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Mimicking Vascular Mechanics: Endothelial Response to Dynamic Deformation on a Hybrid Membrane

https://doi.org/10.1515/cdbme-2025-0203

Abstract: Endothelial cells (ECs) are constantly exposed to mechanical forces including shear stress and cyclic strain *in vivo*. This study explored how ECs respond to radial and 3D cyclic stretching using a collagen-based hybrid membrane system and a pneumatic control (MPSstimulus) to mimic dynamic mechanical conditions. Although deformation of the cell substrate was very low and occurred mainly at the polymer mesh that stabilized the collagen matrix, ECs still showed notable changes such as increased stress fiber formation and cell number. These results highlight the sensitivity of ECs to even minor mechanical cues in 3D environments. Overall, the study underscores the importance of integrating three-dimensional mechanical forces in the design of *in vitro* vascular models.

Keywords: Endothelial cells, cyclic stretching, mechanobiology, vascular barrier, micro physiological systems, hybrid membrane, collagen

1 Introduction

Endothelial cells (ECs) are continuously exposed to mechanical forces *in vivo*, including shear stress from flowing blood and repetitive stretching caused by the heartbeat. The cells respond with changes in functional, biological and phenotypic features. As such, mechanical stretch is an important component of physiological relevant *in vitro* models and has been applied to reconstitute physiological conditions with magnitude stretches of 5-10 % as well as pathological environment with high magnitude stretches of 20 % and more [1]. In a previous study, we were able to confirm that the geometry of the substrate plays a decisive role in sensing and reacting to mechanical cues. ECs growing in a three-dimensional (3D), radial environment responded to shear stresses much lower than shown so far [2]. This study investigates the effect of low radial 3D cyclic stretching on ECs to help understanding how these cells respond to three-dimensional mechanical deformation. This knowledge is crucial for further development of *in vitro* models of vascular barriers.

2 Material and Methods

2.1 Hybrid membrane and deformation

In this study, ECs were cultivated on a thin hybrid membrane and subjected to radial cyclic stretching. The hybrid membrane is composed of a polymer mesh (Netwell insert bottom) and a collagen membrane. Collagen coating of the mesh was done by adapting protocols from previous work [3]. In brief, a 2 mg/ml human collagen solution from fibroblasts (Advanced BioMatrix) was mixed with fibrillation buffer (50 mM Tris base, 250 mM NaCl, pH 10, collagen-buffer ratio 2:1) and pipetted on the lower side of the bottom of a Netwell by turning the insert upside down. After fibrillation and air-drying, collagen was cross-linked in 70% ethanol and 2% (w/v) 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) for 30 min at RT. After washing with ultrapure water, collagen was again air-dried for 24 h under the hood.
An electropneumatic control (MPSstimulus, Fraunhofer IWS) was used for mechanical deformation of membranes by means of pressure gradients [3]. The system provides four pneumatic outlets. These are precisely set with electronic pressure regulators and monitored with sensors. Each output can

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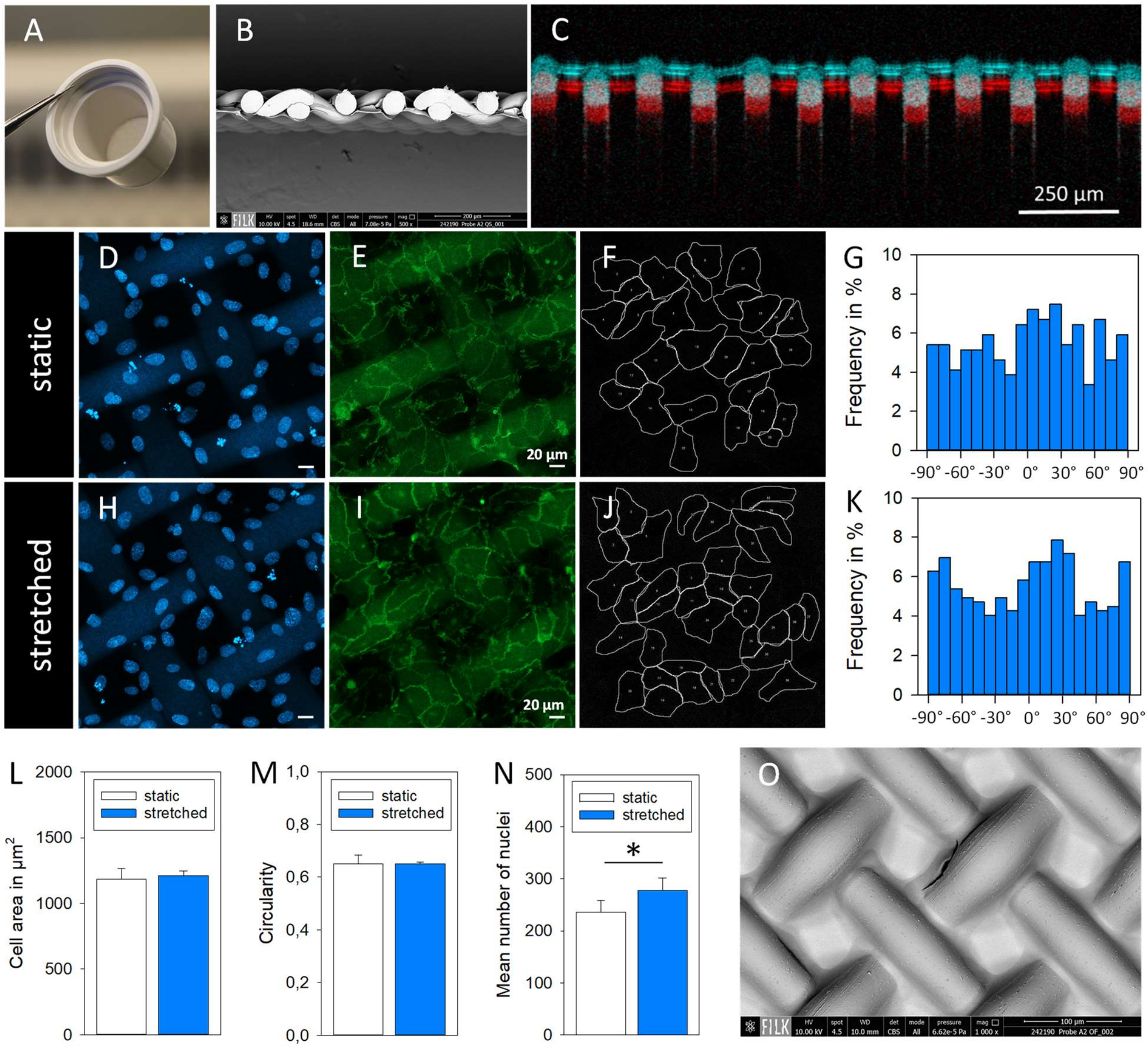

FIGURE 1. Radial cyclic stretching of HUVECs does not affect cell orientation on the hybrid membrane but increases cell number. **A and B)** Photograph and scanning electron microscope (SEM) image of a Netwell insert and the hybrid membrane at the bottom of the insert. Scale bar = 200 µm. **C)** Overlay of two OCT cross-sections, showing the deformation of the hybrid membrane at different differential pressures, red: 0 mbar, magenta: 5 mbar. **D, E, H, I)** Confocal images of VE-cadherin staining and DAPI nuclear marker in HUVECs 72 h after cultivation under radial stretch versus static conditions. Scale bar = 20 µm. **F and J)** Output image after labeling clearly identified cell borders of HUVECs. Cells whose boundaries could not be clearly assigned were excluded from the measurement. **G and K)** Quantification of cell orientation angle in stretched (upper) versus static (lower row) conditions. Data represent frequency of cells per class (with 9 classes, each 10°; -90° to 0° to 90°; n = 3). Data not significant different. **L)** Mean cell area of stretched HUVECs versus static controls. Data not significant. **M)** Quantification of circularity of HUVECs under stretched versus static conditions. 1.0 represent fully round cells. Data not significant. **N)** Mean number of cell nuclei quantified in confocal images with 200-fold magnification. Student's *t*-test; $*p < 0.05$; for the alternative hypothesis specifies that group static is smaller than group stretched. **O)** SEM image of the hybrid membrane. View on top of the collagen matrix. Scale bar = 100 µm.

provide defined pressure curves (periodic trapezoidal, sinusoidal, rectangular or free-form) in the range from 0 to 200 mbar. In this study, ECs were stimulated with sinusoidal pressure curve at 0.2 Hz and 5 mbar pressure.

OCT imaging of the hybrid membranes was performed with a commercial spectrometer-based device at a central wavelength of 880 nm (Thorlabs Ganymede GAN332 with OCTP-900 and OCT-LK2-BB). The resolution is 2.2 µm in axial and 4 µm in lateral direction. The OCT volumes were processed and visualized using the manufacturer's software (ThorImageOCT 5.8.0, Thorlabs), and further evaluated using custom Python scripts [4].

2.2 Cell cultivation and staining

Human umbilical vein endothelial cells (HUVECs; Lonza) were cultured in EGM-2 (EBM-2 supplemented with Single Quots; Lonza) according to the supplier's instructions, passaged every 2–3 days using Accutase (Sigma-Aldrich) and cultured for up to 10 passages. For radial cyclic stretch experiments, 400.000 HUVECs per Netwell were seeded on the collagen membrane by turning the Netwell upside down. After 1 h adhesion time, the Netwells were placed into the corresponding holder of the stretching device, incubated for 24 h at 37°C under static conditions and then radial cycling stretched with a magnitude of 5 mbar and a frequency of 0.2 Hz for 72 h at 37°C. Static cells were cultured in parallel as controls. The stretching experiments were performed in three independent experiments. The data show the results of a representative experiment.

Cellular response was characterized using fluorescence analyses. Phalloidin-DAPI and VE-cadherin staining was performed according to protocols published earlier [2]. Samples were analyzed using a cLSM (Zeiss, LSM800; magnification 200- and 400-fold).

2.3 Biological response

Endothelial cell response was characterized after three days of cultivation under dynamic conditions. Strain-induced orientation of cells was measured in immunofluorescence images of cells stained with VE-cadherin (Cell Signaling Technologies) and DAPI (Sigma-Aldrich) as described earlier [2]. Orientation angles were categorized in classes of 10°. The frequency of angle classes represents the number of cell in the corresponding class.

Cell area and circularity of the cells were measured after manual retracing the cellular border of ECs stained with VE-cadherin using the circle tool and automated calculation with ImageJ software. The underlying confocal images were taken at 400-fold magnification.

The number of nuclei of ECs was counted in confocal images showing cells stained for DAPI at a magnification of 200-fold. Intensity of stress fiber formation was measured in confocal images (200-fold magnification) of ECs stained using Phalloidin-TRITC. Data are expressed in %, with the fluorescent area of ECs cultured under static conditions set to 100 %.

3 Results and Discussions

To investigate the response of ECs to radial 3D cyclic stretch, HUVECs were seeded on a hybrid membrane which is deformed in the direction of Z using a pneumatic control (MPSstimulus, Fraunhofer IWS). The hybrid membrane is part of a Netwell insert that has a polymer mesh at the bottom of the insert with a fibre distance of 74 µm. Human collagen was used to fully cover the mesh and to fill the spaces between the fibres (Fig. 1 B and O). Due to the prominent structure of the mesh, the surface of the hybrid membrane is non-planar. Dynamic measurements show that at a differential pressure of 5 mbar, it is primarily the polymer mesh that is tensioned. Figure 1 C shows the change in position of the hybrid membrane between 0 and 5 mbar differential pressure. The deformation of the collagen membranes between the reticular fibers, on the other hand, is less than 1 percent. Regardless of this, when a differential pressure of 5 mbar is applied, a force of 600 Nanonewtons (nN) acts on endothelial cells with an average surface area of 1212,8 ± 34,8 µm².

HUVECs were subjected to radial cyclic stretching over a period of 72 h. Cells were fixed and microscopically analysed after immunofluorescence staining for the adherence junction molecule VE-cadherin (Fig. D to J). Cell orientation measurements indicated no changes in the cellular growth direction. However, counting cell nuclei revealed that the number of cells on the stretched hybrid membrane was significantly higher than on static control membranes (Fig. 1 N) indicating an increase in proliferation. This is in line with previous studies showing that HUVECs respond to cyclic stretching with an activation of the ERK1/2 (MAPK) pathway associated with cell proliferation and an increase in DNA synthesis [5]. It is known that ECs reorganize their cytoskeleton, particularly actin stress fibers under radial stretch [6]. These data inspired to characterize the actin cytoskeleton organisation in cells stretched on the hybrid membrane. There was no obvious change in actin stress fibre orientation (Fig. 2 A to H). Unlike uniaxial stretch, where cells align perpendicular to the stretch direction, radial stretch often leads to a more isotropic or circumferential reorientation or even no alignment preference [6]. Additionally, it became obvious that actin cytoskeleton strongly arranges alongside the polymer mesh fibre orientation. This was very prominent in stretched samples where polymer fibre orientation could be estimated from the orientation of the cytoskeleton (Fig. 2 E to H). These observation indicates that the polymer mesh restricts the reorganisation of the cells on top of the hybrid membrane. Despite these restrictions it was still possible to detect an

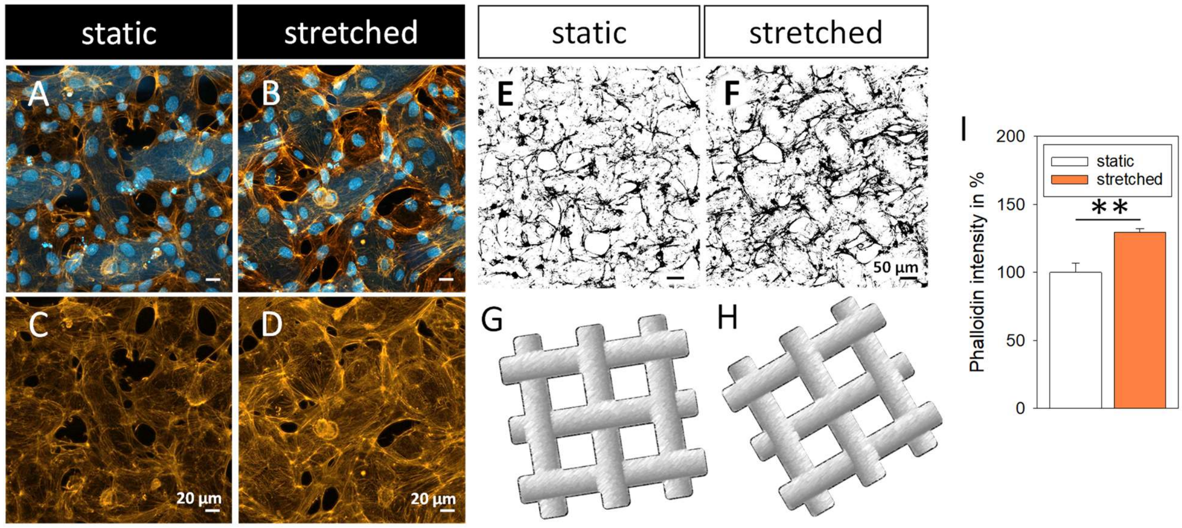

FIGURE 2. Radial cyclic stretching of HUVECs increases stress fiber formation but not fiber orientation. **A to D)** Confocal images of HUVECs cultured under stretched versus static conditions on the hybrid membrane. Orange signal represents actin cytoskeleton stained with Phalloidin-TRITC, blue staining indicates DAPI nuclear staining. Magnification: 200-fold. Scale bar = 20 µm. **E and F)** Processed images from actin cytoskeleton measurements. ECs stained with Phalloidin-TRITC are black on white background. The results indicate cytoskeleton organization depending on culture condition. Scale bar = 50 µm. **G and H)** Schematic representation of the orientation of the underlying polymer mesh. **I)** Quantification of actin fibre formation expressed as Phalloidin fluorescence intensity in stretched cells in % compared to static controls. Data are based on confocal images at 200-fold magnification. Student's *t*-test; **p < 0.005.

increase in actin cytoskeleton formation in HUVECs subjected to radial cyclic stretching (Fig. 2 I) indicating that cells were able to sense and to react on mechanical deformation of the membrane.

These results show that ECs exhibited distinct morphological changes and cellular growth as a response to small-scale deformation. These findings are consistent with our previous observations that endothelial cells are highly sensitive to low mechanical forces when cultured in 3D, suggesting that substrate geometry plays a key role in EC mechanotransduction. Nevertheless, there is a need for the development of more sophisticated hybrid membranes for *in vitro* cell models.

4 Conclusion

This study confirms that even subtle deflections from the horizontal plane can influence endothelial cell behavior, emphasizing the importance of incorporating three-dimensional mechanical forces into *in vitro* models of vascular endothelium. Our findings provide valuable insights into endothelial mechanobiology and the design of advanced barrier models.

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