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Contractility as a global regulator of cellular morphology, velocity, and directionality in low-adhesive fibrillary micro-environments

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ABSTRACT

Recent reports demonstrated that migration in fibrillary environments can be mimicked by spatial confinement achieved with micro-patterning [1]. Here we investigated whether a model system based on linearly structured surfaces allows to draw conclusions about migration of endothelial cells (ECs) in fibrillary 3D environments. We found that ECs on 3 μm wide tracks (termed as 1D) migrate less efficient in comparison to ECs on broader tracks in regard to velocity and directional persistence. The frequent changes of direction in ECs on narrow tracks are accompanied by pronounced cell rounding and membrane blebbing, while cells migrating with an elongated morphology display a single lamellipodium. This behavior is contractility-dependent as both modes can be provoked by manipulating activity of myosin II (blebbistatin or calyculin A, respectively). The comparison between 1D and 3D migrating cells revealed a striking similarity in actin architecture and in switching between two morphologies. ECs move more directed but slower upon inhibition of contractility in 1D and 3D, in contrast to 2D cell culture. We conclude that micro-patterning can be used to study morphological switches in a controlled manner with a prognostic value for 3D environments. Moreover, we identified blebbing as a new aspect of EC migration.

1. Introduction

Endothelial cells (ECs), like other cell types, need to orchestrate substrate-adhesion, signaling, and cytoskeletal dynamics in order to regulate their motile capacities in angiogenesis [2]. An important factor for the complex process of migration is the regulation of myosin based contractility of cells *via* the actin cytoskeleton [3]. The tension created by the actin-myosin system is important for retraction of the trailing edge, as well as for leading edge dynamics [4] [5]. The location and functionality of myosin is mainly regulated by RhoA, a small GTPase [6].

Contractility and small GTPases are furthermore relevant for the formation of various cell protrusions, like filopodia, lamellipodia or membrane blebs. Switching between these protrusions is pivotal for the motility in complex 3D environments where the cell has to be morphologically highly dynamic in order to face the versatile obstacles present in inhomogeneous environments [7].

Plasma membrane blebs in particular become more and more important in cell biology, being crucial for cellular migration of leukocytes [8], or as an alternative migration mode of cancer cells [9]. Recently, they turned out to be an underestimated factor for cell migration through 3D matrices [10], and not just a sign of apoptotic processes [11].

Membrane blebs expand as a result of intracellular hydrostatic pressure [12]. This is in contrast to other cellular protrusions like lamellipodia, where actin polymerization is the driving force [10]. Resorption of the membrane blebs occurs through polymerization of actin at the bleb cortex and subsequent retraction of the bleb towards the cell body in an actin-myosin contractile-dependent manner [13]. To date, many molecular players associated with the actin cytoskeleton have been identified to be important for bleb formation (reviewed in Ref. [14]). One major regulator of membrane blebbing is the Rho-ROCK signaling pathway due to its potential to provide cellular contractility. Beside the necessity of

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contractility in bleb retraction as mentioned before, contractility has been shown to be a critical inducer of membrane blebs due to induction of local ruptures in the cell cortex [13,15]. In contrast to the knowledge about intracellular mechanisms, extracellular cues provoking bleb formation are rather unknown. To date, the best analyzed extracellular cause of plasma membrane bleb formation is the loss or limitation of cell-substrate adhesion, for example during cell spreading or as an alternative mode of migration [8]. In endothelial cells in particular, plasma membrane blebbing has been exclusively described in the context of cell spreading [16,17], which can be likewise seen as a temporary low adhesive state.

In this study, we demonstrate that plasma membrane blebbing is inducible in endothelial cells through spatial confinement and enhancement of contractility in a defined setup. Additionally, we show that blebbing plays a role in 3D migration of endothelial cells as a dynamic and important reaction to low-adhesive environments.

Earlier reports showed that aspects of 3D migration in aligned collagen might be imitated by thin artificial adhesion cues (termed as 1D migration), produced with lithographic techniques [1].

In the present study we investigated whether it is possible to provoke a dynamic switch of protrusions of ECs in fibrillary and thus low adhesive environments. We used micro contact printing (μCP) for the creation of linear protein micro-tracks for human umbilical vein endothelial cell (HUVEC) migration, and found that migration morphology on 1D structures highly correlates with phenomena observed in a fibrillary 3D environment. We thereby identify blebbing as a novel and important aspect of endothelial cell migration, and contractility as main driver of this process.

2. Material and methods

2.1. Cell culture

HUVEC (= human umbilical vein endothelial cells) were purchased from Promocell (Heidelberg, Germany). Cells were cultivated with ECGM 2 (Promocell, Heidelberg, Germany), supplemented with 1% penicillin/streptavidin/amphotericin B (purchased from PAN-Biotech, Aidenbach, Germany) and 10% FCS (PAA, Pasching, Germany). Cells were incubated at 37 °C under 5% CO_2 atmosphere. For all experiments cells were used in passage # 6. Prior to experiments and for passaging, cells were covered with 1× trypsin/EDTA (PAN-Biotech, Aidenbach, Germany) until detachment of the cell layer. Subsequently, trypsin/EDTA was inactivated by DMEM, supplemented with 10% FCS. After inactivation, cells were spun down and the cell pellet was diluted to desired concentrations for further use in experimental procedures.

2.2. Transfection

We used a HUVEC transfection kit (Targeting Systems, El Cajon, CA, USA) and transfected cells according to manufacturer's instructions.

Plasmids: pLifeAct-TagRFP (Ibidi, Martinried, Germany); pGFP-cortactin (Addgene # 50728).

2.3. Microcontactprinting (μCP)

Microstructured wafers were produced under clean room conditions in the physics department of the LMU Munich using photolithography. Silicon wafers were covered with Sylgard Silicone Elastomere 184 (Dow Corning, Midland, MI, USA) in a 1:10 ratio and PDMS was allowed to polymerize overnight at 60 °C after vacuum-based removal of air bubbles. PDMS stamps were cut out from the silicone wafer and treated in a PSD-UV ozone/UV-cleaner (Novascan, Ames, IA, USA) for 20 min. Subsequently, PDMS stamps were incubated in a protein solution containing 50 μg/ml fibronectin (BD, Franklin Lakes, NJ, USA) diluted in sterile ultrapure water. After 2 h of incubation, stamps were removed from protein solution, washed twice with ultrapure water, and left in laminar flow for drying. 8 10 minutes later, stamps were completely dried and ready for further use. As printing substrate, we exclusively used untreated 8-Well slides from Ibidi (Martinsried, Germany). 8-well slides were pretreated with UV/Ozone for 8 min and PDMS stamps were positioned into a well with the structured side on the surface. For proper protein transfer we left the stamp on the surface for 1 h and then passivated the non-adhesive regions with 1 mg/ml PLL-(2)-PEG diluted in PBS (Surface Solutions, Dübendorf, Switzerland) for 20 min. Subsequently, wells were washed twice with PBS before cell seeding in order to remove unbound PLL-(2)-PEG.

2.4. Laser scanning confocal microscopy

Laser scanning confocal microscopy was performed using a Leica SP8 SMD microscope. Pinhole size was at 1.0 airy unit for all experiments. Following excitation laser wave lengths were used: 405 nm, 488 nm, 561 nm, 647 nm. The detectors were individually adjusted to the specific spectra of the dyes. To avoid cross-talk between the channels only sequential scans of single channels were made in all experiments. In order to reduce background noise, an average of four frames was obtained for every channel. Scanning frequency was at 400 Hz in most of the experiments. For fast cellular processes scan frequency was set to 1400 Hz or to 7000 Hz (resonance scanner). Objectives used in this study: 63× oil, 40× oil, 63× water.

2.5. Live cell imaging

Live Cell Imaging was performed using either a Nikon Eclipse Ti Inverted Microscope (Nikon, Düsseldorf, Germany) or a Leica SP8 confocal microscope (Leica, Wetzlar, Germany). For the Nikon microscope we used a heating and incubation system from Ibidi (Martinsried, Germany) and for Leica SP8 an Okolab H301-EC-LG-BL incubation chamber (Pozzuoli, NA, Italy) and a Okolab bold line incubation system. In all experiments, cells were kept under constant 37 °C, 5% CO_2 atmosphere and 80% humidity. In tracking/migration experiments, we used a 4× phase contrast objective and for closer observation a 20× DIC (differential interference contrast) objective. For observation of transfected cells in the confocal setting we used a 63× oil objective.

2.6. Migration experiments

Migration experiments on micro-tracks were performed using adhesive linear fibronectin patterns, manufactured with μCP as described above. To ensure printing quality, a mixture of 40 μg/ml unlabeled fibronectin and 10 μg/ml labeled fibronectin was used for μCP. Fibronectin was labeled using a DyLight488 antibody and protein labeling Kit from Pierce/ThermoScientific (Waltham, MA, USA). Cells were seeded in a density of 25×10^3/well, and were allowed to attach to the micro-tracks for 1.5–2 h.

2D migration experiments were performed using 8-well slides (Ibidi, Martinsried, Germany). Slides were coated with 50 μg/μl fibronectin for 1 h. Cells were seeded in a density of 25×10^3/well, and were allowed to attach to the surface for 1.5–2 h.

3D migration experiments were performed using Ibidi chemotaxis slides without applying a chemotactic gradient. Cells were embedded in a rat tail collagen I gel following the manufacturer's instructions. Both reservoirs were filled with ECGM 2 (Promo Cell,