The Influence of Substrate Elasticity on Adhesion and Phenotype of Re-differentiating Human Articular Chondrocytes

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Abstract

Articular cartilage damages caused by trauma have very limited abilities for self repair and are often followed by self degeneration of the cartilage. Conventional microfracturing treatment of the defect has been poorly successful in older patients. Even though clinical trials have not shown any advantage till today, autologous chondrocyte transplantation emerges as an alternative, very promising technique. The poor clinical results are partly due to the very limited capacity of expanded, dedifferentiated articular chondrocytes to redifferentiate into their original phenotype. Recent studies have shown that the substrate stiffness is influencing the differentiation of human mesenchymal stem cells, which are progenitor cells of human articular chondrocytes (HAC). We showed, that the substrate stiffness influenced the redifferentiation capacity of HAC when cultured in chondrogenic medium containing TGFβ3. Real-time PCR as well as morphology and actin cytoskeleton organization studies confirmed that HAC cultivated on soft (0.3kPa) polyacrylamide hydrogels (PA) showed an increase in the redifferentiation compared to those cultured on stiffer (21kPa, 75kPa) PA. Furthermore, the initial adhesion of HAC on the PA, characterized by AFM force spectroscopy and a centrifugation assay, showed no significant difference. However, a slightly non significant faster adhesion was found on softer substrates then on stiffer ones, which might be due to a slightly higher ligand density.

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Contents

1 Introduction

The condensation of chondrogenic progenitor cells at the future bone sites during skeltogenesis results in a primitive cartilaginous skeleton. Most chondrocytes in this early tissue proliferate, synthesize large amount of extra cellular matrix and become hypertrophic [1]. They induce cartilage matrix mineralization [2], which leads to vascularization, invasion of bone marrow cells and finally to bone formation [3]. In contrast to this so called transient cartilage, a persistent form of cartilage may be found at the epiphyseal ends [4]. The chondrocytes in this type of cartilage have a reduced proliferation rate, remain biosynthetically active and do not maturate into the hypertrophic state [5]. They express mainly the type II, IX and XI collagen as well as aggrecan and stay in a round shaped morphology [6].

Articular cartilage is persistent and consists mainly of water, type II collagen and proteoglycans [7]. The human articular chondrocytes (HAC) do not only synthesize the main components of this cartilage, but they also organize and maintain it. Injuries may lead to physical or biochemical changes in the environment and can result in a change of the phenotype of HAC towards more fibroblast like phenotype. A similar transition happens when HAC are cultured in 2D [8]. This process is called dedifferentiation and initially meant, that the HAC loose their functionality rather then to regain abitlities of their progenitor cells [9]. However, recent studies of Barbero *et al.* [10] showed, that growth factor stimulated differentiation of dedifferentiated HAC toward various mesenchymal cell lines was possible. Therefore, dedifferentiated HAC showed mesenchymal progenitor cell like multilinage differentiation capacity. The proliferation of dedifferentiated HAC is increased compared to native ones, the expression of type II collagen decreased and the one of type I collagen increased and so far it is not possible to retain the native chondrocytic phenotype during expansion [11].

Once damaged, articular cartilage undergous limited natural healing. On one hand, distinct chondral or partial thickness fractures lead to tissue necrosis, followed by proliferation of the suviving chondrocytes. These chondrocytes increase temporarily the type II collagen synthesis. The resulting long term cartilage shows a lost of its characteristic hyaline structure and may result in osteoartritic diseases [12].

On the other hand, osteochondral or full thickness fractures lead to an invasion of bone marrow derived mesenchymal progenitor cells, which differentiate after several other steps into chondrocyte like cells. The defect is completly refilled with new bone and cartilage tissue. This new cartilage shows a more

fibrous cartilage structure, which does not match the properties of native cartilage. Furthermore, the new formed tissue has a lower durability [13].

In general, both natural healing mechanisms have a very limited ability for self repair [14] especialy in older patients. A long term stable treatment would help millions of patients each year [15]. Up to date microfracturing is still the most often used treatment. It is cheap and fairly successful in patients under 50 years of age. Promising other treatments like an autologous chondrocyte implantation (ACI) or matrix assisted autologous chondrocyte implantation (MAACI) depend strongly on the ability of expanded HAC to perform its chondrocytic phenotype.

Since chondrocyte expansion can not be done without loosing the phenotype, the goal should be to re-differentiate the HAC back to their native phenotype after expansion [11]. The differentiation of human mesenchymal stem cells (MSC), a chondrogenic progenitor cell, is known to depend on biochemical factors such as soluble factors [16], surface ligand density [17], identity [18] and accessibility [19, 20] as well as on more recently studied physical factors such as substrate stiffness [21]. We hypothesized therefore, that apart from the biochemical factors also the substrate stiffness may play an important role in the redifferentiation of expanded, dedifferentiated HAC, which are of MSC like plasticity. Recent studies on pork chondrocytes [22] showed that the HAC remained closer to their native phenotype if cultured on substrates with a low stiffness.

Furthermore, it is known that these biochemical factors [16] as well as physical factors [23] stated above also modulate the adhesion characteristics between the cells and the substrate. The adhesion of mesenchymal lineage cells to biomaterial surfaces itself is again important, since it may direct the cell morphology and proliferation. Dedifferentiated HAC seeded in agarose hydrogels showed a more round morphology and also an increased type II collagen expression [24]. Since they can not form strong substrate adhesions like focal adhesion complexes with agarose, it is accepted that a lack of adhesion leads to a change in the morphology and therefore also in the phenotype of HAC.

On the other hand it was shown that spread rabbit articular chondrocytes with an organized actin cytoskeleton tethered more strongly to polystyrene beads than more round shaped ones [25]. The morphology may therefore modulate the capacity of HAC to form adhesion structures. It is therefore difficult to determine if the adhesion has been influenced by the phenotype or vice versa. However, the change of the phenotype takes some time. If the adhesion is characterized before the cell has time to change its phenotype, the adhesion may lead to a phenotype change but not vice versa. Therefore, we characterized the

initial adhesion of HAC to biomaterials and hypothesized that redifferentiation capacity of dedifferentiated HAC on elastic substrate is modulated by the initial adhesion.

The most common model for 2D substrate stiffness studies is a polyacrylamide hydrogel (PA) [21, 23, 26, 27]. Its elasticity can easily be tuned by changing the ratio of the monomer acrylamide to the crosslinker N,N'-methylene bisacrylamide from the sub kPa level to around 100kPa [26]. It is not autofluorescent and thus allows for fluorescent microscopy. PA is inert for cell adhesion leading to no unspecific cell surface interactions, but proteins can be covalently linked to the surface allowing a controlled ligand protein dependent cell substrate adhesion [28].

It is still under discussion if the ligand density is similar on soft as on hard substrates. The penetration depth of the functionalization agent and ligand might be higher on soft PA than on harder ones leading to a higher ligand density on softer substrates. Bigger pores may be the cause to this deeper penetration. Some researchers found no difference in stiffness depending ligand density [29], while Lo *et al.* [27] showed that the softer substrates stained for type I collagen had a slightly higher intensity than harder substrates. However, they could also show by using beads with $1\mu m$ micrometer diameter and immunofluorescence, that particle with a size smaller than a cell could not penetrate this deeper PA and showed no stiffness depending difference in staining.

Different approaches to characterize cell substrate adhesion have previously been used. Among them are spreading area determination [23], spinning disc method [30], micro pipetting [31], microfluidic laminar flow [32], traction force [27], centrifugation [33] and force spectroscopy [34]. We chose two different approaches to quantitatively determine the adhesion of living cells. We employed force spectroscopy by atomic force microscopy (AFM) to test single cells as well as an in-house established centrifugation assay to characterize adhesion of entire cell populations.

The AFM is a very sensitive instrument which allows nearly direct measurement of forces in the pico to nanonewton range. The bending of a thin silicon bar (called cantilever) is approximately linear to the force applied to it. This bending can be measured via the deflection of a laser beam on this cantilever. Force spectroscopy by AFM allows to characterize interactions in the range from single receptor binding [35] to stronger cell surface bindings [36]. However, only single cells can be tested, which leads to a strong dependence on the homogenity of the cell population. As previously shown [37], the detachment work measured by AFM on the same HAC population varied greatly from cell to cell. This was

thought to be due to two reasons: i) The cells were in different cell cycle phases [38]. ii) The cells were from different zonal origins (due to the mode of harvesting HAC from cartilage biopsis) [11].

The centrifugation assay on the other hand is less sensitive. During an up side down centrifugation cells are pulled away from substrate by the centrifugation force. The adhesion characteristics can only be measured indirectly by cell counting. The advantage however is, that a mean can be measured directly, which is more robust than a single cell measurement. Furthermore, this assay allows only to measure one dependence at the time. Detachment force dependence [33] as well as ligand density dependence [39] was previously characterized by centrifugation essaies. However, a time dependent characterization of the adherent fraction seems more interesting to characterize the diffent initial response of HAC to the substrate.

2 Material and Methods

2.1 Cell Culture

Human Articular Chondrocytes (HAC) were frozen after passage P2 and stored in liquid nitrogen at the University Hospital of Basel. The HAC were thawed in a water bath at 37°C one week before the experiment. Immediately after thawing the suspension was pipetted to the complete medium (CM) containing Dulbecco's modified Eagle's medium (DMEM, Gibco Invitrogen, 10930, Paisley UK) supplemented with 4.5mg/ml D-glucose, 0.1mM nonessential amino acids, 10% fetal bovine serum (FBS), 1mM sodium pyruvate (Gibco Invitrogen, 11360, Paisley UK), 10mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer (HEPES, Gibco Invitrogen, 15630, Paisley UK), 100units/ml penicillin, 100μg/ml streptomycin and 0.29mg/ml L-glutamine (Pen Strep Glutamine, Gibco Invitrogen, 10378, Paisley UK). The HAC were centrifuged at 1400rpm for 4min and the supernatant was aspirated. The HAC were resuspended in CM supplemented with (TFP) 1ng/ml transforming growth factor β1 (TGFβ1), 10ng/ml platelet-derived growth factor (PDGF) and 5ng/ml fibroblast growth factor 2 (FGF2) and seeded to culture flask with a density of 5000 cells/cm^2.

The HAC were expanded in a humidified incubator at 37°C and 5% CO_2 and the medium was changed every two to three days. As soon as they grew confluent they were detached by treatment of 0.3% collagenase type II followed by 0.05% trypsin in a 0.53mM EDTA solution (Trypsin-EDTA, Gibco Invitrogen, 25300, Paisley UK). After trypsin blocking with CM, centrifugation at 1400rpm and aspiration of the supernatant, the cells were resuspended in serum free chondrogenic medium (SFM) containing DMEM supplemented with ITS^{+1} (10μg/ml insulin, 5.5μg transferrin, 5ng/ml selenium, 0.5mg/ml bovine serum albumin, 4.7μg/ml linoleic acid, Sigma Aldrich, I2521, Steinheim DE), 10mM HEPES, 100units/ml penicillin, 100μg/ml streptomycin and 0.29mg/ml L-glutamine, 1mM sodium pyruvate, 0.1mM ascorbic acid 2-phosphate, 1.25mg/ml human serum albumin (HSA), 10^{-7}mM dexamethasone and 1ng/ml transforming growth factor β3 (TGFβ3). Eventually, passage P3 HAC were seeded onto the substrate with different stiffness at a density of 20k cells/cm^2.

2.2 Substrate Preparation

The substrates were prepared as described earlier [40]. In brief, the slides (cover slides round, 23mm, nr 1, Thermo Scientific, Woltham USA) were washed with a solution of 2% (V/V) Neodisher LM30 (Dr. Weigert GmbH & Co., Hamburg

DE) in tap water for 5min in an ultrasonic bath (W375, Heat Systems, Ultrasonic INC.), rindsed with MilliQ water and dried at 50°C. The activation of the glass surface was done with a solution of 5ml/l 3-(Trimethoxysilyl)propyl methacrylate (Sigma-Aldrich, M6514, St. Louis USA) and 30ml/l acetic acid (10%) in water free ethanol (containing Ketone). The slides were placed in this solution for 5min, rinsed with water free ethanol and dried at room temperature. This activation enabled a covalent linkage of the PA to the glass surface during polymerization.

The cover plates were passivated to enable the lift of after polymerization. Therefore, these glass plates were covered with 0.1M Sodiumhydroxide (NaOH) and dried at 50°C. Some droplets of Dichlorodiethylsilane (Merck-Schuchardt, Art. 803452, Hohenbrunn Germany) were pipetted on a plate and an other plate was laid on top. After 10min the plates were separated, allowed to dry in the hood and fixated at 200°C for another 10min. Afterwards the plates were rinsed repeatedly with soap and tap water.

Three different concentrations of an acrylamide solution (AAS, 40%, Fluka, Buchs CH) and N,N'-methylene bisacrylamide (BIS, Bio-Rad Laboratories, Richmond USA) in milliQ water were prepared to reach contrasting in substrate stiffness as reported earlier by Haupt [40]. The concentrations are listed in table 1.

Table 1: The three different mixtures of acrylamide monomer and crosslinker BIS are listed.

label	acrylamide (monomer) [%] (V/V)	BIS (corsslinker) [%] (V/V)
soft	5	0.100
intermediately stiff	10	0.050
stiff	20	0.033

The spacer thickness was reduced compared to previouse work [40] to decrease gel thickness and therefore, reduce detachment of the gels from the glass surface. The activated slides were placed between the spacers on the cover plate. Just prior to use, the polymerization starter ammoniumperoxodisulfate (APS, final concentration 0.5mg/ml, Merck, Darmstadt Germany) and N,N,N',N'-tetramethylethylenediamine (TEMED, final concentration 0.5μl/ml, Fluka, Buchs Switzerland) were added to the prepared solutions, followed by pipetting the solution to the activated sides. Immediately afterwards a second cover plate was placed on top of the first forming a sandwich. This should lead to a thickness of the polyacrylamide hydrogel (PA) similar to the thickness of a

spacer slide, which is of 0.13mm to 0.16mm. Cryo scanning electron microscopy confirmed a thickness of approximately $100\mu m$ (data not shown).

After at least 4h of polymerization the cover plate was removed, the slides were lifted off and immediately immersedsed in milliQ water, followed by rinsing and finally stored in PBS at 4°C.

The PA surface was functionalized using the protocol of Beningo *et al.* [28]. In brief, the surface was activated using the photosensitive, heterobifunctional protein crosslinker Sulfosuccinimidyl-6-[4'-azido-2'-nitrophenylamino]hexanoate (Sulfo-SANPAH, Proteo Chem, Denver USA). It was dissolved in dimethyl sulfoxide (DMSO, Fluka, 41640, Buchs CH) and diluted with 50mM HEPES (pH8.5, Simga-Aldrich, H4034, St Louis USA) to a final concentration of 1mM Sulfo-SANPAH in 50mM HEPES supplemented with 0.5% (v/v) DMSO. This solution was pipetted on the PA surface and activated with a UV lamp (TL-900, CAMAG, Muttens Switzerland) at a wavelength of 350nm for 8min. The solution darkened form red to brownish during this step. This photo activation was repeated. The gels were washed afterwards three times with PBS for 15min. A solution containing 0.2mg/ml type I collagen (Rat tail type I collagen, BD Bioscience, 354236, Bedford UK) in PBS was pipetted onto the surface and let to react for at least 12h at 4°C. The slides were rinsed three times with PBS and stored in the fridge for maximally three days.

One hour prior to use, the slides were sterilised using the UV lamp in the hood for 30min. DMEM was added approximately 30min prior to seeding to equilibrate the gels.

The elasticity of the substrates was previously characterized by Vonwil [41] using rotational rheometry. The Young modulus for the in this work used PA were $0.26\pm0.08kPA$ (*soft*), $21.3\pm0.8kPa$ (*intermediately stiff*) and $75\pm5kPa$ (*stiff*). For an even stiffer control, collagen coated tissue culture treated poly styrene (TCPS) served as *infinitely stiff* substrate. TCPS was not feasible for fluorescence imaging due to high auto-fluorescence. Instead collagen coated glass slides were used as *infinitely stiff* substrate for fluorescence microscopy.

2.3 Gene Expression

HAC were harvested after 7days in culture using collagenase and trypsin as described above. The pellet was washed with firtst DMEM, then PBS both at 4°C. Immediately after aspiring the supernatant, 250ul Trizol (Life Technologies, Basel Switzerland) was added to block RNase and to extract proteins. The tubes were stored at -20°C.

After thawing the samples were sonicated, vortexed with 50ul chloroform,

incubated on ice for 10min, and centrifuged (11000rpm, 4°C) for 15min. The upper phase was extracted and vortexed with 2ul glycogen (Invitrogen, 10814, Carlsbad USA) and 125ul isopropanol. After 10min of incubation on ice they were centrifuged (11000rpm, 4°C) for 10min and the upper phase was discard by inversion. The remaining glycogen RNA pellet was washed three times with 75% ethanol. After the last washing step the pellet was resolved in 35ul RNase free water and placed on ice.

Real-time quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) assay was preformed to quantify the gene expression. Therefore, the instructions of the RNeasy Kit (Ambion, Austin TX) were followed. The SuperScript III reverse transcriptase (Invitrogen, 18080, Carlsbad USA) was used to create cDNA. Random primers (Promega, C1181, Madison WI USA) enabled a transcription of the whole RNA. The real-time PCR was performed with a 7300 Real Time PCR System (Applied Biosystems). Primer sequences and probes for housekeeping gene (18S rRNA), type I collagen and II were used as previously described by Barbero *et al.* [10].

A duplicate was preformed for each sample and the mRNA was normalized to the housekeeping gene.

2.4 Fluorescence Staining

HAC were fixed in 4% (w/w) formaldehyde in phosphate buffer (pH 7.4, University Hospital Pharmacy Basel) at 4°C over night, rinsed three times with PBS and permeabilized with permeabilization solution (PerS) containing 0.02% (w/w) Triton X100 (Fluka, 93426, Buchs CH) in PBS for 10min on ice. Immediatly after aspiration of the PerS the samples were blocked for 1h at room temperature in PBS containing 30mg/ml alumin from bovine serum (BSA, Sigma-Aldrich, A3803, St Louise USA). Then, the specimens were rinsed with labelling buffer (LB) containing 15mg/ml BSA in PBS and incubated with the primary antibody for 1h at room temperature. Subsequently, the specimens were rinsed with LB four times for 5min each and incubated with the secondary antibody for 1h at room temperature. Finally, the slides were washed again with LB four times for 5min each, rinsed with autoclaved milliQ water, mounted with Aqueous Mounting Media (AbD SeroTec, Oxford, UK) and sealed with Klarlack (Lady Manhattan Cosmetics, Germany)

The antibodies and labelling agents were diluted in LB. Vinculin was labelled with primary antibodies (1:400 dilution, monoclonal anti-vinculin antibody produced in mouse, Sigma-Aldrich, St. Louis USA) followed by secondary antibodies (1:800 dilution, Cy3 conjugated anti mouse IgG produced in goat,

Acris Antibodies, Herford Germany).

F-actin was stained with phalloidin (1:400 dilution, phalloidin conjugated with Alexa488, Invitrogen, Oregon USA) and the nuclei with DAPI (1:48000 dilution, 4',6-diamidino-2-phenylindole, Invitrogen, Oregon USA).

Type II collagen was detected by primary antibodies, followed by eather Cy3 conjucated antibodies (see vinculin labelling) or Alexa 546 conjucated antibodies (1:200 dilution, Alexa 546 conjugated anti mouse IgG produced in goat, Invitrogen, Oregon USA).

Microscopy images were auqired with a confocal laser scanning microscope (LSM 710, Zeiss MicroImaging GmbH).

2.5 Protein Expression

The protein expression was analyzed with samples stained for type II collagen and nuclei. 8bit z-stack images were recorded on three different spots on each substrate with a 63x oil inversion objective. All conditions were kept constant during image recording. The pixels with an intensity bigger than 20 were counted on each z-plane using the Zen2008 software (version 5.0, Zeiss MicroImaging GmbH) and normalized on the cell number. The highest value of the z-stack served as a quantitative amount for type II.

2.6 Centrifugation Force on Particles

In the following calculations the HAC are assumed to be spherical, static particles. The centrifugal force F_{cen} is calculated by the following formula:

$$F_{cen} = r\omega^2 m \tag{1}$$

Where r is the radius of rotator, ω is the angular speed and m is the mass of the cell.

Since the cell is in a medium with a density ρ_m there is also a lift force F_{cen} produced by the displacement of medium:

$$F_{lift} = am_m = -r\omega^2 V_c \rho_m \tag{2}$$

With a the acceleration, m_m the from the cell displaced mass of medium and V_c the volume of the cell.

The force F acting on the cell is :

$$F = F_{cen} + F_{lift} = r\omega^2 V_c \left(\rho_c - \rho_m\right) \tag{3}$$

Where ρ_c is the mean density of the intracellular space.

The radius r was not equal inside a sample nor between the wells due to the geometry of the six well plates. But the maximal relative error of the force ΔF was calculated to be less than 2%. Furthermore, the detachment force was reduced by the spacer ring, which decreased the radius and therefore also the force.

We assumed, that the volume of the cell V_c corresponds to a sphere with a diameter of $13\mu m$(according to the microscopy observation), the density of the cell ρ_c was 1.075g/ml and that of the medium ρ_m was 1.00g/ml. An angular speed of 3044rpm was applied leading to a relative gravity force (RCF) of 2000g. This RCF was corrected by the reduction in radius to 1627g (see Appendix). Under these assumptions the force F acting on a cell was 1.38nN.

The speed v of particles in a viscose medium is given by:

$$v = \frac{2d_c^2(\rho_c - \rho_m)RCF}{9\eta} \tag{4}$$

where d_c is the diameter of the cell and η is the viscosity of the medium. This formula was used to estimate the time, which the cells need to settle onto the substrate atfer seeding and the time, which they need during centrifugation to reach a distance 5mm apart from the substrate.

For the parameters used in the experiments all the cells should be in contact with the substrate 13min after seeding. With 5min the first time point was before all the cells were in contact with the substrate.

A detached cell reached a distance of 5mm apart from the substrate within less then a second. A centrifugation time of 5min is therefore more than sufficient to separate the detached from the adherent cells.

2.7 Centrifugation Assay

The centrifugation assay was performed according to the protocol in the Appendix. In brief, for each stiffness four substrates were prepared. HAC were seeded onto the substrates with a density of $20kcells/cm^2$. At seven different timepoints (5-300min) two of the substrates (static control) were fixed with 4% (w/w) formaldehyde in phosphate buffer (pH 7.4) on ice. The other two were placed up side down on Teflon^R rings in six well plates containing PBS, centrifuged at 2000g (Heraeus Multifuge 3SR+, Thermo Science, Waltham USA) and also fixed over night. Figure 1 illustrates the centrifugation.

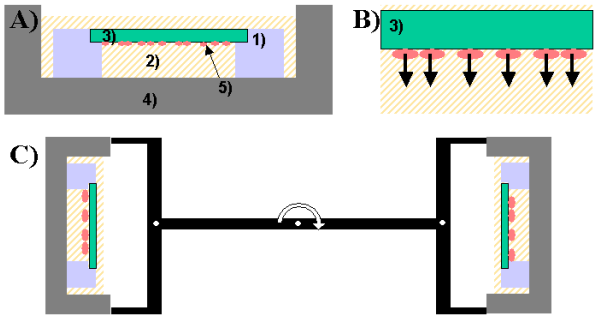


Figure 1: Schematic drawing of centrifugatrion assay. A) A Teflon spacer ring (1) was placed in a dish (4) containing PBS (2). The substrate (3) was placed up side down on this ring. Previously cells (5) were grown on the substrate. B) The centrifugation force preceived by the cells was perpendicular to the surface, as indicated by the arrows. C) The dish was centrifuged, which increased the force 2000 times.

2.8 Evaluation of the Centrifugation Assay

The number of adherent HAC was determined semi automatically by counting DAPI stained nuclei. Fluorescence images were taken on each slide at four random positions by a TS100 (10x objective, Nikon) microscope.

We used an in house built macro for the freeware ImageJ (v1.43, Wayne Rasband) to count the cells. Figure 2 shows counted cells. Since the TCPS substrates showed a strong back ground noise (auto fluorescence), a different macro was used to count cells thereon. All the counts were double-checked manualy by an overlay of an outline of the counted nucleis with a phase contrast immage as shown in figure 2.

The adherent cell fraction was determined by normalizing the cell number on the centrifuged samples to the static controls and plotted over time. In general, these plots showed a monotone increasing function with a plateau at later time points. Reyes *et al.* [39] showed that the adherent cell fraction over surface ligand density showed a sigmoidal characteristc. We adapted this formula to our needs to the following sigmoidal curve:

$$acf(t) = \frac{acf(t=\infty)}{1 + exp\left(-\frac{t-t_{50}}{b}\right)} \tag{5}$$

Where $acf(t)$ is the adherent cell fraction at time point t, b is the maximal slope of the curve and t_{50} is the time point, at which 50% of the cells were adherent after centrifugation. The time t_{50} and served as quantitative value for adhesion and was lower, the faster cells were able to adhere.

The relative rate of detached cells $rdc(t)$ was defined as the newly adherent

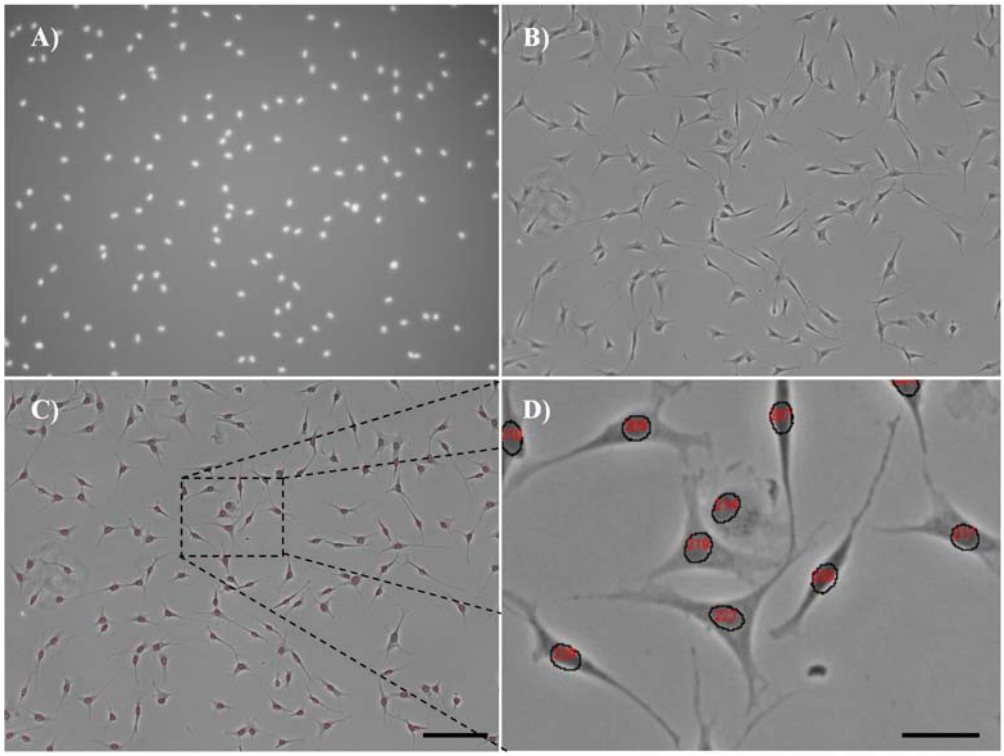


Figure 2: Semi automatically counting of cells on the substrates by fluorescence microscopy. A) Cell nuclei stained with DAPI and B) the corresponding phase contrast image. C) ImageJ was used to count the cell, outline (and numbere) the nuclei and overlay this outline with the phasecontast image. This image was used to mannualy double check the counts. D) is a zoomed region of C). (scalebar size: (A-C) 200μm; (D) 50μm)

cells per a certain time increment and equaled to the deviation of the acf:

$$rdc(t) = \frac{dacf(t)}{dt} \tag{6}$$

The function showed a continuouse curve with one maximum and no minimum.

Furthermore, the attachment was determined from the static controls of the same experiment. The attachment was defined as ratio of adherent fo seeded cells.

2.9 Cantilever Funtionalization

The protocol of Wojcikiewicz *et al.* [34] was used for cantilever functionalization. In brief, cantilevers were washed in acetone for 5min and UV iradiated for 10min. Afterwards, 50μl biotinylated Bovine Serum Albumin (biotin-BSA, Sigma-Aldrich, A8549, St. Louis USA) at 1 mg/ml in 0.1M sodium bicarbonate was adsorbed to the cantilever surface over night at 37°C. After rinsing two times in phosphate buffered saline (1x PBS, 10mM PO_4^{3-}, 150mM NaCl) and one time in 0.01x PBS, the cantilevers were incubated in 50μl of 0.5mg/ml streptavidin in 0.01x PBS (Sigma-Aldrich, 85878, St. Louis USA) at room temperature for 10min. The streptavidin solution was removed, the cantilever were washed three times with PBS and incubated in 50μl 0.2mg/ml Biotin Concanavalin A (Biotin-ConA, Sigma-Aldrich, C2272, St. Louis USA) in PBS for 10min at room temperature. After washing with PBS the cantilevers were stored in the fridge and used within 24h. This cantilever coating is shown in figure 3.

To check the coating, fluorescent labelled streptavidin was applied to biotin-BSA coated cantilevers. A fluorescent image showed a continuous staining on the cantilever surface indicating that the first two steps of the functionalization were successful.

2.10 AFM Adhesion Measurement

Since drying the cantilever could damage the funtionalization, it was kept wet during the mounting process of the cantilever to the AFM (NanoWizard, JPK Instruments AG, DE). Immediately before picking up a single cell, 10μl of cell suspension was added to a surface, which was agarose coated to keep the cells from adhering to the surface. The cantilever was lowered to the cell and pressed to the cell with a force of 1nN. After a few seconds the cantilever was lifted up and the cell stuck to the cantilever.

During the experiment the cell was lowered to the material surface till a force of 500pN was reached. This position was held for one to ten seconds using

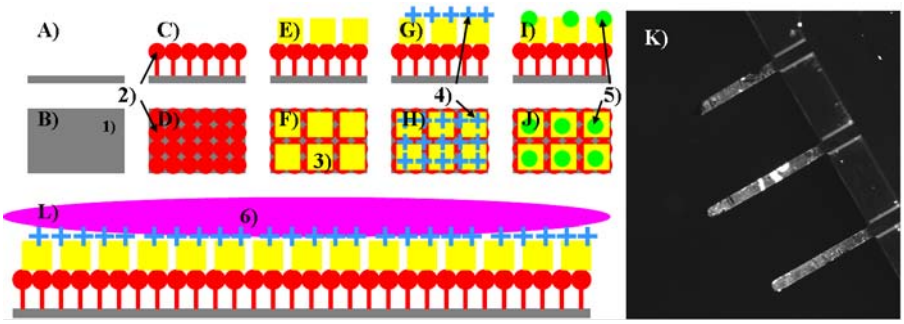


Figure 3: Schematic drawing of the cantilever functionalization in a side view (A,C,E,G,I,K) and a top view (B,D,F,H,J). (A,B) shows the uncoated silicon surface (1) of the cantilever. In (C,D) the cantilever is coated with biotin-BSA (2), followed by an incubation with streptavidin (3) in (E,F). And finally the biotinylated concanavalin A (4) functionalized cantilever is shown in (G,H). (L) Shows the binding of a cell (6) to the coat. (I,J) Shows the fluorescence labeling of the cantilevers with conjucated streptavidine (5). (K) The flourescence image of cantilevers with stained streptavidin (5) confirmed that the coating was present.

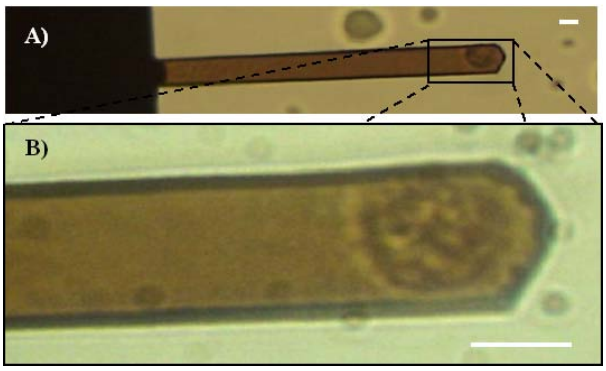


Figure 4: Picking up of a HAC onto a cantilever. A),B) A cell is picked up from an agarose coated surface and attached to the cantilever. The cell's diameter was smaller than the cantilever width. ((A+B) bar=$10\mu m$)

the constant height mode of the AFM. The deflection *vs.* z-piezo position force curves were recorded. The retrace as well as the trace speed was kept constant at 2μm/s. For each condition 15-30 force curves were collected from different locations.

3 Results

3.1 Gene Expression

The expression of type I and II collagen mRNA in HAC was performed after 7days of culture under redifferentiation conditions. The experiment was repeated fifteen times with four different donors and statistically analysed.

In contrast to the type I collagen mRNA expression (data not shown), the one of type II collagen was altered by the substrate stiffness. HAC cultured on *soft* substrates showed the same type II collagen mRNA expression level as the aggregate cultures, but a significant higher level than on the *stiff* PA and TCPS (Kurskal-Wallis paired (Conover) $p<0.05$). Furthermore, no significant differences could be found between HAC cultured on *stiff* and *infinitely stiff* substrates. On *intermediately stiff* substrates HAC showed a significantly lower type II collagen mRNA expression than on the *infinitely stiff* substrates (Kurskal-Wallis paired (Conover) $p<0.05$). The results shown in figure 5 are reproduced by Vonwil [41].

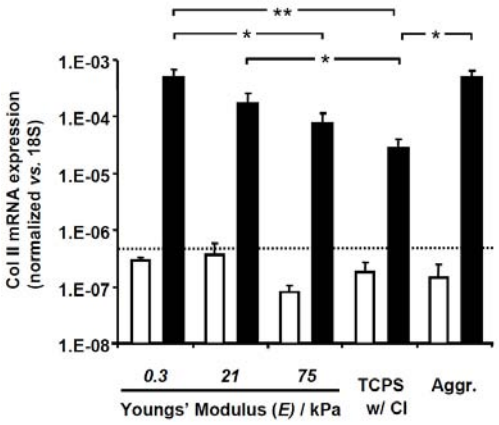


Figure 5: The expression of type II collagen of re-differentiating HAC after 7days of culture in chondrogenic medium containing TGFβ3 (black bars) or not (white bars). The values are normalized to the housekeeping gene 18S. A general trend towards more type II collagen expression on softer substrates in presence of TGFβ3, but not in absence was found. The significant differences (Kurskal-Wallis paired (Conover), * $p<0.05$, ** $p<0.01$) are indicated by asterisks above the bars. The dashed line represents the expression in expanded, dedifferentiated HAC. The graph was reproduced from Vonwil [41].

Even though the expression of type II collagen could be increased by up to 18 times on the *soft* substrate, the absolute amount of type I collagen mRNA was still over 500times higher than the one of type II collagen mRNA. In absence of TGFβ3 the type II collagen expression was several hundred times lower and

seemed not to be influenced by the substrate stiffness.

3.2 Morphology

HAC grown on the *soft* substrate showed a round shaped morphology, with limited spreading. Cells grown on all the stiffer substrates showed greater degree of spreading. Figure 6 shows typical morphologies on the different substrates. Recent studies done by Vonwil [41] quantified this change in morphology and spreading. It confirmed, that the HAC on *soft* substrates showed a significant higher shape factor as well as a significant smaller spreading area compared to the ones cultured on the *intermediately stiff*, *stiff* and *infinitely stiff* substrates. These results are shown in table 2.

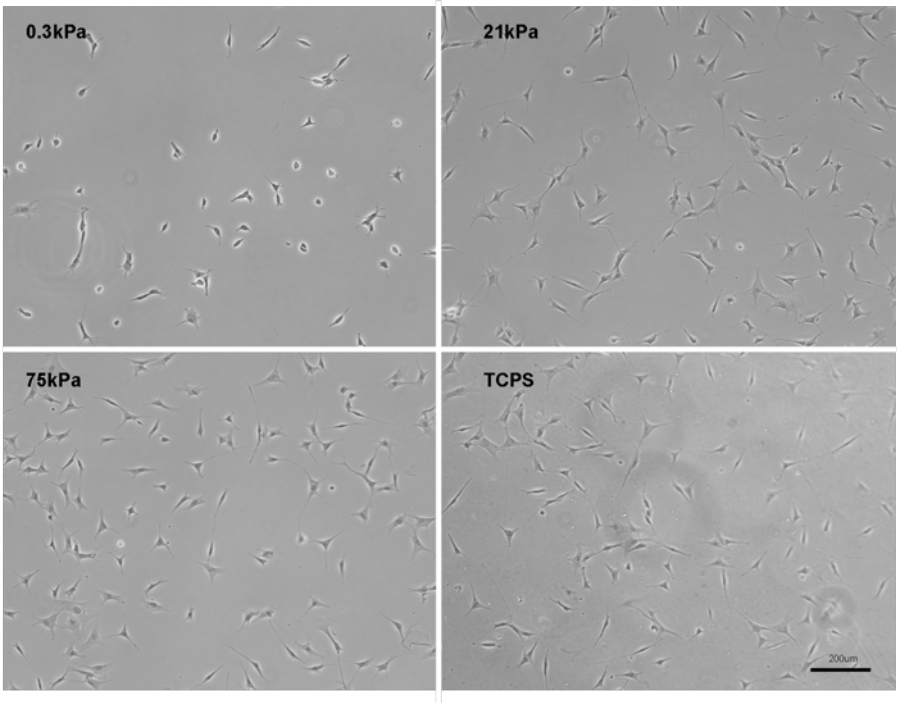


Figure 6: Phase contrast images of HAC cultrued 5h on PA. HAC on the *soft* substrate showed a more round shaped morphology, while those on the *intermediately stiff*, *stiff* and *infinitely stiff* substrate did not differ from each other in their morphology.

Table 2: The spreading area A and the shape factor ϕ of HAC cultured in chondrogenic medium for 7d. The shape factor is defined as $\phi = \frac{4\pi \cdot A}{p^2}$, with the perimeter of the cell p.

substrate	spreading area $[1000\mu m^2]$	shape factor ϕ
soft	0.40±0.02	0.35±0.03
intermediat stiff	1.34±0.06	0.25±0.02
stiff	1.50±0.07	0.23±0.02
infinitely stiff	1.28±0.05	0.25±0.02

3.3 Actin Cytoskeletal Organization

The actin cytoskeleton of HAC cultured 5h under chondrogenic conditions showed the beginning of the formation of stress fibers on the *intermediately stiff* and *stiff* substrates, while these were absent on *soft* substrate. The images are shown in figure 7.

This trend was confirmed after 7d in culture, as shown in figure 8. Actin stress fibers were formed by HAC if grown on the *infinitely stiff* to *intermediately stiff* substrate. The actin cytoskeleton on the *intermediately stiff* substrate was slightly less organized. On the *soft* substrate the cells did hardly form any fibers. It appears that the higher the substrate stiffness was, the more organized were the fibers.

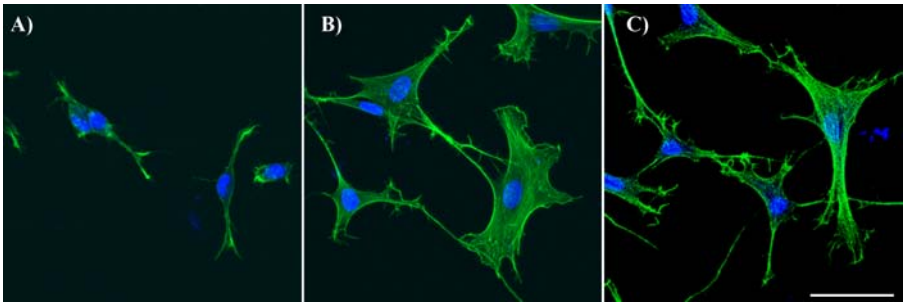


Figure 7: Actin cytoskelleton of HAC curtured for 5h in chondrogenic medium on PA. A) *soft* (0.3kPa), B) *intermediately stiff* (21kPa) and C) *stiff* (75kPa) PA. The actin is stained green and the nucleus blue. (scalebar size: $50\mu m$)

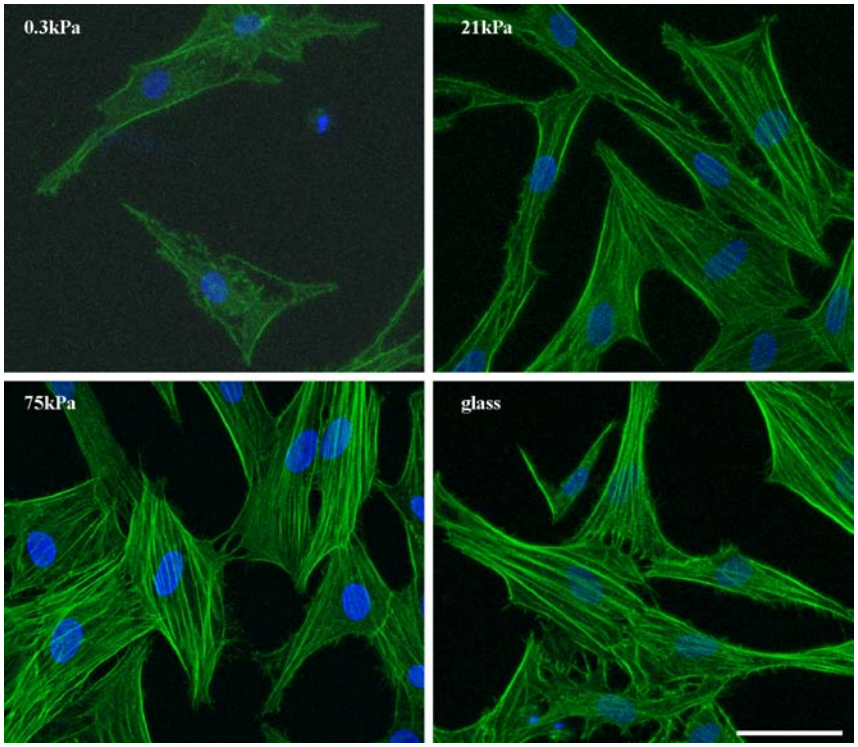


Figure 8: Actin cytoskelleton of HAC cultured for 7d in chondrogenic medium on *soft* (0.3kPa), *intermediately stiff* (21kPa), *stiff* (75kPa) PA and *infinitely stiff* (glass). The actin is stained green and the nucleus blue. (scalebar size: $50\mu m$)

3.4 Focal Adhesion Formation

Vinculin staining showed that after 5h of culturing in chondrogenic redifferentiation culture, focal adhesions were formed on the *stiff* substrate as shown in figure 9. On the *soft* substrate no focal adhesions nor focal complexes could be found (data not shown).

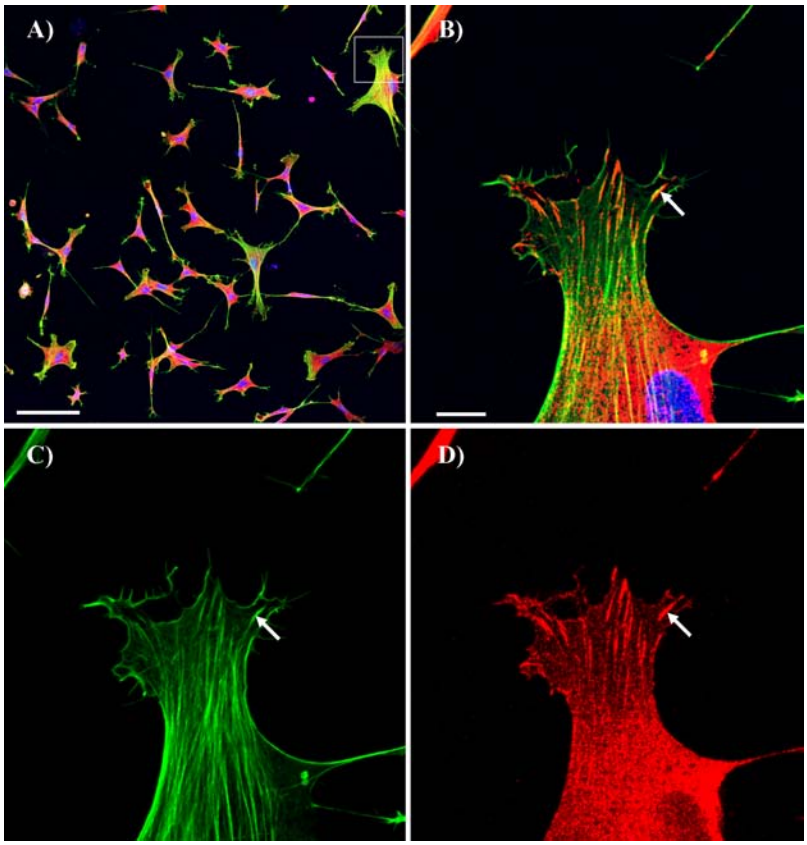


Figure 9: Focal adhesion sites of HAC on *stiff* PA. HAC cultured for 5h in chondrogenic medium on the *stiff* (75kPa) PA. An overview of the region is shown in A). B)-D) are zoomed in at the region indicated by the white rectangle. The nucleus is stained blue, the actin cytoskeleton green (C) and the vinculin red (D). A focal adhesion contact is indicated by an arrow. (scalebar size: (A) $100\mu m$; (B,C,D) $10\mu m$)

3.5 Type II Collagen Protein Expression

HAC cultured for 7days in chondrogenic medium on the *soft* PA stained for type II collagen showed a positive intracellular staining, while HAC cultured on the stiffer PA and on glass showed a much smaller intensity. Representative images are shown in figure 10. However, the staining seemed independent of the primary antibody (mouse anti human type II collagen), thus not the type II collagen was stained. Using a different secondary goat anti mouse IgG antibody showed a similar primary antibody independent staining. The staining never the less showed reproducible strong differences between the cells on the different substrates and was therefore considered as a semi-artifact.

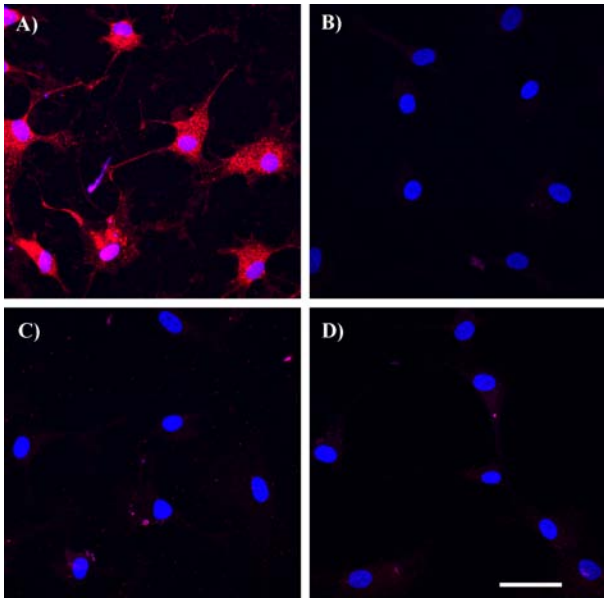


Figure 10: Fluorescent images of type II collagen (red) and nuclei (blue) stained HAC after 7days culture in chondrogenic medium on *soft* (A, 0.3kPa), *intermediately stiff* (B, 21kPa), *stiff* (C, 75kPa) PA and *infinitely stiff* (D, glass) substrates. Note, that the type II collagen staining was primary antibody insensitive and therefore at least semi-artificial. (scalebar size: $50\mu m$)

The type II collagen labeling was repeated with two different donors at different time points and the staining was quantified. The results are shown in figure 11. The imaging conditions for the second donor (donor 2) were changed to increase the sensitivity (reduced resolution, increased exposure time and increased pinhole diameter). Therefore, the absolute pixel counts could not be directly compared between donor 1 and 2. The staining was up to 50times stronger on the *soft* substrate than on the *stiff* one. Interestingly, the staining showed not only an influence of the substrate stiffness, but also a strong influ-

ence of time. The longer the cells were in contact with the substrate, the weaker was the staining.

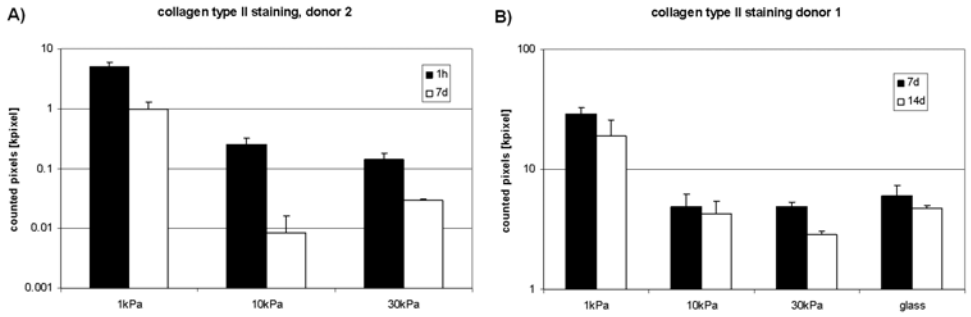


Figure 11: Quantitative analysis of the semi-artificial staining of type II collagen proteins for two donors. A+B)The imaging properties were changed from the first donor (B) to the second one (A) to increase the sensitivity of the quantification. Therefore, the absolute pixel counts between the two donors can not be directly compared. Still the same trend can be seen for both donors.

3.6 Centrifugation Assay

The centrifugation assay was performed at seven different time points between 5 to 300min after seeding HAC onto the corresponding surface. The experiment was repeated four times with two different donors. During the first hour, the fraction of adherent cells was higher on soft as compared to stiffer substrates. After about 1h, the fraction reached its maximum around 100%. In other words a force of 1.38nN was not high enough to detach cells on the substrate any more. Figure 12 illustrates these results. However, the error bars were quite high.

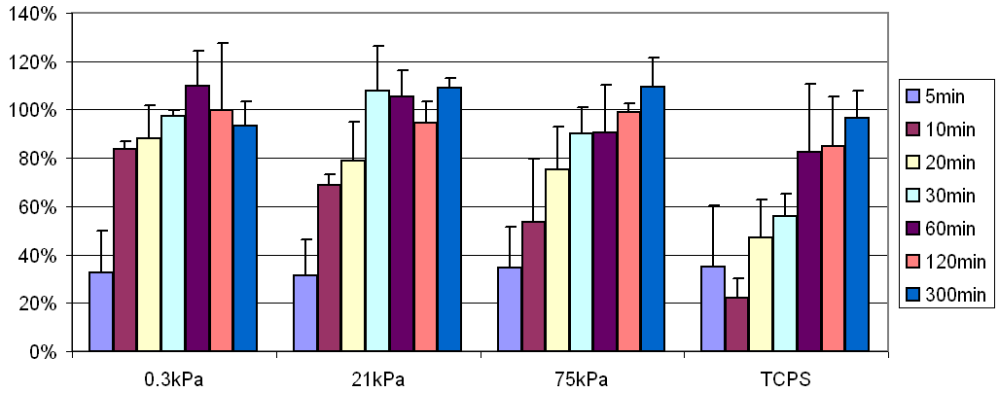


Figure 12: The adherent fraction of cells after centrifugation with 3044rpm at different time points after seeding onto substrates of contrasting stiffness. Each bar is a mean value of three to four measurements with two donors. The error bars are the standart deviations.

The attachment was determined by the static controls. No significant difference was found between the substrates after two and five hours of culturing. Furthermore, the attachment was only 10% for the first time point.

The Sigmoidal fitted curves from the adhesion measurements lead to the *rdc* distribution shown in figure 13. From these fits the time point at which 50% of the cells detached (t50) was evaluated. Even though, there was no significant difference from the *soft* to the *stiff* gel, the t50 value was in line with the general observations of the adherent cell fraction over time. The difference in t50 was significant between the *infinite stiff* substrate to the *stiff* one ((Kruskal Wallis paired (Conover) $p<0.05$)) as well as to the *intermediately stiff* ((Kruskal Wallis paired (Conover) $p<0.01$)) and to the *soft* one ((Kruskal Wallis paired (Conover) $p<0.01$)). These results are presented in figure 14.

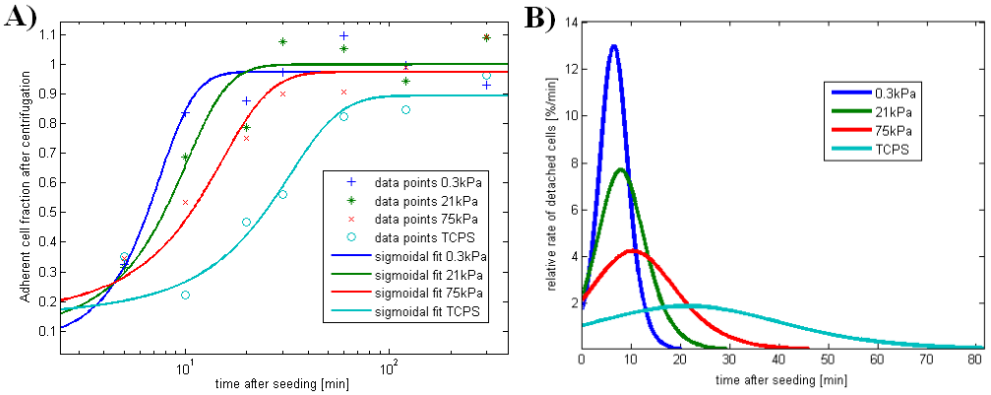


Figure 13: The fitted results from the centrifugation assay of the tested substrates. A) The results were fitted with a Sigmoidal curve. B) The *rdc* distributions were derived from Sigmoidal curves and showed the distribution of the rate of detached cells *rdc*. The maximum of this rate is at the time t_{50} were 50% of all the cells kept adherent after centrifugation.

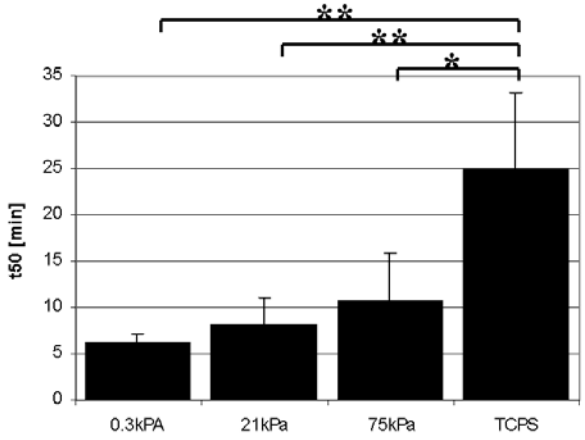


Figure 14: t_{50} times of the tested substrates. The t50 times indicate a tendency towards faster adhesion on softer substrates than on harder one. The error bars indicate the standard deviation. Significant differences (Kurskal-Wallis paired (Conover), * $p<0.05$, ** $p<0.01$) were indicated by the asterisks above the bars.

3.7 Detachment Work and Tetherings

The initial detachment work of one single HAC on the *soft* as well as on the *stiff* gel was measured at two time points (1s, 10s) using the AFM. The detachment work was calculated by integrating the force distance curve as shown in figure 15. The later time point allowed cells to interact longer with the substrate which lead to an increase in the detachment work on both tested substrates. This detachment work was measured to be in the sub femto Joule or keV range. It showed a trend towards higher initial detachment work on the softer substrate at both time points. These results are shown in figure 16. The detachment work on both gels was around 50times bigger than the one measured on agarose coated TCPS, which served as a negative control. The absolute value of the detachment work may vary with experimental conditions such as removing speed and should not be considered too much. But the relative values can be compared with each other.

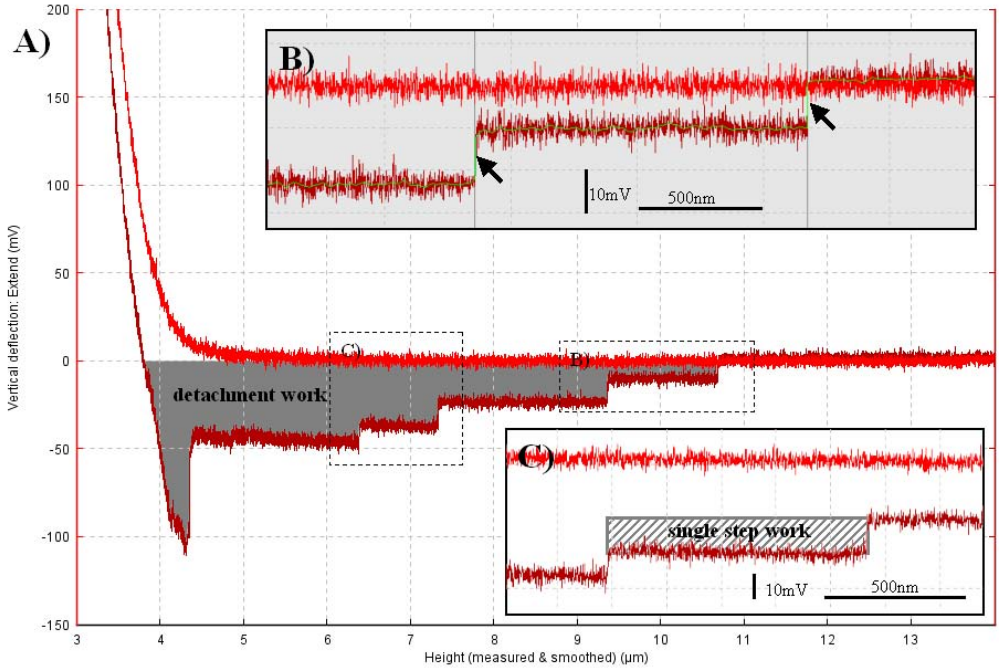


Figure 15: Force curve of HAC on *soft* substrate. A) From the typical force curve with trace (red) and retrace (dark red) the detachment work was determined by integrating the retrace (grey area). B) The tethering events (arrows) were semi-automatically quantified using JPK image processing software. C) The tethering work was calculated by multiplying the length of the event with its height.

The step like pattern in the retrace is known as tethering. The number of these tethering events per curve confirmed the detachment work results. The

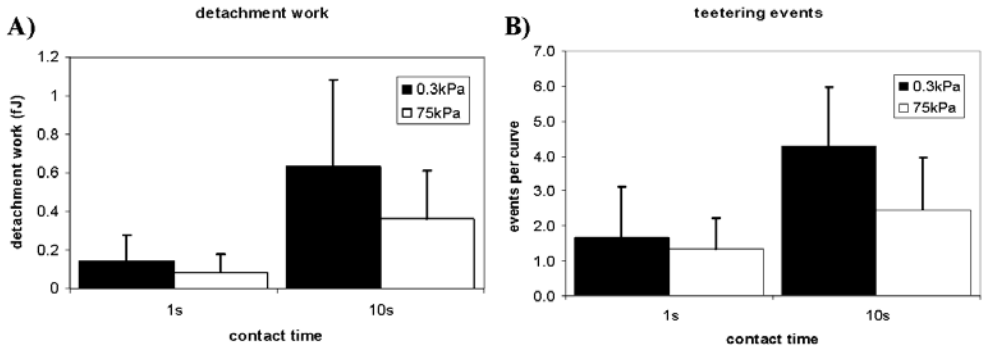


Figure 16: Detachment work and tethering events per curve. A) The work needed to detach a single HAC after 1s and 10s in contact with a *soft* (0.3kPa) and a *stiff* (75kPa) PA indicated a non significant trend in adhesion strength on the softer gel. B) A similar trend was seen by counting the tethering events per curve (B).

soft substrate showed more tethering events per curve than the *stiff* one. On both substrates an increase in the tethering events per curve with the contact time could be seen. No tethering events could be found on agarose as shown in a typical force curve in figure 17. The single step energy was calculated by multiplying the step height with the tethering length as shown in figure 15 and ranged from 0.001 to 0.1fJ. The mean of these energies for the *soft* substrate was with 0.08±0.1fJ and for the *stiff* one with 0.07±0.09fJ not distinguishable.

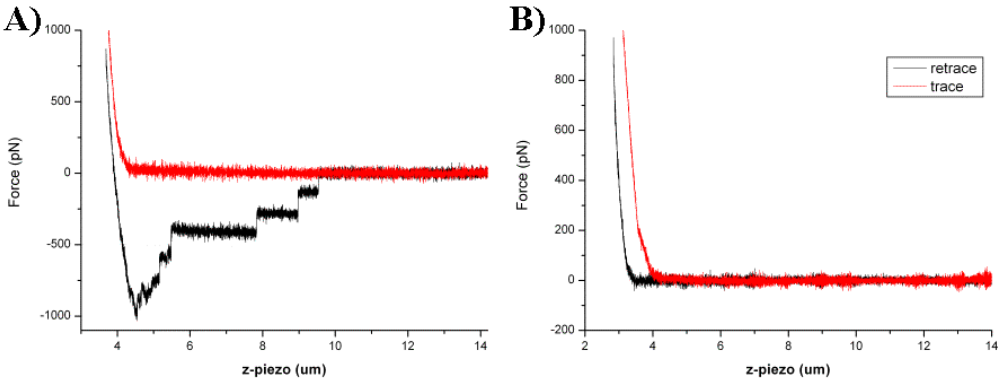


Figure 17: The force curve of HAC on the collagen coated hydrogel (A) showed tethering events, whereas the ones on agarose (B) did not.

4 Discussion

4.1 Redifferentiation Capacity

As previously shown, the actin cytoskeleton has an effect on the phenotype of chondrocytes. Treatment of cells with cytochasin, which inhibits actin polymerization, forces the cell to a round shape [44]. It could be shown, that cytochasin treatment promotes re-differentiation [45] of dedifferentiated adult chondrocytes. From the cytoskeleton point of view our results demonstrated, that the re-differentiation of HAC is preferable on soft PA compared to stiffer PA in two dimensional cultures. Furthermore, the actin cytoskeleton of the dedifferentiated HAC after 7d in chondrogenic medium was similar organized as the one of not expanded porcine articular chondrocytes cultured on PA with corresponding stiffness [22]. The actin organization of the HAC indicates that soft substrates are more supportive for re-differentiating dedifferentiated chondrocytes.

The gene expression of type I and type II collagen confirmed that the substrate stiffness had a strong influence on the redifferentiation capacity of dedifferentiated HAC in presence of TGFβ3. Our results showed an increase in type II collagen expression for up to 18fold on the softest substrates compared to the stiffest. Freshly isolated porcine articular chondrocytes cultivated for 7days on PA [22] showed an 2-fold up-regulation only. This might be due to the fact, that our substrates were about ten times softer. However, the gene expression of the redifferentiated HAC showed that the synthesis of proteins was still far away from that of native, *in vivo* HAC.

Morphology, cytoskeleton organization as well as gene expression analysis all showed the same tendency towards higher redifferentation capacity of dedifferentiated HAC in chondrogenic medium on softer gels. The stiffness of these *soft* PA was similar to the stiffness of human mesenchymal stem cells [46]. The chondrogenic condensation of hMSCs in the early stage of skeletogenesis is therefore thought to match the stiffness of our *soft* PA. During condensation stage these chondrogenic progenitor cells undergo differentiation towards the chondrognic cell line. This may explain the increased chondrogenic redifferentiation on softer gels.

So far, it is not fully understood how cells feel the stiffness. Promising explanations are, that the stress produced by the cell result in stress sensitive conformational change in ion channels [47], higher dissociation rate of ligand receptor bindings [48], domain unfolding of extra cellular [49] and/or intracellular proteins [50], which results in new receptor binding sites. Jiang *et al.* [51]

could show, that the stiffness response of cells on fibronectin coated PA could be blocked in RPTPα deficient cell lines, while the stiffness respond to collagen coated PA was not influenced. Taken together, this indicates that there exists more than just one independent mechano-sensing mechanism.

The type II collagen staining of HAC on the *soft* substrate was in good agreement with the one found by von der Mark *et al.* [42]. However, the staining was consideret to be a semi-artifact and has to be interpreted carefully. Further experiments as described in the outlook are needed.

4.2 Initial Adhesion

During the AFM adhesion measurements the contact area between the cell and the substrate could not be estimated. It may be suggested that the HAC indented the *soft* PA more than the stiff. This would lead to an increased contact area on the *soft* substrate compared to the *stiff* one. A bigger contact area would of course also lead to a bigger chance of building a tethering event and therefore also to an increase in the work needed to detach a cell. Therefore, the stronger detachment work and higher number of tethering events per curve of dedifferentiated HAC on PA indicated by the force spectroscopy measurements might be due to a difference in contact area.

An other explanation for the higher detachment work on softer substrate may be a difference in ligand penetration depth on the substrate. Even if it was shown that spheres with diameter of $1\mu m$ could not penetrate the PA [27], cells may act dynamically on the surface and achieve a deeper penetration of the gel, resulting in the ability to access more ligands. However, the results from the AFM esperiments should be interpreted carefully, since they are based on a single experiment with one cell only.

It is not yet fully understood, which interactions formed the measured tethering events. The work of Puech *et al.* [43] showed that these steps vanished upon addition of soluble RGD. Since it is known that RGD binds to integrins they concluded, that these steps were due to integrin substrate interactions. We found no difference in single tethering event energy and concluded, that a single interaction between the cell and the substrate on the *soft* PA was not distinguishable from the one on the *stiff* PA.

The low attachment of cells to the substrates 5min after seeding confirmed on one hand the theoretical calculations from the introduction and explained on the other hand the considerable error bars of the 5min time point measurement.

The adhesion characteristics determined by the centrifugation assay showed a significantly faster adhesion on the PA than on the collagen coated TCPS.

Since TCPS was a totally different system, there were most probably other factors (like ligand density, accessibility and presentation) than only substrate stiffness, which lead to the increase in t50. No significant stiffness depending change in t50 was found on the PA. However, the slightly faster adherence on softer PA might be due to the slightly higher collagen ligand penetration of the PA, resulting in a higher ligand density. Still, higher ligand density should result in a more spread morphology, therefore cells should spread less on the harder PA. Our results showed the opposite, indicating that the effect of ligand density, if present, played a significantly less important role than the substrate stiffness.

The sharpness of the *rdc* distribution was also influenced by the resolution of the time axis. With a higher t50, the curve was automatically flatter, because less time points were analized. Therefore, it was hard to determine how much of the curve shape was influenceded by the experimental design and how much by the cell substrate interaction. To eliminate this experimental designe influence one might design an experiment in which the time points are linear distributed over time. However, the t50 time should not be affected by this artifact.

Previously it was shown that the traction forces of T3T fibroblasts were lower on softer substrates than on stiffer ones [27]. Our findings were that the dedifferentiated HAC initially adhered slightly faster on the softer PA. They were made at the initial few minutes of cell-surface contact. We assumed, that during this time no change in membrane proteins occurred. The traction force was measured much later and was therefore more a long term response of the cells to the substrate. The cells had time to upregulate and express genes and form more complex adhesion interactions.

The cell cycle plays an important role in the cell substrate adhesion. Osteosarcoma cells in S-phase showed an increased detachment work compared to those in G_1 or G_2M phase [38]. We tested the attachment time for a population of cells with the centrifugation assay rather than measuring single cell detachment work. Over 1500 cells were counted in average per condition and experiment, leading to an overall of about 150k cells. Most likely there were some cells in S as well as in other phases. Since the experiments were done in parallel, we assumed that the relative distribution of cell cycle phase was similar in all conditions. Therefore, the cell cycle should not affect these results.

The force, which leads to the detaching of the cell during centrifugation, was attacking equaly distriubted within the cell rather than on one part of the surface. Therefore, this centrifugation assay to characterize the adhesion of the cells might be cell friendly. It might be possible to cultivate the not adhesive or the

adhesive fraction of the cells for further studies after centrifugation. However, the viability of HAC after centrifugation was not tested.

5 Conclusion

We could show that the substrate stiffness had a strong influence on the chondrogenic redifferentiation capacity of HAC in presence of TGFβ3. Dedifferentiated HAC cultured on *soft* (0.3kPa) substrate showed an increase in type II collagen gene expression, a more native round shaped morphology and a less organised actin cytoskeleton than those cultured on stiffer (21kPa, 75kPa) polyacrylamide hydrogels or on TCPS.

No significant difference was found in the initial adhesion of dedifferentiated HAC on *soft* (0.3kPA), *intermediately stiff* (21kPA) and *stiff* (75kPa) PA, the initial adhesion on the *infinitively stiff* TCPS was significant slower. What lead to this slower adhesion remains unknown. The initial adhesion did not significantly modulate the redifferention capacity of dedifferentiated HAC into their native phenotyp on our substrates.

It is known that blocking of the adhesion of HAC to a substrate lead to a maintenance of their round morphology. We could show, that the cell could adhere to all the PA quite fast and without significant difference accoridng to the t_{50} times. Therefore, the lack of adhesion ligand proteins is not an explanation for the increase of the re-differentiation capacity of the dedifferentiated HAC on soft substrates.

While matrix elasticity in combination with TGFβ3 as a soluble signal may be an important prameter to influence chondrogenic differentiation of chondrogenic progenitor cells, initial adhesion appears largly unaffected by this parameter.

This new knowledge combined with other factors may help to design an optimal redifferentiation assay of HAC in a more rational way, which brings us one step closer to a successful treatment of articular cartilage defects by autologous chondrocyte implantation or matrix assisted autologous chondrocyte implantation.

6 Outlook

In future studies, HAC might be TFP expanded right on the substrate followed by a medium change to serum free chondrogenic differentiation meduim containing TGFβ3. Furthermore, it might be a good idea to change the ligand coating to type II collagen, which is natively the most abundant in cartilage. Other important factors for future experiments might be the cell density and the zonal origin of the HAC.

Type II collagen labeling at earlier time points or even before seeding might help to find out, whenever the controverse results have been a full artifact or not. There would be three possible results:

1) The cells on the *soft* substrates are stained stronger already from the beginning, which indicates an experimental artifact most probably due to fixation or the staining itself.

2) All the cells are stained equally strong at the beginning, but the ones on stiffer substrate loose the staining after some time. This would indicate a maintenance of a cell property on the *soft* substrate, but a loss of this property in the cells grown on stiffer substrates.

3) All the cells are stained equally weak at the beginning, but the staining for the ones on the *soft* substrate increases before it starts falling again. This would indicate a change in a cell property selectively for the cells grown on the *soft* substrate.

Furthermore, the substrate could be blocked with a goat serum instead of the bovine serum for the type II collagen labeling, because both secondary antibodies were expressed in goat and a goat specific blocking might decrease the unspecific binding of the antibody even more.

To increase the sensitivity of the centrifugation assay, the RCF might be increased to higher values. However, this might prove dangerous since the force may increase the deformation of the gels, damage the glass slides or even the 6-well plate. Linear time points might increase the time resolution of the *rdc*.

To determine the viability of HAC after centrifugation (e.g. by Evans blue staining) may help to see if the centrifugation essay is cell friendly or not and to find out if this centrifugation assay might allow the sorting of cells corresponding to their adhesion properties.

Synchronizing the HAC as well as the use of monoclonal HAC may decrease the variability of the adhesion onto substrate, since the intercellular difference would be reduced. This may therefore increase the sharpness of the *rdc* distribution and make AFM studies more feasible.

The polyacrylamide hydrogel system proofed to be a valuable tool, but as

it is a 2D system, the cells are polarized, which may lead to a lack of their native morphology. Future studies should therefore be performed in a 3D system, where cells are able to form 3D contacts with the surrounding matrix as well as cell-cell contacts. However, the translation of this system from 2D to 3D might prove difficult, since it is not possible to embed cells into the gel during polymerization due to the toxicity of the monomer. There are promising new 3D models currently under investigation like a gel made of fibrinogen and polyethylene glycol [52], which allow the control of mechanical properties independent of biochemical properties.

A Appendix

A.1 Reduction of the Centrifugal Force

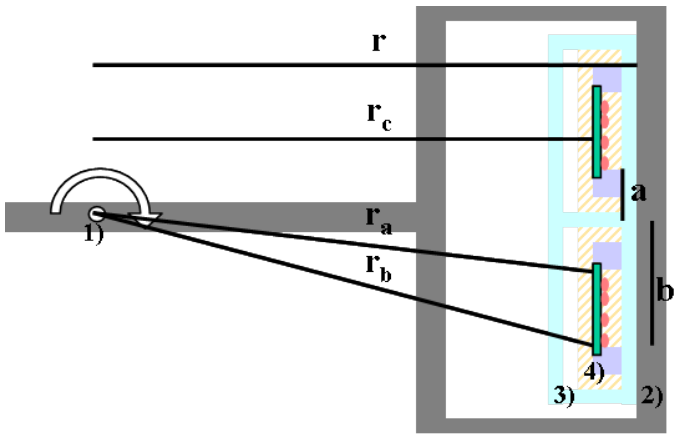


Figure 18: The geometry of the experimental setup leaded to reduction in the radius r, which is definded as the distance from the middel axis (1), to the centrifugal basket (2). This was due to the organization of the single wells (4) in the 6-well-plate (3) and due to the organization of the sample in a single well.

There were changes in the radius r due to geometry. Figure 18 ilustrates these changes. A general reduction of the radius $r = 15.9cm$ to $r_c = 15.6cm$ was due to the spacer rings. The sample itself had a extension is space, which leaded to a minimal radius r_a and a maximal radius r_b inside each sample. These radius where calculated to with the following formula:

$$r_a = \sqrt{r_c^2 + a^2} \tag{7}$$

$$r_b = \sqrt{r_c^2 + b^2} \tag{8}$$

With $a = 0.72cm$ and $b = 3.02cm$ the results were $r_a = 15.6cm$ and $r_b = 15.9cm$, which leaded to a maximal error in radius inside a sample of less than 2%, and therefore due to equation (3) also in force.

Because each well of the 6-well-plate had the same distance to the middle axis of the centrifuge, there was no difference in radius nor in force between the different sampels.

The effective radius in the centre of each sample was according to equations (7,8) $r_{effectiv} = 15.7cm$, which leaded with equation (3) to a force of $1.38nN$ and a RCF of 1627g.

A.2 Speed of Particals in Viscose Liquids

The speed of a particle in viscose medium is calculated from the stokes equation:

$$F_{stokes} = 6\pi\eta r v \tag{9}$$

A partical during centrigugation is in a balance of forces. The centrifugal force, the lift force and the stokes force sum up to zero:

$$F_{stokes} + F_{cen} + F_{lift} = 0 = \frac{4}{3}\pi RCF \cdot r^3((\rho_c - \rho_m) - 6\pi\eta r v \tag{10}$$

Solving this equation to the velocity v gives:

$$v = \frac{2d_c^2(\rho_c - \rho_m)RCF}{9\eta} \tag{11}$$

This equation is similar to the one described above (4). To find out, whenever the flow was viscose or not, we calculated the Raynolds number Re:

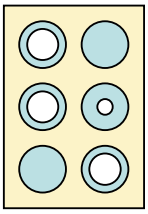
$$Re = \frac{v\rho_m d_c}{\eta} \tag{12}$$

This calculation resulted in $Re = 3 \cdot 10^{-3}$ for the seeding part (RCF=1g) respectively $Re = 0.15$ for the centrifugation part (RCF=1627g). Since both $Re < 1$ was true for both conditions, we assumed a mainly viscose characteristics, which confirmed these results.

A.3 Centrifugation Assay

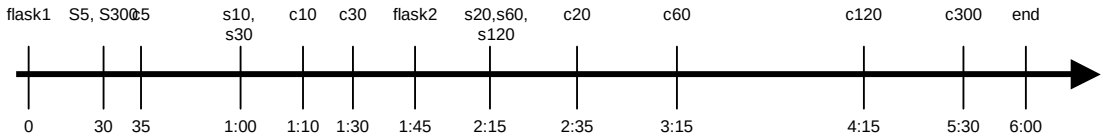
Centrifugation Assay: Cell Substrate Adhesion

Preparation

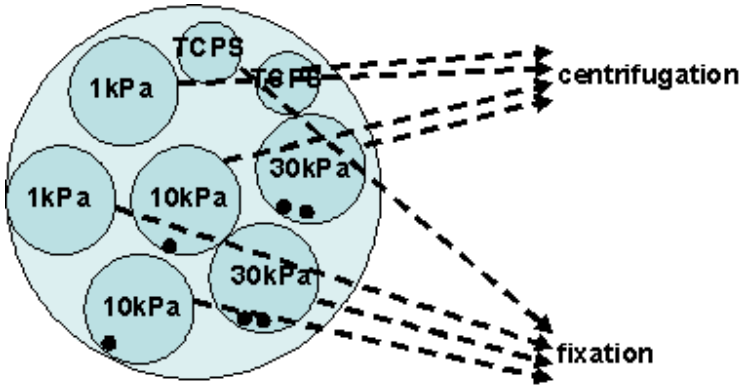


- Prepare substrate: Prepare four gels on 23mm round glass coverslides for each condition (e.g. 7 time points,4diff. substrates->112 samples).
- Prepare Petri dishes: label two Petri dish for each time point (eg: 5a, 5b, 10a, 10b, 20a, 20b,...)
- Prepare two 6-well plates for centrifugation: Add three Teflon rings for the glass slides and one for the TCPS per 6-well plate (see image). Add 3ml PBS to the rings for glass slides and 1.5ml PBS for the ones for TCPS. Label one plate A, and the other B.
- Prepare plates for fixation: Add 2ml of Formaldehyde in PBS per well of a 6-well plate for the glass slides and 1ml Formaldehyde in PBS per well of a 12-well plate for the TCPS. Label the plates and wells and put them on ice.
- Prepare one 50ml Falcon tubes containing 15ml CM per time point (7 tubes for standard time points).
- Prepare 200ml SFM+T.

- Have a plan ready, otherwise you may miss a time point. An example (c means centrifugation and s seeding, flask means harvesting flask):



- Mark the stiffness of the gel on the slides with a pen on the back side (e.g. one dot for 21kPa, two dots for 75kPa and no dot for 0.3kPa gels).



- Pipette 12ml PBS to the Petri dishes.
- Put two 1kPa, two 10kPa, two 30kPa and two TCPS slides in each Petri dish. Two Petri dishes are needed for one time point (duplicate).
- Make sure that the slides do not overlay each other and UV radiate them in the hood for 30min.

Experiment

Seeding:

- Aspirate the PBS from the Petri dishes.
- Add 12ml DMEM to each Petri dishes and place them into the incubator (approx. 30min before seeding).
- Harvest the cells with collagenase II and 0.05% Trypsin in EDTA. Centrifuge, aspire the supernatant, resuspend in **CM** and count them.
- Add 2.43 million cells into each prepared Falcon tube. If you do additional slides for microscopy add cells for them as well to a separate falcon tube (1.22M per Petri dish). Store the falcon tube at 37°C.
- Just before seeding centrifuge cells, aspire supernatant and resuspend them in 24ml SFM+T
- Seed 12ml of the cells suspension to each of the two Petri dishes, set timer (5min,10min,…)

Centrifugation:

- As soon as the alarm clock rings, put the slides up side down to the prepared centrifugation 6-well plates
- Immediately start the centrifugation (2000g, accelerating 6, decelerating 6, temp 25°C)
- Immediately put the not centrifugation slides into the fixation solution on ice.
- After centrifugation put the slides into the fixation solution on ice.
- Renew the PBS in the centrifugation 6-well plates (3ml for 23mm glass slides, 1.5ml for TCPS.
- Put the fixed slides into the fridge **over night**.

- Repeat this step for all the other time points.

Evaluation

Prepare PerS and LS:

- PerS 80ml: 170.4mg Trition X100 in 80ml PBS (or 2x 85.2mg Triton X100 in 40ml PBS)
- LS 14.7ml: 300ul DAPI (48x) in 14.4ml PBS

Staining:

- Wash two times with PBS
- Permeabilize cells with PerS 10min on ice (0.8ml for 23mm glass slides, 0.3ml for TCPS).
- Wash three times with PBS.
- Aspire the PBS and add the LS to the slides for 30min at room temperature (150ul for 23mm glass slide, 70ul for TCPS).
- Wash three times with PBS.

Imaging:

- Use a fluorescence microscope to visualize the staining.
- Use 10x magnification.
- Make four fluorescence pictures per sample form different spots on it and label the images clear (e.g.: sample 23, duplicate a, spot three, fluorescence=23a3f.tif)

The TCPS slides have strong background. Therefore take a glass slide, paint a circle on it with the Darko PEN, add PBS to it and put the TSPC slide up side down on the droplet. The circle with the pen should enable to keep the PBS on one spot.

Counting:

The counting can be done with the freeware ImageJ. Use the macros newexp for the TCPS samples and the cenX for the glass slides. The program will give you a list of the counted cells for each slide position on the slide.

A.4 List of Abbreviations

- AAS: acrylamide solution, monomer solution
- acf: adherent cell fraction
- ACI: autologous chondrocyte implantation
- AFM: atomic force microscope
- APS: ammoniumperoxodisulfate, polymerization starter
- BB: blocking buffer
- BIS: N,N'-methylene bisacrylamide, crosslinker
- BSA: bovine serum albumin
- CM: complete medium
- DAPI: 4',6-diamidino-2-phenylindole
- DMEM: Dulbecco modified Eagle's medium
- DMSO: dimethly sulfoxide
- ECM: extracellular matrix
- EDTA: ethylenediaminetetraacetic acid
- FBS: fetal bovine serum
- FGF2: fibroblast growth factor 2
- HAC: human articular chondrocyte
- HEPES: 4-(2-hydroxyethyl)-1-piperazineethansulfonic acid, buffer
- hMSC: human mesenchymal stem cell
- HSA: human serum albumin
- ITS: medium supplement containing insulin, transferrin and selenium
- LB: labelling buffer
- MAACI: matrix assisted autologous chondrocyte implantation
- PA: polyacrylamide hydrogel
- PBS: phosphate buffered saline
- PDGF: platelet-derived growth factor
- PerS: permeabilization solution
- RCF: relative centrifugal force [g]

- rdc: relative rate of detached cells $[min^{-1}]$
- RGD: amino acid code for Arginine-Glycine-Aspartic acid
- SEM: scanning electron microscopy
- SFM: serum free chondrogenic medium
- TCPS: tissue culture treated polystyrene
- TEMED: N,N,N',N'-tetramethylethylenediamine, polymerization starter
- TFP: growth factors TGFβ1, FGF2 and PDGF.
- TGFβ1: transforming growth factor β1
- TGFβ3: transforming growth factor β3
- TIRM: total internal reflexion microscopy

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