamm*. 2014;2014:561459.
8. Wang Q, Rozelle AL, Lepus CM, et al. Identification of a central role for complement in osteoarthritis. *Nat Med*. 2011;17:1674–9.
9. Carroll MV, Sim RB. Complement in health and disease. *Adv Drug Deliv Rev*. 2011;63:965–75.
10. Klos A, Tenner AJ, Johswich KO, Ager RR, Reis ES, Kohl J. The role of the anaphylatoxins in health and disease. *Mol Immunol*. 2009;46:2753–66.
11. Makrides SC. Therapeutic inhibition of the complement system. *Pharmacol Rev*. 1998;50:59–87.
12. Hyc A, Osiecka-Iwan A, Strzelczyk P, Moskalewski S. Effect of IL-1beta, TNF-alpha and IL-4 on complement regulatory protein mRNA expression in human articular chondrocytes. *Int J Mol Med*. 2003;11:91–4.
13. Onuma H, Masuko-Hongo K, Yuan G, et al. Expression of the anaphylatoxin receptor C5aR (CD88) by human articular chondrocytes. *Rheumatol Int*. 2002;22:52–5.
14. John T, Stahel PF, Morgan SJ, Schulze-Tanzil G. Impact of the complement cascade on posttraumatic cartilage inflammation and degradation. *Histol Histopathol*. 2007;22:781–90.
15. Schulze-Tanzil G, Kohl B, El Sayed K, et al. Anaphylatoxin receptors and complement regulatory proteins in human articular and non-articular chondrocytes: interrelation with cytokines. *Cell Tissue Res*. 2012;350:465–75.
16. Busch C, Girke G, Kohl B, et al. Complement gene expression is regulated by pro-inflammatory cytokines and the anaphylatoxin C3a in human tenocytes. *Mol Immunol*. 2013;53:363–73.
17. Girke G, Kohl B, Busch C, et al. Tenocyte activation and regulation of complement factors in response to *in vitro* cell injury. *Mol Immunol*. 2014;60:14–22.
18. Szulc P, Beck TJ, Marchand F, Delmas PD. Low skeletal muscle mass is associated with poor structural parameters of bone and impaired balance in elderly men – the MINOS study. *J Bone Miner Res*. 2005;20:721–9.
19. Warriner AH, Patkar NM, Curtis JR, et al. Which fractures are most attributable to osteoporosis? *J Clin Epidemiol*. 2011;64:46–53.
20. Kleemann RU, Krocker D, Cedraro A, Tuischer J, Duda GN. Altered cartilage mechanics and histology in knee osteoarthritis: relation to clinical assessment (ICRS Grade). *Osteoarthritis Cartilage*. 2005;13:958–63.
21. van den Borne MP, Raijmakers NJ, Vanlauwe J, et al. International Cartilage Repair Society (ICRS) and Oswestry macroscopic cartilage evaluation scores validated for use in Autologous Chondrocyte Implantation (ACI) and microfracture. *Osteoarthritis Cartilage*. 2007;15:1397–402.
22. Goebel L, Orth P, Muller A, et al. Experimental scoring systems for macroscopic articular cartilage repair correlate with the MOCART score assessed by a high-field MRI at 9.4 T – comparative evaluation of five macroscopic scoring systems in a large animal cartilage defect model. *Osteoarthritis Cartilage*. 2012;20:1046–55.
23. Goebel L, Zurakowski D, Muller A, Pape D, Cucchiarini M, Madry H. 2D and 3D MOCART scoring systems assessed by 9.4 T high-field MRI correlate with elementary and complex histological scoring systems in a translational model of osteochondral repair. *Osteoarthritis Cartilage*. 2014;22:1386–95.
24. Mankin HJ, Dorfman H, Lippiell L, Zarins A. Biochemical and metabolic abnormalities in articular cartilage from osteoarthritic human hips. 2. Correlation of morphology with biochemical and metabolic data. *Arthritis Rheum*. 1971;14:400.
25. Aigner T, Cook JL, Gerwin N, et al. Histopathology atlas of animal model systems – overview of guiding principles. *Osteoarthritis Cartilage*. 2010;18(suppl 3):S2–6.
26. O'Connor MI. Osteoarthritis of the hip and knee: sex and gender differences. *Orthop Clin North Am*. 2006;37:559–68.
27. Mark R. Disabling hip osteoarthritis: gender, body mass, health and functional status correlates. *Health*. 2010;2(7):696–704.
28. Pearson RG, Kurien T, Shu KS, Scammell BE. Histopathology grading systems for characterisation of human knee osteoarthritis – reproducibility, variability, reliability, correlation, and validity. *Osteoarthritis Cartilage*. 2011;19:324–31.
29. Pritzker KP, Gay S, Jimenez SA, et al. Osteoarthritis cartilage histopathology: grading and staging. *Osteoarthritis Cartilage*. 2006;14:13–29.
30. Mankin HJ. Biochemical and metabolic abnormalities in osteoarthritic human cartilage. *Fed Proc*. 1973;32:1478–80.
31. Pauli C, Grogan SP, Patil S, et al. Macroscopic and histopathologic analysis of human knee menisci in aging and osteoarthritis. *Osteoarthritis Cartilage*. 2011;19:1132–41.
32. Holloway I, Kayser M, Lee DA, Bader DL, Bentley G, Knight MM. Increased presence of cells with multiple elongated processes in osteoarthritic femoral head cartilage. *Osteoarthritis Cartilage*. 2004;12:17–24.
33. Kim HA, Lee YJ, Seong SC, Choe KW, Song YW. Apoptotic chondrocyte death in human osteoarthritis. *J Rheumatol*. 2000;27:455–62.
34. Mobasheri A, Matta C, Zakany R, Musumeci G. Chondrosenescence: definition, hallmarks and potential role in the pathogenesis of osteoarthritis. *Maturitas*. 2015;80:237–44.
35. Huber-Lang M, Ignatius A, Brenner RE. Role of complement on broken surfaces after trauma. *Adv Exp Med Biol*. 2015;865:43–55.
36. Piccoli AK, Alegretti AP, Schneider L, Lora PS, Xavier RM. Expression of complement regulatory proteins CD55, CD59, CD35, and CD46 in rheumatoid arthritis. *Rev Bras Reumatol*. 2011;51:503–10.
37. Schulze-Tanzil G, Muller RD, Kohl B, et al. Differing in vitro biology of equine, ovine, porcine and human articular chondrocytes derived from the knee joint: an immunomorphological study. *Histochem Cell Biol*. 2009;131:219–29.
38. Muller C, Marzahn U, Kohl B, et al. Hybrid pig versus Gottingen minipig-derived cartilage and chondrocytes show pig line-dependent differences. *Exp Biol Med*. 2013;238:1210–22.

Lebenslauf

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

Komplette Publikationsliste

Publikation:

1. **Huang Z**, Nooeaid P, Kohl B, Roether JA, Schubert DW, Meier C, Boccaccini AR, Godkin O, Ertel W, Arens S, Schulze-Tanzil G. Chondrogenesis of human bone marrow mesenchymal stromal cells in highly porous alginate-foams supplemented with chondroitin sulfate. Mater Sci Eng C Mater Biol Appl. 2015 May; 50:160-72. http://dx.doi.org/10.1016/j.msec.2015.01.082.

2. **Huang Z**, Kohl B, Kokozidou M, Arens S, Schulze-Tanzil G. Establishment of a cytocompatible cell-free intervertebral disc matrix for chondrogenesis with human bone marrow mesenchymal stromal cells. Cells, tissues, organs. 2016; 201:354-365. http://dx.doi.org/10.1159/000444521.

3. Jessica Badendick*, Owen Godkin*, Benjamin Kohl, Carola Meier, Michal Jagielski, **Zhao Huang**, Stephan Arens, Tobias Schneider, Gundula Schulze-Tanzil. Macroscopical, histological, and in vitro characterization of nonosteoarthritic versus osteoarthritic hip joint cartilage. Clin Med Insights Arthritis Musculoskelet Disord. 2016 May 3;9:65-74. http://dx.doi.org/10.4137/CMAMD.S29844. Co-authorship.

Kongressbeiträge:

1. **Zhao Huang**, Benjamin Kohl, Gundula Schulze-Tanzil. Optimized Human Intervertebral Disc De- and Recellularization using Allogeneic Disc Cells or Mesenchymal Stromal Cells. 110th Annual Meeting of the Anatomical Society, Würzburg, Germany, September 23th-25th, 2015, *Poster & Kurzvortrag.*

2. **Zhao Huang**, Benjamin Kohl, Gundula Schulze-Tanzil. Intervertebral disc tissue engineering on the basis of a cell-free extracellular matrix. Deutscher Kongress für Orthopädie und Unfallchirurgie, Berlin, Oktober 20-23, 2015, *Poster & Kurzvortrag.*

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