Cytoskeleton remodelling of confluent epithelial cells cultured on porous substrates

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The impact of substrate topography on the morphological and mechanical properties of confluent MDCK-II cells cultured on porous substrates was scrutinized by means of various imaging techniques as well as atomic force microscopy comprising force volume and microrheology measurements. Regardless of the pore size, ranging from 450 to 5500 nm in diameter, cells were able to span the pores. They did not crawl into the holes or grow around the pores. Generally, we found that cells cultured on non-porous surfaces are stiffer, i.e. cortical tension rises from 0.1 to 0.3 mN m⁻², and less fluid than cells grown over pores. The mechanical data are corroborated by electron microscopy imaging showing more cytoskeletal filaments on flat samples in comparison to porous ones. By contrast, cellular compliance increases with pore size and cells display a more fluid-like behaviour on larger pores. Interestingly, cells on pores larger than 3500 nm produce thick actin bundles that bridge the pores and thereby strengthen the contact zone of the cells.

1. Introduction

Structure and function of eukaryotic cells heavily depend on their cellular microenvironment leading to a close coupling between cellular properties and the surroundings. Sensing of the environment is usually accomplished through a defined molecular contact between a protein network—the extracellular matrix (ECM)—and specified transmembrane proteins such as integrins that connect the ECM network to the cytoskeleton allowing the transmission of force. Adhesion of cells is the initial step that precedes cell spreading, proliferation, differentiation and cell–cell contact formation. Cellular adhesion determines the function and fate of eukaryotic cells to a much larger extent than initially expected. In particular, environmental cues such as those emanating from the substrate itself, like topography, elasticity or surface functionalization, govern a large number of cellular responses encompassing cell growth, gene expression, apoptosis all accompanied by substantial cytoskeletal remodelling [1]. Strikingly, also the differentiation of stem cells is guided by mechanical and adhesive properties of the culture dish [1]. Cells are capable of sensing the underlying substrate and respond to variations in elasticity or topography as first shown by Pelham & Wang [2]. Since then, many studies have shown an influence of substrate rigidity on cellular migration, proliferation, cell stiffness and even differentiation [3–5]. Cells gather information about mechanical properties of the surroundings via mechanotransduction [6–8], in which they sense their mechanical environment mostly via cell–cell or cell–substrate connections. These mechanosensory elements rely predominantly on the actin cytoskeleton. The actin cytoskeleton is tightly linked to the membrane and is able to generate forces via motor proteins of the myosin family. It is therefore conceivable that the amount of tension that can be generated due to the deformability of the substrate is responsible for the integration of the mechanical signal. While the impact of substrate stiffness on cell morphology and more particularly on cell adhesion has been well examined, less attention has been paid to substrate...
topography of rigid surfaces. Especially in the context of cell attachment and growth on implant materials, surface roughness and topography are decisive parameters that might foster or reduce the degree of differentiation and polarization of epithelial cells. Numerous hard materials ranging from metals to silicon are used by the healthcare industry to serve as artificial joint replacements, stents or dental implants. Surface treatment to change the roughness has been proved to modulate adhesion of cells, cytokine release and gene expression of osteoblastic cells [9]. Spatz and co-workers [10] used colloidal lithography to provide specific attachment sites for integrins in a defined geometry, thus addressing topographical effects on the nanoscale. The study helped to shine light on the universal length scale that defines the optimal spacing of RGD sequences found in ECM proteins such as collagen to match the intrinsic spacing of integrins in the basal cell membrane. Recently, it has also been found that topographical cues can determine the fate of stem cell differentiation [11,12]. Additionally, the response of cells to their environment depends heavily on the cell type. So far, mostly mesenchymal cells such as fibroblasts have been studied. These cells have been demonstrated to bridge non-adhesive areas via actin stress fibres and myosin II [13]. Similarly, growths of epithelial cell monolayers over large non-adhesive gaps simulating wound healing of skin have been shown to rely on tension generated by the actomyosin network [14]. Supports of microneedles or pillars used for traction force microscopy also show that cells can span large non-adhesive areas connected via contractile elements [15]. Consequences of substrate topography for cell morphology, polarity and mechanics in established cell monolayers have only sparsely been addressed, although surface topology is crucial to identify the right scaffolds for epithelial tissue engineering that requires a fundamental understanding how cell adhesion and mechanics are coupled to environmental cues and how cells respond collectively to changes in substrate topography.

Here, we systematically investigated the morphological and viscoelastic properties of MDCK-II cells grown to confluence on hard porous substrates with varying pore sizes. We found that cells generally appear softer and more liquid-like with increasing pore size up to 5.5 μm in diameter and remodel their actin cytoskeleton to span larger pores. Additionally, cells generally become smaller and higher when grown on pores. Cultured on substrates with pore sizes larger than 5 μm in diameter, MDCK-II cells mirror the cubic organization of the underlying substrate and also display a larger amount of excess surface area and localization of ezrin only at the apical membrane of the cells. The study shows how subtle changes in substrate topography substantially influence the morphology and mechanics of epithelial cell layers by forcing the cell to remodel the actin cytoskeleton in response to the altered environment. As softness and morphology of the cells can be adjusted by changing the porosity and pore size of the surface, it is conceivable to use this technique also in implants, stents and wound healing to adapt to the biological requirements.

2. Material and methods

2.1. Substrates

Substrates with pore diameters of 0.45, 0.8, 1.2 and 2 μm were purchased from fluXXion B.V. (Eindhoven, The Netherlands). The porous membrane of these substrates consists of silicon nitride and displays a regular hexagonal pore pattern (figure 1, insets). The porosity varies between 20% and 30%. To produce substrates with larger pore diameters (3.5 μm, 5.5 μm, we used (1-0-0) oriented SOI wafers as described previously [16]. To achieve a hydrophilic surface, the pores were covered with a SiO2 layer of 500 nm thickness by means of wet-thermal oxidation. After cleaning the substrates in argon plasma, they were coated with a 30–35 nm thick gold layer by argon sputtering (Balzers, BAE 250 coating system) or were first sputtered with a thin titanium layer (Cressington 108auto Sputter coater, Watford, UK) and gold-coated afterwards (BAL-TEC MED 020 Coating System). Before use, the substrates were sterilized in pure ethanol and incubated with cell culture medium before inoculation of the cells.

2.2. Cell culture

All experiments were carried out using epithelial Madin–Darby canine kidney cells (MDCK-II cells) purchased from the Health Protection Agency (Salisbury, UK). The cells were cultured in minimal essential medium with Earle’s salts (Biochrom, Berlin, Germany) containing 4 mM L-glutamine, 2.2 g l−1 NaHCO3 and 10% FCS (M10F medium) at 37 C in a 5% CO2-humidified incubator. Confluent cell layers were subcultured weekly by trypsinization (Biochrom). For experiments, substrates were placed into a Petri dish (2.5 cm, TPP, Switzerland). Cells were seeded in a density of 500 000 cells per Petri dish and incubated for 2 days at 37 C in a 5% CO2-humidified incubator. Experiments were carried out in M10F medium supplemented with penicillin–streptomycin and HEPES. For actin depolymerization experiments, the medium additionally contained 2 μM cytochalasin D (see the electronic supplementary material).

2.3. Atomic force microscopy

Atomic force microscopy (AFM) experiments were carried out using a MFP-3D (Asylum Research, Santa Barbara, CA, USA) set-up equipped with a BioHeater mounted on an inverted Olympus IX 51 microscope (Olympus, Tokio, Japan). MLCT cantilevers (C-lever, nominal spring constant 10 pN nm−1, length 200 μm, tip height 8 μm, Bruker, Camarillo, CA, USA) with a pyramidal tip were used for imaging and force spectroscopic experiments. Prior to each experiment, the spring constant of the cantilever and the hydrodynamic drag force acting on the cantilever were determined on a flat glass slide. The spring constant was calibrated using the thermal noise method [17]. The hydrodynamic coefficient was calculated by a method previously described by Alcaraz et al. [18]. Afterwards, the glass slide was replaced by the substrates used for cell culturing. A homemade holder of spring steel fixed the substrates during measurement inside the BioHeater. The temperature was set to 37 C throughout measurement. Before each force spectroscopic measurement, the area of interest was imaged in contact mode (constant force, see figure 1).

Force spectroscopy and frequency-dependent rheological data were acquired by the cantilever approaching the surface with a velocity of 3 μm s−1. When the pre-set cantilever deflection was reached, the z-piezo movement was stopped for 0.5 s before it was excited to oscillate with frequencies between 5 and 100 Hz at small oscillation amplitudes (A0 = 40 nm, peak to peak). After an additional quiescent period of 0.5 s, it was retracted from the surface. Per area of interest, 1024 of these force–distance curves where recorded in a 32 × 32 point grid, thus the individual positions where the force curves have been measured have a distance of 2 μm. Each experiment has been independently conducted at least two times probing several cells.
Figure 1. Morphology of MDCK-II cells grown on substrates with different pore sizes. Images show AFM deflection maps of the cell surface of living MDCK-II cells. The diagrams below are height profiles of the cells shown in the picture (red line, average over five scan lines). Inlets show 10 × 10 μm² AFM height images of the porous substrates (approx. twofold magnification compared with the corresponding deflection images of the cell surface). (Online version in colour.)
for a maximum of 3 days. Prior to labelling, the samples were washed two times with washing buffer (0.1% bovine serum albumin (BSA, Sigma-Aldrich, Munich, Germany) in PBS) and incubated for 45 min in blocking buffer (5% BSA, 0.3% Triton-X-100 in PBS) at room temperature. To stain actin filaments, samples were incubated for 1 h at room temperature with 165 nM solution of AlexaFlour546-labelled phalloidin (Invitrogen, Germany) in dilution buffer (1% BSA, 0.3% Triton-X-100). After incubation, cells were rinsed twice for 5 min in washing buffer. To label DNA, samples were treated with a 25 ng ml⁻¹ solution of 4',6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich) in PBS for 10 min at room temperature. Finally, samples were washed two times with PBS for 5 min.

2.4.2. Ezrin and ZO-1 staining

The cells were grown for 2 days on the corresponding substrate and then fixed as described in §2.4 using paraformaldehyde (PFA). Prior to labelling, the samples were washed two times with washing buffer (0.1% BSA (Sigma-Aldrich) in PBS) and incubated for 30 min in blocking buffer (5% BSA, 0.3% Triton-X-100 in PBS) at room temperature. After rinsing the sample three times with PBS, cells were incubated with mouse anti-ezrin antibody (5 µg ml⁻¹, BD Biosciences) for 1 h at room temperature. In the next step, the cells were again rinsed three times for at least 5 min using PBS and then treated with the secondary antibody (5 µg ml⁻¹ AlexaFlour546-coupled goat anti-mouse, Invitrogen) for 1 h. After staining ezrin and rinsing three times, ZO-1 was stained using an AlexaFlour488-coupled mouse anti-ZO-1 antibody (5 µg ml⁻¹, Invitrogen, 339188). Finally, the sample was washed three times and DNA was stained using a 50 ng ml⁻¹ solution of DAPI. All antibodies and DAPI were solved in a PBS buffer containing 0.1% BSA, 0.3% Triton-X. Electronic supplementary material, figure S7, shows MDCK-II cells grown in part on the porous region of the substrate and in part on the flat region. The samples were imaged using a LSM710 confocal laser scanning microscopy (Zeiss, Göttingen, Germany) equipped with a 63× objective (Zeiss) and an Argon-LASER (LASCOS Lasertechnik GmbH, Jena, Germany). The open source software FIJI (homepage: www.fiji.sc) was used for image representation.

2.5. Scanning electron microscopy and image analysis

MDCK-II cells grown on different substrates were washed with PBS before they were treated for 2 min with a 4% Triton X-100 solution in PBS. After rinsing the samples in PBS, the cells were fixed using a 2.5% glutaraldehyde solution (Acros Organics, Belgium) in PBS. The samples were incubated for 1 h at room temperature. Then cells were again washed in PBS and dehydrated using an ethanol series with an increasing ethanol proportion (50%, 70%, 80% and 90%). The samples were incubated in each ethanol solution for 1 h at room temperature and stored overnight in ethanol p.a. at 4 °C. Finally, the samples were dried using a gentle nitrogen stream and subsequently coated with a 5 nm gold layer by argon sputtering. Line densities of filaments were determined by analysing SEM images of the same category. For each pore size, a spatially linear sequence of at least 20 images was recorded. Furthermore, lines with an average length of 2 µm and random orientation were drawn into each image. Finally, the number of single filaments crossing an individual line was counted yielding the line densities in 1 nm⁻¹. This was repeated for at least 100 lines resulting in line density distributions for the different pore sizes (see figure 3 and electronic supplementary material).

2.6. Finite-element simulation

In fluorescence images of the actin cytoskeleton of MDCK-II cells grown on 5.5 µm pores, we observed that many pores were interconnected by two distinct thick actin bundles. To find an explanation for this effect, we conducted finite-element analysis. For numerical simulations, we used COMSOL multiphysics (Göttingen, Germany). A long, 200 nm thin sheet crossed by two orthogonal sheets simulated the actin cytoskeleton. The sheet's elastic modulus was set to 1 MPa and the Poisson ratio to 0.49. To account for the pores, we drew circular holes at the two crossing points of the three sheets. An isotropic inwards-directed pressure of 5 Pa was applied to the boundaries of the pores.

3. Results

MDCK-II cells were grown to confluence on substrates displaying regular pores ranging from 0.45 to 5.5 µm in diameter. Morphology, cytoskeleton organization and viscoelasticity of MDCK-II cells were investigated by techniques with high spatial resolution to quantify how substrate topology is mirrored in cellular structure and mechanics. Notably, MDCK-II cells grow on all substrates to confluence bridging the pores.

3.1. Morphology of MDCK-II cells grown on flat and porous substrates

MDCK-II cells belong to normal epithelia. They polarize when cultured on a Petri dish and exhibit a high density of cell–cell contacts (tight junctions), which is also expressed in a high barrier resistance (Rₒ) of 30 Ω × cm² measured by electric cell–substrate impedance sensing [22]. Figure 1 shows the influence of substrate topography on the morphology of confluent MDCK-II cells. MDCK-II cells grown on the gold-coated, non-porous glass substrate possess an ordinary cobblestone-like morphology with well-developed cell–cell contacts and have a size of approximately 20 × 20 µm². ECM proteins deposited on a preformed cytoine monolayer mediate adhesion of cells. The line profile shows rather flat cells with a height difference of 1.5 µm from cell–cell contacts to their highest point at the centre of the cell. With increasing pore size from 0.45 to 5.5 µm, an increase in the average height of the apical cap and a decrease in the spreading area of the MDCK-II cells can be observed. If cells are grown on a substrate with a pore size of 5.5 µm in diameter, they exhibit a height difference from their lowest point in the cell periphery to their highest point of more than 3 µm. At the same time, the cell’s footprint decreases to an area of 13 × 13 µm². A similar trend has been observed for vascular endothelial cells [23]. It should be noted that it is not possible to determine the overall cell height from substrate to apex with AFM-contact imaging but only the height of the apical cap of the cell. However, confocal laser scanning microscopy images (z-stacks) reveal that MDCK-II cells cultured on pores are generally higher than those cultured on flat substrates (see also table 1) [24].

3.2. Structure of the actin cytoskeleton

Actin filaments are stained using AlexaFlour546-labelled phalloidin and imaged by confocal laser scanning microscopy. Figure 2 shows the structure of the actin cytoskeleton at the basal level of MDCK-II cells cultured on substrates with different pore sizes. On the flat surface, the actin cytoskeleton is well developed (figure 2a). A large amount of stress fibres, which traverse the entire length of the cells, is observable. By contrast,
Table 1. Cell height of MDCK-II cells cultured on substrates with different pore size (diameter) measured by confocal laser scanning microscopy.

<table>
<thead>
<tr>
<th>Pore Size (µm)</th>
<th>h ± s.d. (µm)</th>
</tr>
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<tbody>
<tr>
<td>0.45</td>
<td>5.6 ± 0.9</td>
</tr>
<tr>
<td>0.80</td>
<td>5.9 ± 0.7</td>
</tr>
<tr>
<td>1.20</td>
<td>4.6 ± 0.8</td>
</tr>
<tr>
<td>3.50</td>
<td>6.5 ± 0.9</td>
</tr>
<tr>
<td>5.00</td>
<td>6.3 ± 2.2</td>
</tr>
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</table>

Cells grown on pores show reduced fluorescence intensity on the porous regions (figure 2h–f). The number of stress fibres traversing the cell also decreases with increasing pore size. Strikingly, however, we observe an accumulation of f-actin traversing the cell also decreases with increasing pore size. The distribution of line densities as a function of substrate topography is shown in figure 3f. The line density allows a rough estimate of the network density. We observe a minimum in the line density of actin filaments for cells grown on the 0.8 µm pores. A value of 0.014 nm^−1 found for cells grown on flat surfaces corresponds to an average filament-to-filament distance of 71 nm, which is in good agreement with mesh sizes of the actin network found for other cells [26]. Cells cultured on a flat support exhibit a significantly denser network compared with cells grown on porous supports. Micrographs of cells grown on the different substrates can be found in electronic supplementary material, figure S2. The images show that in all cases, the actin network covered the pores. In the case of the larger pores, the actin is located slightly under the plane of the pore rims. The reason for this might be that the cells extend into the pore interior (partial wetting the pore interior) if pores are large enough (see also Scheme in figure 7) [27].

### 3.3. Cellular mechanics

To determine the influence of different pore sizes on the cellular mechanics, we conducted AFM force–indentation curves and AFM-based microrheological experiments. Force–distance curves were described quantitatively using a modified tension model first introduced by Sen et al. [19] and refined by Pietuch and colleagues [20,28] as this model has been shown to be indenter invariant and delivers more universal mechanical parameters compared with the Hertz, Sneddon or comparable contact mechanical models neglecting the shell structure of the cells [19,20,28]. The tension model describes the cell as an isotropic elastic shell with a constant surface tension. The model assumes that the restoring force originates solely from a tension T, which is the sum of the cortical and the membrane tension T0 and a dynamic contribution from stretching of the plasma membrane (equation (3.1)).

The contribution of stretching to the tension is dependent on the projected cell surface area A0 and the area compressibility modulus K_A, which needs to be replaced by the apparent area compressibility modulus K' _A if the projected cell surface area is smaller than the actual cell surface area due to folds and wrinkles in the membrane in the nanometre scale.

\[
T = T_0 + K_A \frac{\Delta A}{A_0} \tag{3.1}
\]

and

\[
K' _A = K_A \frac{A_0}{A_0 + A_{ex}} \tag{3.2}
\]

A_0 is the projected cell surface area before indentation, \(\Delta A\) is the change of surface area due to stretching and A_{ex} is the area of the excess membrane. If the excess cell membrane stored in folds like caveolae or microvilli is very small, K_A approaches K' _A. T_0 dominates the tension at low indentation depth, while at large strains stretching of the membrane becomes the main contributor to the overall tension—a consequence of the inextensibility of lipid bilayers. As a consequence, the restoring force increases nonlinearly with the indentation depth. Recently, Pietuch et al. [29] showed that isolated apical membrane sheets display the same mechanical
Apical membranes were removed from confluent living MDCK-II cells and placed on a porous mesh. The plasma membrane patches were subject to indentation with an AFM tip and the force curves fitted with a tension model neglecting bending resistance. Area compressibility modules were similar than those found for living cells suggesting that the tension model used in this study is appropriate for polarized confluent cells. It was also demonstrated that contact models such as those based on Hertzian mechanics fail to deliver results independent of indenter geometry. Indenting one and the same cell with two different indenters (sphere and pyramid) attached to an AFM cantilever produced apparent Young’s modules originating from fitting the contact models to the indentation curves that are over one order of magnitude apart. By contrast, applying the tension model to fit the indentation curves gave almost identical results (pre-stress and area compressibility modulus) for both indenter geometries [29]. Schneider et al. [20]

Figure 2. (a–f) Confocal micrographs of MDCK-II cells grown on substrates with different pore sizes. Images show the actin cytoskeleton (Alexa-Fluor546-labelled phalloidin, Invitrogen) of the cells at the level of the pores (pseudocoloured) and corresponding orthogonal views below (scale bar, 20 μm). (g) Confocal micrograph of the actin cytoskeleton of MDCK-II cells grown on 5.5 μm substrates above pore level (grey scaled). (h) Magnification showing an area of six pores (green filled circles). (i) Line profiles of the interconnections between pores shown in H (grey lines) and mean intensity value (red squares, mean ± s.d.). (j) Finite-element simulation of a thin elastic sheet representing the actin cytoskeleton between two pores. Pores are represented by holes in the sheet at the crossing. Black lines indicate shape of the sheet before deformation. An inward-directed pressure (arrows) is applied to the pore boundaries causing deformation and occurrence of stress compensating tension in the membrane. Blue colour indicates low stress, green and yellow intermediate stress and red high stress). (Online version in colour.)
as well as Pietuch et al. [28] found that individual cells not being part of a confluent monolayer display different mechanical properties as those found for confluent cells. In single cells, the whole cell seems to participate in the mechanical response and stress fibres generate appreciably higher tension. The tension measured for single cells was found to be almost one order of magnitude larger than the cortical tension measured for cells within a confluent monolayer.

To model the force–distance curves with the tension model above, the projected cell surface area needs to be calculated using the parametrization described by Sen et al. [19]. The radius of the cap (apical surface of cells), $R_1$ and the contact angle $\varphi$ are determined from AFM–images (see figure 1 and electronic supplementary material, figure S3). Assuming that both, the curvature and the volume stay constant during indentation, one can calculate the restoring force $F$ for different indentation depths. The mechanical parameters $T_0$ and $\tilde{K}_A$ can thus be obtained by fitting the result to the measured force–indentation curves. Figure 4a exemplarily shows two force–indentation curves for MDCK-II cells cultured on flat substrates (grey circles) and substrates with 5.5 µm pores (magenta triangles). We generally observed that cells grown on the flat surface show a steeper increase of the force with increasing indentation depth compared with cells on porous substrates. At small indentation, depth cells grown on larger pores show a weaker increase of force and therefore a lower cortical tension. This observation is reflected in changes of the tension $T_0$, which is the sum of membrane and cortical tension as the dominating contribution and thus the response to externally applied forces (equation...
Figure 5. (a,b) Real part $G'$ (a) and imaginary part $G''$ (b) of the complex shear modulus as a function of frequency $f$. The grey circles represent data from MDCK-II cells cultured on flat substrates, while the magenta triangles are obtained from samples with 5.5 µm-sized pores. Lines show the fits of the frequency-dependent complex shear modulus by the power-law structural damping model. Parameters are compiled in table 2. (c) Loss tangent ($G''/G'$) of cells cultured on the two samples (grey circles: flat substrate; magenta triangles: 5.50 µm pores) as a function of oscillation frequency. Data (median values) are obtained from more than 100 curves. The corresponding lines are the fits of the power-law structural damping model. (Online version in colour.)

Table 2. Mechanical parameters of MDCK-II cells grown on porous substrates with different pore size (diameter).

<table>
<thead>
<tr>
<th>liquid droplet model</th>
<th>AFM-based microrheology</th>
<th>image analysis</th>
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<tbody>
<tr>
<td>$T_0$ ± s.d. (mN m$^{-3}$)</td>
<td>$G_0$ ± s.e. (Pa)</td>
<td>$\alpha$ ± s.e.</td>
</tr>
<tr>
<td>flat</td>
<td>0.31 ± 0.07</td>
<td>188 ± 21</td>
</tr>
<tr>
<td>0.45 µm</td>
<td>0.28 ± 0.08</td>
<td>128 ± 9</td>
</tr>
<tr>
<td>0.80 µm</td>
<td>0.28 ± 0.08</td>
<td>127 ± 26</td>
</tr>
<tr>
<td>1.20 µm</td>
<td>0.18 ± 0.08</td>
<td>137 ± 27</td>
</tr>
<tr>
<td>2.00 µm</td>
<td>0.19 ± 0.07</td>
<td>180 ± 9</td>
</tr>
<tr>
<td>3.50 µm</td>
<td>0.21 ± 0.08</td>
<td>134 ± 15</td>
</tr>
<tr>
<td>5.00 µm</td>
<td>0.11 ± 0.05</td>
<td>120 ± 37</td>
</tr>
</tbody>
</table>

(3.1)) at low indentation depth. $T_0$ decreases monotonically when cells are cultured on substrates with increasing pore diameter (figure 4b). Also, the apparent area compressibility modulus $k_A$ decreases with increasing pore size, indicative of the presence of excess membrane area (table 2). This behaviour can be directly guessed from the fact that the slope of the force-indentation curves at high indentation depths for cells on larger pores is lower than that of cells cultured on the plane surface (figure 4a).

Time- and frequency-dependent mechanical data are obtained by AFM-based microrheological experiments. The method first described by Shroff et al. [30] uses small amplitude oscillations of the cantilever, which is in contact with the sample. By application of a contact mechanical model the complex shear modulus $G^*$ can be calculated [31,32]. The real part of the complex shear modulus $G'$, the storage modulus, represents the energy stored in the sample during oscillation of the cantilever. The energy that is dissipated during the measurement is represented by the imaginary part of the complex shear modulus $G''$ and is therefore called loss modulus. In figure 5a,b, $G'$ and $G''$ are exemplarily shown as a function of the oscillation frequency $f$ for MDCK-II cells cultured on flat substrate (grey circles) compared to cells cultured on substrates exhibiting 5.5 µm pores (magenta triangles). At all frequencies, the cells cultured on flat substrates appear stiffer than those grown on porous samples. In general, $G'$ increases according to a weak power law with the frequency (linear increase in an double logarithmic diagram), whereas $G''$ shows a stronger dependency on the oscillation frequency, which leads to a crossing point of $G'$ and $G''$ at a certain frequency. At this crossing point, the oscillation of $F(\omega)$ and $\delta(\omega)$ are 45° (or $\pi/2$) out of phase and both quantities, $G'$ and $G''$, exhibit the same value. Therefore, the loss tangent ($G''/G'$) equals 1 at this frequency. A loss tangent smaller than 1 means that $G' > G''$ and the elastic properties of the sample dominate the rheological behaviour. Inversely, if $G' < G''$, the loss tangent exceeds 1 and viscous properties dominate the rheology of the cells showing a more fluid-like behavior. For MDCK-II cells grown on a flat surface the crossing point of $G'$ and $G''$ is found at a frequency of around 40 Hz (figure 5c, grey), while cells grown on large pores the crossing point is found already at 20 Hz (magenta). Generally, we found that for cells cultured on porous substrates the frequency, at which $G'$ and $G''$ match, is smaller as $G''$ is largely unaffected by the substrate, especially at higher frequency. Thus, cells cultured on porous substrates show a more fluid-like behaviour at smaller frequencies compared with cells on a non-porous support. We used the power-law structural
damping model, first used by Fabry and colleagues to analyse the spectra of $G^*$ in cellular microrheology [21,33,34]. Table 2 summarizes all parameters of the power-law structural damping model obtained from fitting the spectra as described recently [32]. Explicitly, we find a power-law coefficient $\alpha$ between 0.2 and 0.3 for all samples (table 2) [33]. The scaling factor $G_0$ shows a similar trend as the storage modulus. Cells on a flat substrate exhibit the highest value (188 ± 21 Pa), cells grown the substrate with the 5.5 μm pores exhibit the lowest value (120 ± 37 Pa).

Figure 6 shows that a correlation (Pearson correlation coefficient $r = 0.81$) between the scaling factor $G_0$ and the line density of actin filaments obtained from SEM analysis exists. The expression level of the actin network is obviously responsible for the reduced stiffness found in microrheology experiments of cells cultured on pores.

4. Discussion
In this study, we investigated the influence of macroporous materials on confluent epithelial cells. These materials either simulate the influence of an ECM consisting of adhesive fibres separated by micrometre-sized gaps or ceramic or metallic implant materials on the response of adherent cells to structural heterogeneity of their environment [13]. First, we investigated the impact of the substrate on the morphology of cells grown to a confluent monolayer. Figure 1 shows the apical surface of MDCK-II cells on substrates with varying pore sizes imaged by AFM. The line profiles show a height increase of the apical cap when cells are cultured on macroporous substrates. Additionally, cells on larger pores occupy a smaller area, which might be due to a decrease in spreading rate due to the limited surface area and/or an increase in proliferation rate like it has been previously shown for hepatocytes cultured on mesoporous anodized aluminium oxide [35]. Hoess et al. used substrates with pore sizes ranging from 57 to 213 nm in diameter and found that the cells on larger pore diameters show a faster proliferation rate, which might also be the case in our experiments although the pores used in this study are in the macroporous range. An explanation for increased proliferation could be a more in vivo like situation concerning the additional nutrient supply also from the basal membrane when cells are cultured on porous material. The decrease in spreading area and the overall shape of the cell found in our experiments might be explained by surface energy considerations. On a flat hydrophilic surface, the reduction in surface free energy of a cell will be large. Thus, the contact angle will be small, which facilitates spreading of the cell. By removing up to 30% of the hydrophilic surface, the gain in free energy is reduced and the contact angle will become larger, which in turn leads to a higher cell body occupying a smaller area if the volume is conserved. Furthermore, we find that cells grown on a cubic pore pattern reproduce this pattern in cell layer organization. The long-range cubic pattern of the cells can also be confirmed by two-dimensional fast Fourier transform analysis of the corresponding AFM height image of the image in figure 1 (electronic supplementary material, figure S4).

The obvious changes in cell morphology are accompanied by substantial changes in the cytoskeletal arrangement. On flat substrates MDCK-II cells form an f-actin network with thick stress fibres traversing the entire cell. Cells on porous substrates, however, show a reduced number of stress fibres, which are also shorter. But nevertheless, up to a pore size of 3.5 μm, the cells are still able to bridge the pores without tremendous remodelling of the actin network. Interestingly, the organization of the actin cytoskeleton changes dramatically from 1.2 to 5.5 μm pores. Cells cultured on 5.5 μm pores show dense actin aggregates inside pores (figure 2f). Occasionally, this effect is also found for cells cultured on 3.5 μm pores. We attribute this behaviour to an increase in tension of the actomyosin network, which is needed to span the pores. A similar behaviour was observed previously by Rossier et al. [13], who researched the growth of single fibroblasts on micropatterned substrates. The authors found that bridging between adhesive contacts is achieved by formation of large stress fibres. This bridging is dependent on active non-muscle myosin II. Like in a chain bridge, it needs a certain tension of the actin fibres generated by myosin motors to span the distance from one side (of the pore) to the other (see also figure 2f). The larger the distance, the higher becomes the tension acting on the filaments. As a consequence, the cell suspends the pores by assembling a large number of actin filaments into strong networks. Additionally, thick actin bundles connect to the aggregates inside the pores on the pore rims. Most of them reach directly from one pore to another. Numerous aggregates are interconnected by exactly two thick actin bundles (figure 2g–i). To explain this phenomenon, we conducted finite-element simulations (figure 2j). The actin cytoskeleton is simulated by a cross shaped elastic material. The porous region is represented by the absence of the material. When applying an inwards-directed homogeneous pressure to the pore boundaries, the edges of the interconnections are the regions, which exhibit the highest stress values. This means for the natural system that these areas need to be strengthened by additional filaments. This supports our previous hypothesis that there is strong inwards-directed force, which might be produced by the contractile force exerted by myosin motorproteins. This high tension also requires a strong adhesion of the cells to the substrate in proximity to the pores. In epithelial cells or fibroblasts adhesion to the substrate is mainly realized by focal adhesion. Wu et al. [36] as well as Rossier et al. [13] observed an accumulation of focal adhesion contacts in regions next to pores or non-adhesive regions du Roure et al. [15] reported that MDCK
cells cultured on micropillars indeed span the region between the posts. Interestingly, in confluent monolayers, the cells do not reflect the posts notably. By use of a mild detergent, we were also able to uncover the cytoskeleton and visualize it using scanning electron microscopy (figure 3; and electronic supplementary material, figure S2). The images showed a reduction of cytoskeletal filaments in cells grown on porous substrates.

These tremendous changes in cytoskeletal arrangement in response to substrate properties also have strong impact on cellular mechanics as shown in our force-indentation and microrheological experiments. The cortical tension of cells obtained from indentation experiments decreases with increasing pore diameter. Compared with cells cultured on 5.5 µm pores, the tension \( T_0 \) of cells on a flat surface is increased by a factor of 3 to 4. The found tension value above. Along the same lines, the findings of Pietuch et al. [30] are of second order. Actin remodelling might also contribute to both mechanical parameters (figure 6). Notably, the observed line densities of actin filaments (figure 3f) show a similar trend as \( \dot{K}_0 \) and \( G' \), leading to the conclusion that the actin cytoskeleton is the main contributor to both mechanical parameters (figure 6). Notably, although \( G' \) of MDCK-II decreases, when cultured on porous substrates, the power-law exponent does not show a clear trend. One might expect that a decrease in cell stiffness is accompanied by a higher power-law exponent as it has been observed for several cell types treated with various drugs [33,38]. In our experiments, however, there’s a maximum power-law coefficient of 0.3 at 0.8 and 1.2 µm sized pores, while larger pores show smaller values comparable to those found on a flat substrate. This might be due to severe cytoskeleton remodelling (figures 2 and 3).

In order to show that the contributions of the actin cytoskeleton are responsible for the observed effects, we treated cells on pores and on a flat substrate with cytochalasin D (see electronic supplementary material, figure S6). By administration of cytochalasin D, the actin cytoskeleton is disrupted. As expected for this case, \( G' \) decreases and shows a stronger dependency with the frequency. \( G'' \) is also reduced at all frequencies. Comparison of cells grown on
Figure 7. Scheme compiling the effects of culturing MDCK-II cells on various macroporous substrates. Cells decrease their spreading area with increasing pore diameter. This goes along with a reduction in cortical tension, which might be a consequence of a reduced contractile tone of the cell due to fewer and shorter stress fibres. Furthermore, on large pores the cells produce thick actin aggregates over the pores, which generate high tension. (Online version in colour.)

5. Conclusion

The extracellular environment is an essential mediator of cellular structure and function by providing biochemical and mechanical stimuli to influence single and collective cell behaviour. Through adhesion, cells sense the mechanical properties of the environment and translate this information into a signalling cascade, which regulates cellular responses such as spreading, migration and growth. While abnormal mechanotransduction is known to be responsible for a number of diseases, the robustness of how cells respond collectively to such stimuli is largely unexplored. Here, we provide structural and mechanical information about the impact of pore size on the mechanical response of confluent epithelial cells to substrates with defined topography.

Interestingly, we found that the cells can distinguish even subtle changes in pore size and develop strategies to remodel their cytoskeleton in order to explore substrates with large pores that would prevent sufficient adhesion area otherwise by spanning and reinforcing the cytoskeleton of the free standing part.

Cells on larger pores up to 5.5 μm respond to the reduced adhesion area and strain in the membrane by a comprehensive remodelling of the cytoskeleton especially in the vicinity of the pores. The cells invade into the pores, form a contractile actin aggregates and thereby manage to maintain an intact cell monolayer covering the entire area. Interestingly, the cell size and arrangement adapt to the underlying porous structure, which we attribute to a self-organization driven by minimizing the area of freestanding membrane over pores. The effect of culturing MDCK-II cells on substrates with different pore sizes are also illustrated in figure 7. A simple change in topography by adding pores to the surface results in a rich variety of phenotypes that partly also mimic softer material and might become an alternative to self-organize cells to a predefined morphology, which also affects cellular elasticity, without using biochemical cues or modifying the stiffness or surface functionalization of the substrate.

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References
