Influence of hyaluronic acid binding on the actin cortex measured by optical forces

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Abstract
Melanoma cells are often surrounded by hyaluronic acid (HA) rich environments, which are considered to promote tumour progression and metastasis. Induced effects in compound materials consisting of cells embedded in an extracellular matrix have been studied, however, alterations of the single cells have never been addressed. Here, we explicitly addressed single cell properties and measured HA-induced biomechanical changes via deformations induced solely by optical forces. With the optical stretcher setup, cells were deformed after culturing them in either the presence or absence of HA revealing the crucial interplay of HA with the CD44 receptor. To assess the role of CD44 in transducing effects of HA, we compared a CD44 expressing variant of RPM-MC with RPM-MC CD44+ variant of the melanoma cell line with CD44 receptor; RPM-MC, melanoma cell line without CD44 receptor (also wild type of cell line); TGF-β, transforming growth factor beta; WT, wild type; μOS, microfluidic optical stretcher.

Abbreviations: ECM, extracellular matrix; ERM, ERM protein family: ezrin, radixin, moesin; FACS, fluorescence-activated cell sorting; FN, fibronectin; HA, hyaluronic acid; LatA, Latrunculin A; n.s., not significant; PDGF, platelet-derived growth factor; RPM-MC CD44+, variant of the melanoma cell line with CD44 receptor; RPM-MC, melanoma cell line without CD44 receptor (also wild type of cell line); TGF-β, transforming growth factor beta; WT, wild type; μOS, microfluidic optical stretcher.

Jörg Schnauß, B. U. S. Schmidt and Christina B. Brazel contributed equally to this study.

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Cells can adjust their internal structure with regard to material properties of the environment they are adhered to [1, 2]. The local microenvironment and its extracellular matrix (ECM) are associated with remodeling of the cytoskeleton of cancer cells, which directly influences motility and force generation of the cells [1, 3]. Mechanical adaption of the cells to changes of the environment is well-known and for example used in diagnostics tools such as elastography [4, 5]. In addition to mechanosensitive responses, structural and functional adjustments also highly depend on the type of adhesion receptor triggering the connection to the substrate [6]. In tissues, cells interact with the ECM via mechanosensitive adhesion sites as well as by receptors activated by components of the ECM. These components do not necessarily have to act as direct binding sites, but can influence the transduction of mechanical cues between cells and the ECM [1].

A prominent ECM component is hyaluronic acid (HA). HA is an unbranched, negatively charged glycosaminoglycan polysaccharide, which is ubiquitously present in vertebrate tissues [7]. The functions and properties of HA in a given tissue depend on its structure, which is modified by interactions with cell surfaces, proteins, proteoglycans, and crosslinking by other components of the ECM [5, 8]. In healthy tissue, the HA amount is balanced by constant synthesis and degradation while over-production of HA or up-regulation of HA receptors promote cell migration, invasion of tumor cells or rapid tumor growth [9–11]. In certain types of tumors, the HA level is prognostic for malignancy due to its important role in tumor growth as well as metastasis. HA levels are thus correlated with poor patient survival in various types of cancer including prostate, breast and ovarian cancer [7, 10–15].

Components of the ECM, including HA, are mainly synthesized and remodeled by fibroblasts. In many types of cancer, fibroblasts show an activated phenotype, which produce increased amounts of HA and other ECM components such as collagen and fibronectin (FN). The phenotype of these cancer-associated fibroblasts is comparable to myofibroblasts, which are activated in the process of wound healing. However, the mechanisms that lead to fibroblast activation in cancer are incompletely understood. In cell culture models, melanoma cells secrete minor amounts of HA, but stimulate HA synthesis of fibroblasts in a paracrine manner by secretion of TGF-β and PDGF [16–19].

It has been recently demonstrated that the concerted interactions of cells with a HA-rich microenvironment lead to a collected contractility of the cells exerting tension on the surrounding matrix [5]. In contrast to these bulk effects, we specifically address HA-induced effects on the single cell level. With this approach we decouple purely cellular responses and effects specifically caused by cell-matrix interactions.

Here, we used the CD44-negative RPM-MC melanoma cell line and a generated, CD44-expressing variant RPM-MC CD44+ to investigate the influence of HA and especially the influence via the CD44 receptor, which is known to mediate between cells and ECM-bound HA [5]. We cultured cells on a pure, thin HA layer to inherently exclude microenvironment or mechanosensitive effects, which are known to be induced by HA via substrate adhesion [15]. Subsequently, the cells were detached and suspended for measurements of single cell mechanics in the optical stretcher (Figure 1). The optical stretcher is a dual laser beam trap, which allows to deform cells in suspension by optical forces without any surrounding microenvironment [20]. Culturing cells on FN instead of HA served as control for cells with CD44 receptors.

CD44 is a class I transmembrane glycoprotein, which is expressed by almost every vertebrate cell type. The highly conserved CD44 gene gives rise to a polymorphic group of proteins by means of alternative splicing and post-translational modifications. The smallest CD44 isoform, CD44s, is the most commonly expressed protein in vertebrate tissues. The extracellular part of CD44s binds to HA and the intracellular tail has binding sites for several proteins, which con-
nect CD44 to the actin cytoskeleton [21]. Ankyrin connects CD44 via spectrin to actin filaments, whereas ERM (ezrin, radixin, moesin) proteins create a direct link between CD44 and actin [22, 23]. Although other HA receptors exist, CD44 has been shown to be the primary receptor for HA in melanoma [24]. While it is well established that CD44 mediates tumor cell migration on HA [25] and HA induced proliferation [24], the influence of CD44-HA interactions on cell mechanics is poorly understood.

2 | MATERIALS AND METHODS

2.1 | Coating of cell culture dishes and coverslips

For HA treatment, dishes were coated with 1.5 mg high molecular weight HA overnight. Coverslips were coated for 15 minutes alternately with 1 mg/mL poly-L-lysine (Sigma-Aldrich) and 1 mg/mL high molecular weight HA in 10 successional layers. For FN-coating, dishes were coated with 20 μg FN; coverslips were coated with 25 μg/mL FN for 30 minutes. HA and FN were obtained from Innovent e.V., and Biochrom, respectively. All coatings were performed at room temperature.

2.2 | Cell culture and drug treatment

Cells were grown in RPMI 1640 (Biochrom) supplemented with 10% FCS (Biochrom) and 1% ZellShield (Minerva BioLabs) at 37°C in 5% CO₂. For optical stretcher measurements, cells were seeded on HA or FN coated dishes and cultured for 4 or 24 hours, respectively. Cells were treated with 0.25 μM Latrunculin-A (Sigma-Aldrich) for 6 hours or 10 μM blebbistatin (Sigma-Aldrich) for 30 minutes prior to stretcher measurements.

2.3 | Generation of stably transfected cells

The cDNA coding for CD44s was cloned into a pSecTag2B vector (Invitrogen). Wild type (WT) RPM-MC cells (Cd44-negative) were transfected with Lipofectamin 2000 (Invitrogen) according to the manufacturer's protocol and cultured in the presence of 200 μg/mL zeocin (InvivoGen) as selection reagent. Stably CD44 expressing cells were selected by FACS (BD FACSVantage SE, Becton Dickinson). Cells were

**FIGURE 1** CD44+ expression enhances cell stiffness after culturing on hyaluronic acid (HA) substrate. A, Schematic of optical stretcher setup. Cells in suspension are pumped to the focus of a dual beam laser trap and stretched by optical forces. B, HA-activated CD44 enhances binding to ankyrin and ERM. This increases the interaction of the actin cortex with the membrane. C, CD44 expression analysis of wild type (RPM-MC; CD44-negative) and CD44 transfected melanoma cell line (RPM-MC CD44+) performed by flow cytometry. D and E, Creep deformation curves of RPM-MC and CD44+ counterpart in the optical stretcher for the whole step stress pattern (left) and for the end of stretch (right, t = 3 seconds). Both cell types were measured after being cultured for 24 hours on HA or fibronectin (FN, control), respectively. The CD44 expressing cells are significantly stiffer when cultured on HA. Creep deformation curves are represented by their median (solid lines) using bootstrapping to estimate a 95% confidence interval as error (shaded areas in respective color). The differences in the bar diagrams were evaluated with a Kolmogorov-Smirnov test (n.s., not significant; *P < .05)
continuously cultured in the presence of zeocin and were regularly tested for CD44-expression via flow cytometry.

### 2.4 | Antibodies

Antibodies used in our study were mouse anti-CD44 (Clone SFF304, ThermoFisher Scientific) and rabbit anti-Vimentin (kindly provided by Prof Dr Thomas M. Magin, Leipzig). Filamentous actin (F-Actin) was visualized using Phalloidin-TRITC (Sigma-Aldrich) as described previously [26, 27]. Fluorophore coupled secondary antibodies used in the study were goat anti-mouse Alexa488 and goat anti-rabbit Alexa647 (Invitrogen).

### 2.5 | Immunofluorescence analysis

Cells were seeded on coverslips coated with HA or FN and cultured for 30, 90 or 180 minutes. Cells were fixed with 4% formaldehyde/PBS for 20 minutes, permeabilized for 10 minutes with PBS/0.1% Triton X-100 and blocked with 1% BSA/PBS for 10 minutes. Subsequently, cells were incubated with primary antibodies for 1 hour, followed by incubation with secondary antibody for 30 minutes. All antibodies were diluted in 1% BSA/PBS and all incubations were performed at room temperature. Coverslips were mounted with MobiGLOW mounting medium (Mobitech). Image acquisition was performed using a Zeiss LSM 780 confocal microscope with 40×/1.3 NA or 63×/1.46 NA objectives as well as a spinning disc confocal microscope (inverted Axio Observer.Z1/Yokogawa CSU-X1A 5000 (Carl Zeiss Microscopy GmbH, Germany). For Image analysis and processing AxioVision 4.8 and Zen 2010 Software (Zeiss) were used.

### 2.6 | Flow cytometry

Cells were detached using 0.025% Trypsin/EDTA (Biochrom) and transferred to 96-well plates (Greiner Bio-One). Cells were stained with primary antibody or isotype control antibody for 1 hour followed by two brief washes with PBS/10% GelaFusal (Serumwerk Bernburg, Germany). Cells were incubated with secondary antibody for 45 minutes and all antibody incubations were performed at 4°C; antibodies were diluted in PBS/10% GelaFusal. Flow cytometry analysis was performed using a Cytomics FC 500 flow cytometer (Beckman Coulter). For data acquisition and analysis, CXP Cytometer and CXP Analysis 2.2 (Beckman Coulter) software was used, respectively.

### 2.7 | Adhesion and spreading assay

For analysis of spreading, cells were seeded on HA coated coverslips, cultured for 30, 90 or 180 minutes and subjected to immunofluorescence staining and image acquisition as described above. Images of F-actin staining were used to determine cell areas with ImageJ. Per cell line, 10 randomly chosen microscopic fields were analyzed in 3 repetitive experiments.

### 2.8 | Cell viability assay

Cells were seeded in FN coated 24-well plates and cultured in medium with or without 500 μg/mL HA for 24 hours. Viability of cells was determined using XTT-cell proliferation Kit II (Roche) according to the manufacturer’s protocol. Cell viability is given as the absorption at 490 nm subtracted for absorption at 690 nm. Three different samples have been analyzed each with five technical replicates.

### 2.9 | Optical stretcher measurements

The cells were detached from the culture flask using 0.025% Trypsin/EDTA (PAA) and resuspended in culture medium. The cell suspension was then added to the reservoir of the microfluidic optical stretcher (μOS) setup, which is based on the setups described previously [28–31]. In brief, single cells are pumped through a capillary to the focus of a microscope. Within the field of view, cells are sequentially trapped by two divergent laser beams at 100 mW and a step stress experiment is performed using an increased laser power of 800 mW for 2 seconds (see Figure 1A). The deformation of cells is captured at 30 fps (PHYTEC FireWire-CAM-111 H camera, PHYTEC, Germany). Experiments were performed at a constant stage and reservoir temperature of 23°C. For each cell type, experiments were performed with the same batch of cells having the same passage number. In order to achieve the highest possible comparability, we measured the cells of the two variants each on the same day with the differing conditions. The start of the culturing of the samples with the different conditions was explicitly adjusted to the measurement times in the optical stretcher to ensure that the cells were subjected
to the same culturing times prior to the measurements. These measurement sequences have been carried out in triplicates.

### 2.10 Deformation data analysis

A self-written Matlab algorithm (The MathWorks, Inc.) was employed to extract the contours for each frame, which were subsequently used to derive the deformation data and curves for every single cell. A creep deformation \( J(t) = \frac{\varepsilon(t)}{\sigma_0} = \frac{\varepsilon(t)}{F_g} \) was used to evaluate the cells, with \( \sigma_0 \) being the optically induced stress depending linearly on the applied laser power \([32]\) and \( \varepsilon(t) = (d(t) - d(0))/d(0) \) the relative deformation along the long axis \( d \) of the cell (\( d(0) \) being the time-dependent diameter of the cell along the laser axis and \( d(0) \) the initial cell diameter before the stretching process started). The relative deformation uses specific assumptions, which vary between different cell lines. The geometric factor \( F_g \) accounts for the used model of the cell, for example a homogenous sphere or a shell-like object. In our standardized analysis, we set \( F_g \) to 1 and continue to use the creep compliance in arbitrary units, since a model requires assumptions that differ for various cell types and a normalization with the laser power \( P \) is sufficient to compare results. Thus, the values in arbitrary units readily differ for different cell types, but the values within one cell line can be compared, when the culturing conditions are comparable. To clearly distinguish between different cell lines, more parameters would have to be accounted for, which are not necessary for the effects presented in this study. All creep deformation curves are represented by their median using bootstrapping to estimate a 95% confidence interval as error.

### 2.11 Statistical analysis

To evaluate the level of significance of curves of the mechanical deformations, the Kolmogorov-Smirnov test was used. This nonparametric test is especially suitable to determine whether two probability distributions differ or come from the same distribution.

The Mann-Whitney \( U \) test is nonparametric test, which can be used to evaluate if two independent samples were selected from populations having the same distribution and that observations from both groups are independent of each other. This test has been used to evaluate whether HA induces significant differences regarding cell viability and during spreading assays between the two variants of the cell line.

### 3 RESULTS AND DISCUSSION

#### 3.1 Effects on cell mechanics

To investigate the influence of CD44 and HA in conjunction with the cell stiffness, we have chosen the melanoma cell lines RPM-MC (CD44 negative) and the variant RPM-MC CD44+, which expresses CD44 due to stable transfection \([33, 34]\). We used FN coating as control and HA coating to activate the CD44 receptor pathways. The HA formed a thin film on the hard plastic substrate so that mechotransduction \([33, 35]\], as for example on hydrogels of lower stiffness, could be excluded as an effect in our experiments. Thus, we were able to study only the signaling pathways in conjunction with exposure to HA in the substrate that work independently from the biomechanical pathways.

We used the optical stretcher for the mechanical measurements of cells in suspension since an atomic force microscope measurement would additionally dependent on adhesion, which is known to be modulated by HA \([35]\). The optical stretcher allowed us to measure the stiffness change of the cells after exposure to HA without having to account for increased adhesion.

For the first set of control experiments CD44 negative WT cells (RPM-MC) where cultured separately on FN or HA coatings for 24 hours. Subsequently, the cells were detached and their mechanical properties measured in the suspended state revealing no significant changes in deformation behavior for the different coatings (Figure 1D). Repeating this procedure with the CD44 expressing RPM-MC CD44+ cell line revealed a significant stiffening of cells cultured on the HA substrate compared to cells cultured on FN (Figure 1E).

#### 3.2 Differences in CD44, actin and vimentin distribution

A change in the deformation behavior of cells should be also reflected in the cytoskeleton. Thus, we employed...
immunofluorescence staining to investigate how the cytoskeletal components change with regard to whole cell deformation. When culturing cells on FN, F-actin and vimentin distribution as well as filament organization were similar for both cell types (Figure 2A). In contrast to the FN coating, actin and vimentin filament organization were drastically altered in both cell types when cultured on HA substrate. We would like to note that the cells of both variants cannot adhere to HA substrates as sufficiently as to FN coated surfaces. Thus, cells appear more roundish and both cell types exhibited less actin stress fibers while vimentin was more diffusely distributed in the cell cytoplasm (Figure 2B). Since cell adhesion was markedly influenced on HA substrates for both cell variants, we conducted spreading assays to evaluate the comparability. These tests revealed no differences in adhesion area and rate (Figure 2C). Furthermore, to ensure that the cells were not permanently damaged due to the experimental procedure, we conducted viability tests showing that the presence of HA during culture slightly increased the viability for both cell types (Figure 2D). For these experiments, the cells had to be cultured on FN surfaces to sufficiently adhere while HA was added to the media in the respective experiments (see Section 2).

Interestingly, the RPM-MC CD44+ cells clearly displayed a co-localization of CD44 and ezrin (see Supporting Information Figure S2), a member of the ERM protein family. These proteins are known to function as general cross-linkers between plasma membrane proteins and the actin cytoskeleton indicating the pronounced role of actin in the apparent cellular changes [22].

3.3 | Influence of the actin cortex

To investigate the influence of the actin cortex, we treated the RPM-MC CD44+ cells with Latrunculin A to disrupt actin filaments [28]. This additional treatment negated the stiffening effect originally observed in the creep deformation experiments after culturing these cells on HA substrates (Figure 3A). Independent of the culture substrate (HA or FN), the deformation curves are within each other’s confidence intervals after the treatment with Latrunculin A. Furthermore, the RPM-MC CD44+ cells are not significantly stiffer at the end of the stretch when grown on HA substrates. This control indicates that the change in creep deformation is mediated via the CD44 receptor, possibly by a stronger binding of the actin cortex to the cell membrane [21, 38].

Our results clearly show that actin structures are key factors for the altered mechanical responses of the RPM-MC CD44+ cells upon HA treatment. To decouple cellular responses such as the structural reorganization of the cytoskeleton and altered protein expression levels, we compared measurements after plating these cells for only 4 hours on the FN or HA substrate, respectively (Figure 3B). This short culture time excludes changes in protein expression levels to a large extend since such a
response would need longer time scales. Interestingly, the effects and changes after this short culturing time are similar to the alteration after the longer culturing time indicating that the different mechanical properties are purely induced via cytoskeletal rearrangements. Creep deformation curves are represented by their median (solid lines) using bootstrapping to estimate a 95% confidence interval as error (shaded areas in respective color). The differences in the bar diagrams were evaluated with a Kolmogorov-Smirnov test (n.s., not significant; \( *P < .05 \)).

4 | CONCLUSION

In conclusion, we were able to show that the interaction of HA and the CD44 receptor can modulate the cell mechanics significantly. This could counteract the effects of mechanotransduction and adaption on softer hydrogels where the cells would become softer. To gain a comprehensive picture, however, different cell types and other substrate coatings such as albumin, poly-lysine or collagen have to be considered in future studies. Based on these findings we suggest that HA contributes to a loss of adhesion via a tighter connection between membrane and actin cortex. This effect also shows that the cytoskeleton is directly capable of adapting the phenotype of the cell to changes in the ECM via CD44 receptors or...
mechanotransduction without necessarily adapting via gene expression. This may trigger metastatic cells to quickly adapt to new environments without going through a full transition between an epithelial and mesenchymal phenotype. Since metastatic cancer cells are rather soft, the HA-rich environment of tumors could stiffen them, thus facilitating a more efficient escape from the tumor. After leaving the tumor and the HA-rich microenvironment, these cells can soften again in order to migrate through dense tissue.

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CONFLICT OF INTEREST
The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS
Jörg Schnauß, B. U. Sebastian Schmidt, Christina B. Brazel, Wolfgang Losert, Ulf Anderegg and Josef A. Käs planned and designed experiments. Jörg Schnauß, B. U. Sebastian Schmidt, Christina B. Brazel and Ulf Anderegg performed experiments. Jörg Schnauß, B. U. Sebastian Schmidt and Christina B. Brazel analyzed the experimental data. Jörg Schnauß and Senol Dogan correlated experimental data to patient databases. Wolfgang Losert, Ulf Anderegg and Josef A. Käs supervised the project. Jörg Schnauß and B. U. Sebastian Schmidt wrote the manuscript. All authors discussed and interpreted results and commented on the manuscript. Jörg Schnauß, B. U. Sebastian Schmidt and Christina B. Brazel contributed equally to this work.

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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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