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Subcellular stretch-induced cytoskeletal response of single fibroblasts within 3D designer scaffolds

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ABSTRACT

In vivo, cells are exposed to mechanical forces in many different ways. These forces can strongly influence cell functions or may even lead to diseases. Through their sensing machinery, cells are able to perceive the physical information of the extracellular matrix and translate it into biochemical signals resulting in cellular responses. Here, by virtue of two-component polymer scaffolds made *via* direct laser writing, we precisely control the cell matrix adhesions regarding their spatial arrangement and size. This leads to highly controlled and uniform cell morphologies, thereby allowing for averaging over the results obtained from several different individual cells, enabling quantitative analysis. We transiently deform these elastic structures by a micromanipulator, which exerts controlled stretching forces on primary fibroblasts grown in these scaffolds on a subcellular level. We find stretch-induced remodeling of both actin cytoskeleton and cell matrix adhesions. The responses to static and periodic stretching are significantly different. The amount of paxillin and phosphorylated focal adhesion kinase increases in cell matrix adhesions at the manipulated pillar after static stretching whereas it decreases after periodic stretching.

1. Introduction

Cell behavior is governed by several intrinsic but also by extrinsic signals. Beside chemical cues, external forces and the properties of the extracellular matrix (ECM) [1] influence cell decisions. To sense the environment, cells mostly use mechanosensory units like stretch-modulated ion channels, primary cilia, or dynamically generated cell matrix adhesions (CMAs) [2–4]. Here, transmembrane integrin receptors bind with their extracellular domains to ECM proteins and cluster, while the cytoplasmic tails are connected with a complex of proteins including paxillin and the focal adhesion kinase (FAK) [5,6]. Next, mechanical signals are transduced into biochemical signals and functional responses, a process known as mechanotransduction [1,7–10]. This connection leads to an activation of intracellular signaling pathways conducting cellular responses like cell migration, cytoskeletal rearrangement, changes in the spatial distribution of CMAs, or alteration of gene expression patterns.

To investigate the influence of mechanical stimuli on cell behavior, various methods have been developed to measure cell responses. Each method has its own advantages and disadvantages [11]. Studies throughout the last decades could show that mechanical forces can be applied onto cells in different ways. Cell populations as well as single cells have been objects of the studies. Many techniques have one thing in common: They can manipulate cells on a cellular but not on a subcellular level. Entire single cells can be deformed using micropipette aspiration assays [12]. A deformation of a single cell can even be achieved by stretching the cell between two pipettes, the spacing of which is increased [13–15]. Mechanical stimuli with the help of shear-induced stress [16–18] or stretchable substrates [19–21] are inexpensive and easy to apply, but the effect of these stimuli is always affecting the entire cell.

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In addition to these methods, biophysical manipulation with the help of optical tweezers, magnetic tweezers, and atomic force microscopy have been locally applied on cells in different studies. Using magnetic tweezers, integrin receptors in single cells or even single molecules have been studied [22,23]. Atomic force microscopy (AFM) is commonly used to analyze cell mechanics in cell-cell adhesions or to study which mechanical forces are necessary to unfold specific protein repeats [24,25]. Another study shows the possibility to exert a force onto single CMAs using magnetic microposts [26]. While all of these studies can apply forces onto single cells, the different cells under investigation may have a varying morphology and a heterogeneous distribution of CMAs. This heterogeneity leads to difficulties in averaging over results on different individual cells, which is highly desirable for further analysis with significant statistics.

In this work, we introduce a new approach to specifically stretch individual CMAs at a subcellular level in single primary fibroblasts exhibiting uniform phenotypes. Our approach is based on dedicated three-dimensional scaffold architectures fabricated by direct laser writing (DLW) [27–31] and controlled deformation of these scaffolds using a precise home-built micromanipulator. The hexagonal scaffold structures allow the cell to attach only on well-defined, bio-functionalized patches without establishing undesired contact sites. This leads to a defined and identical geometry of each single cell. We achieve this by using two different photoresists, one with protein-binding and the other one with non-protein-binding properties [29]. This combination is crucial to control the spatial distribution and size of cell matrix adhesion sites in three dimensions. As a result, the external forces are only applied *via* these adhesion sites.

The objective of the present study is to characterize and understand how this mechanical manipulation affects the morphology of the actin cytoskeleton as well as the molecular composition of single CMAs in comparison to other non-manipulated CMAs within the same cell.

2. Material and methods

2.1. Direct laser writing

For the fabrication of the 3D structures we used a commercially available DLW system (Photonic Professional, Nanoscribe GmbH). It is equipped with a 100×, numerical aperture NA = 1.4 oil immersion objective. This lens also allows for adjusting the sample for the second lithography step. Typical average laser powers in the DLW process were 10–20 mW (in front of the microscope lens) and the typical piezo scanning velocity was 200 μm s^{-1}.

2.2. Scaffold preparation

To enhance the adhesion of the photoresists to the glass-substrate surface, plasma-cleaned coverslips were functionalized with 3-(trimethoxysilyl) propyl methacrylate (Sigma Aldrich; 1 mM in toluene) for 1 h, rinsed in water, and dried with nitrogen [32]. For the first writing step, the photoresist was drop-cast onto the coverslips. After exposure, the scaffolds and an additional marker structure at the glass–photoresist interface were developed in a 1:1 mixture of methyl isobutyl ketone (MIBK) and isopropyl alcohol, rinsed in isopropyl alcohol, and allowed to dry in air. The second photoresist was then drop-cast onto the coverslips carrying the scaffolds. The marker structure was then used to align the cover slip in the lateral plane for the next DLW step. The vertical position was automatically adjusted by the Photonic Professional system by finding the glass-photoresist interface. After the second DLW step, the composite scaffolds were developed, rinsed in isopropyl alcohol, and finally dried with nitrogen gas.

The used photoresists were Ormocomp (micro resist technology) and a home-made resist [29] composed of the monomer polyethylene glycol diacrylate (PEG-DA) with 4.8% (wt) pentaerythritol tetraacrylate (PETTA) as a mechanical stabilizer for the resulting polymer and 3.0% (wt) Irgacure 369 as a photoinitiator.

2.3. Cell culture

Coverslips holding 3D composite scaffolds were first washed in 70% ethanol. After rinsing in phosphate buffered saline (PBS), they were incubated for 30 min at room temperature with 200 μl of 10 μg ml^{-1} fibronectin (life technologies) in PBS, which preferentially binds to the Ormocomp parts of the scaffolds. Then they were rinsed with PBS and placed in small petri dishes containing 2 ml F12 supplemented media as described below.

Chicken embryonic fibroblasts (CEFs) were derived from the dermis of 8-day-old embryos and grown on tissue culture plastic in F12 medium supplemented with 10% bovine growth serum (HyClone), 2% chicken serum (life technologies), and 0.2% Pen/Strep (20 U ml^{-1} and 20 μg ml^{-1}, Sigma Aldrich) under a humidified atmosphere containing 5% CO_2. For our studies CEFs were used from passages 5–10.

To remove cells from tissue culture dishes, they were washed twice with PBS and treated with Trypsin (0.17%)/EDTA (1.6 mM) in Hank's balanced salt solution (HBSS) free of Ca^{2+} and Mg^{2+} (life technologies) for 2 min. Dissociated cells were washed in F12 medium and centrifuged, 40.000 cells in suspension (about 50 μl) were drop cast into the petri dishes holding the scaffolds. Then, the samples were placed into the incubator for 3 h to allow cell spreading.

To obtain optimal imaging quality and sufficient magnification (40×) during the micromanipulation experiments, immediately prior to the experiments the coverslips holding the scaffolds and cells were transferred into sample holders for microscopy. They consist of a steel bottom plate with a circular hole for imaging holding the cover slip, a silicone O-ring, and a magnetic upper body to hold the culture medium (life cell instrument). During the experiments 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) was added to the culture medium as a buffer.

2.4. Micromanipulator

The custom-made micromanipulation-setup consists of a pulled glass capillary with a tip diameter of about 1 μm mounted on a 3D piezoelectric actuator for precise positioning with a range of 100 μm in each direction and a mechanical 3D positioning stage with a travel range of 13 mm along all three axes.

The micromanipulator tip was positioned next to a structure with a cell growing symmetrically inside of it. The structure was then deformed by pushing the tip against it in such a way that the displacement of the adhesive cube was 5.3 μm. The displacement was either applied statically or sinusoidally with a frequency of 0.5 Hz for a duration of up to 15 min.

2.5. Biological sample preparation

Cells were fixed immediately after micromanipulation using 4% paraformaldehyde (PFA) in PBS for 10 min. Thereafter, they were permeabilized using 0.1% Triton X-100 in PBS for 3 × 5 min. Cells were incubated in primary antibody solution for 1 h at room temperature. Then they were rinsed with 0.1% Triton X-100 in PBS for 3 × 5 min and incubated in secondary antibody solution for 1 h at room temperature containing Phalloidin Alexa 488 (Molecular Probes, 1:100) and DAPI (Roth, 1:1200).

Primary antibodies and dilutions used were mouse anti-paxillin (BD Transduction Labs, 1:500), rabbit anti-phosphorylated paxillin (Tyr118) (Life technologies, 1:500), rabbit anti-phosphorylated FAK (Tyr 397) (Invitrogen, 1:300). Secondary antibodies used were goat anti-mouse Alexa 647 (Dianova, 1:200) and goat anti-rabbit Alexa 568 (Invitrogen, 1:300). After rinsing with PBS for 3 × 5 min the cells were embedded in a few droplets of Mowiol (Hoechst) on microscope slides.

2.6. Microscopy

Scanning electron microscopy (SEM) images were obtained with a Supra 55 scanning electron microscope (Zeiss). Biological samples were analyzed using a laser scanning microscope (LSM 510 Meta, Zeiss) equipped with a 63×, 1.4 Oil DIC objective (Zeiss). Corresponding manipulated and control cells were scanned with the same settings.

2.7. Image analysis

From the LSM image stacks a maximum intensity projection was calculated. Then images were centered and rotated such that all cells had the same orientation (manipulated pillar on the left). The intensities of each image and channel were divided by their respective mean values. Out of these normalized intensities the averaged intensity distribution for each channel was calculated for all cells of one set of parameters. Resulting false-color plots of one channel are represented on the same scale.

We calculate the asymmetry factors of the distributions according to

$$A = \frac{I_m - I_0}{I_m + I_0}. \quad (1)$$

Here, I_m are the intensities at the manipulated region of the cell and I_0 the intensities at the non-manipulated region. To define which pixels belong to I_m and I_0, we define regions of interest (ROIs) as follows: As the cell is confined in the volume between the pillars it adheres to, we choose a circle C_1 around the center of the structure with a radius R_1 defined by the distance of the pillars to this center as a ROI for the actin cytoskeleton. In order to compare the affected side of the cell with the non-affected one, we divide this ROI into two halves by a vertical line through the center. The CMAs are located in an area close to the adhesion sites the structure is offering. Thus, we define additional circles around the pillars with a radius R_2 that is half the distance of one pillar to the next. The ROIs for the CMAs are then confined by