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## **Generation and distribution of endothelium-derived contractile forces and their impact on vascular permeability**

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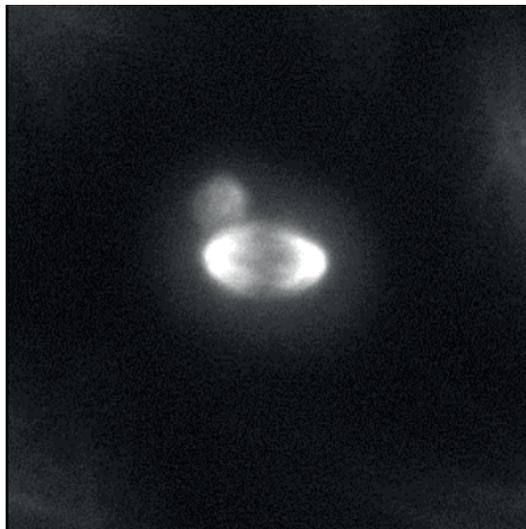
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# Chapter 8

General discussion





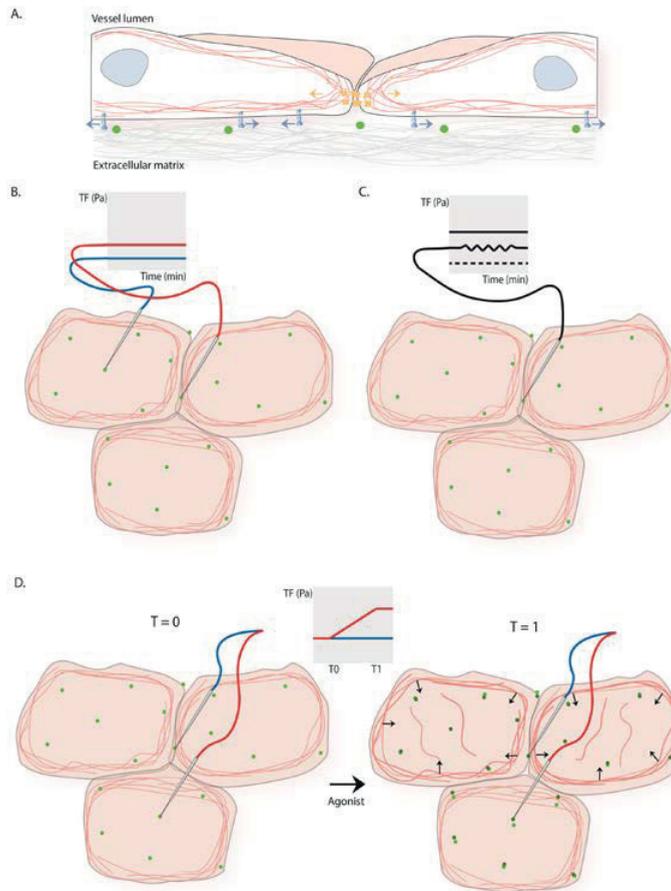
## General discussion

The aim of the studies described in this thesis was to evaluate the distribution and dynamics of contractile forces within the endothelium at high resolution in resting and activated endothelial monolayers. Through the successful implementation of the traction force microscopy (TFM) technique - with the help of our collaborators in Boston - we succeeded in conducting biophysical measurements on confluent endothelial monolayers, which we will address in the first part of this chapter. In addition to data on unstimulated endothelial monolayers, we will also address traction force changes within the activated endothelium. In the second part, we focus on the relation between contractile traction forces and the formation inter-endothelial gaps, and discuss the contribution of specific force-modulating proteins. Finally, we will place our results in the context of the vascular permeability field, discuss some limitations of our work and provide future directions.

### Contractile traction forces underneath confluent endothelial monolayers

By the application of the TFM method, we were able to study the source of local shape changes (i.e. the traction forces) rather than measuring only cell movement. The TFM technique is based on the displacement of cross-linked fiducial markers at the top of a deformable substrate. The spatial differences of these markers between the cell-loaded (Figure 1A) and -unloaded situation, provides the specific displacement field. Subsequently, with this input and the knowledge about the physical properties of the substrate, it is possible to calculate the exerted traction forces. In the past, TFM was frequently used to study the interaction of single cells or small groups of cells with the extracellular substrate.<sup>1-6</sup> However, with the introduction of monolayer traction microscopy it become possible to conduct experiments with a continuous cell monolayer.<sup>7</sup> This was conceivable after circumventing, by the introduction of a new algorithm, the important problem of boundary artifacts from unbalanced forces outside the field of view. Hereby, TFM provided a new way to investigate the vascular endothelium in a more physiologically relevant manner and the option to study the heterogeneous and asynchronous distribution of these innate physical forces underneath the endothelium.

From the experiments conducted with TFM - in most detail presented in **Chapter 3** - we learned that the traction force landscape is characterized by specific punctuated force hot spots with magnitudes multiple times larger than the mean, which preferentially cluster in proximity of tri-cellular corners. Moreover, traction forces are highly dynamic, fluctuate over time and co-localize with specific cell structures such as adherens junctions and the F-actin cytoskeleton (Figure 1B+C). Also, they can be elevated significantly upon stimulation with a contraction-inducing agonist such as thrombin (Figure 1D). Under these conditions, especially the increase in the number- and size of force hotspots can be detected, of which the force vectors can be asynchronous and sometimes even opposing the junctions of neighboring cells. This process continues until part of the junctions no longer can bear the cell-generated forces, resulting in the formation of intercellular gaps. In general, this takes place around 10 minutes after the administration of thrombin, followed by the appearance of high contractile forces at the gap boundaries and low contractile forces in the center of the gap. These biophysical insights are new for confluent endothelial monolayers and were not able to be revealed without the application of the TFM technique.



**Figure 1. Monolayer traction forces of the endothelium.** (A) Representation of a cross-section of two resting endothelial cells, which are connected via VE-cadherin junctions (orange) and adhere to the extracellular matrix by integrin-mediated interactions (blue). Both structures are indirectly coupled to the F-actin cytoskeleton in which myosin-motor generated tension is exerted via inside-out transmission on neighboring cells and the substrate. This last pathway comprises a category of forces (traction forces, blue arrows) that can be quantified by studying displacement of fiducial markers (green) linked to the extracellular matrix on a deformable hydrogel. (B) A top view of three interacting endothelial cells in which at two locations within this simplified monolayer, traction forces (TF, in Pascal) are monitored. The measured forces at center of the cell (nuclear), indicated in blue, are relatively low compared to the forces at marginal region of the cell, displayed in red. (C) Manipulation of specific force-modulating proteins can positively (solid black line) or negatively (dashed black line) influence the endothelial monolayer tension. Moreover, in some conditions also the monolayer stability is affected, causing fluctuations in the traction force signal (middle black line). (D) When the endothelium is exposed to agonistic substances the cells become activated (right), leading to the contraction of the cortical actin ring, the formation of stress fibers and intercellular gaps. This results in additional displacement of the markers (T=0, in light green vs T=1, in dark green), however only in places were the force distribution is imbalanced (red). The blue sensor does not register any changes in force since both cells pull with the same magnitude in exactly opposite directions, thereby balancing tension over the displacement marker.

### **Vulnerable loci are not directly linked via traction forces to inter-endothelial gaps**

To test our first hypothesis, i.e. that contractile forces can predict vulnerable loci within the endothelium, we studied the formation of hundreds of gaps within thrombin-stimulated monolayers on the TMF set-up. Our first working model was based on data from the study of Krishnan *et al.*, which suggested that agonist-induced gaps will form at positions where traction force are largest.<sup>8</sup> In different experiments conducted at high spatiotemporal resolution we observed the appearance of inter-endothelial gaps at locations of high traction forces, however frequently, junction disruption also occurred in the absence of specific force hotspots. For this reason we were not convinced by our initial supposition and concluded that there is not a one-to-one relationship between the force magnitude and the disruption of barrier integrity. In a subsequent study - see **Chapter 3** of this thesis - the previously incomplete model was updated by the introduction of the term force fluctuations, defined as the variation of normalized traction force over 15 consecutive time points/minutes. We showed the importance of force fluctuations for monolayer stability by the ability to better predict the formation of gaps by fluctuation of forces rather than by the magnitude of the traction forces alone. With this additional parameter the correlation between traction force and permeability improved significantly, however we were still not able to predict all agonist-induced gaps on the basis of the resting monolayer characteristics.

The inability to predict all agonist-induced gaps is mainly due to the complexity of the relation between contractile forces and endothelial barrier function, which becomes even clearer when a comparison is made including all data presented in this thesis (Table 1). Here, for every different possible permeability outcome: unaffected, protective or disruptive, we found examples of conditions in which traction forces were either increased or reduced. For instance, in DMOG- and hypoxia-treated HUVECs (**Chapter 7**), both conditions show reduced permeability despite opposing effects on contractile traction forces (reduced in hypoxia vs strongly increased by DMOG). Moreover, by relating the highly contractile response induced by the tyrosine kinase inhibitor Imatinib with that of the strongly traction force reducing effects of the ROCK inhibitor Y27632, it can deduced that these different contractile phenotypes both do not influence basal permeability. A final example can be found in two conditions that increase basal permeability by 10 to 25%; the siRNA knock-down of ROCK1/2 and that of the Rho-GAP, Deleted in Liver Cancer 1 (DLC-1) (Unpublished data in collaboration with Dr. S. Huvneers). The siRNA knock-down of ROCK1/2 reduced traction forces whereas, in contrast, knock-down of DLC-1 strongly (more than 50%) induced contractility. Thus, traction force generation does not directly correlate with increased permeability.

From Table 1, we found further support to reject the hypothesis of high traction-induced permeability. In both DMOG- and Imatinib-stimulated confluent monolayers, basal traction forces were significantly enhanced in combination with a strongly protective effect against endothelial barrier disruption. Furthermore, this argues against a uniform endothelial force-threshold at which cell-cell junctions disassemble, since in some conditions forces in a confluent resting cells exceed the agonist-induced values of others. Moreover, also the proposed ROCK2 mechanism (i.e. SLX2119-sensitive, **Chapter 4**), is put in a less generalizing perspective. The barrier protective effect of ROCK2 was suggested to be mediated by a reduction in basal traction forces, however this link seems less predictive when considering the variety of measured effects on basal tension in combination with reduced endothelial

permeability.

One of the reasons why we think that the relation between traction forces and gap formation is not as straightforward as we originally predict, is that with the monolayer TFM method not all forces are detected. Only forces which are not directly balanced within the monolayer lead to substrate deformations and thereby displacement of the fiducial markers (Figure 1D). This results in an underestimation of the actual contractile forces transmitted within the monolayer, which could explain formation of inter-endothelial gaps in a way that does not fit in the current model.

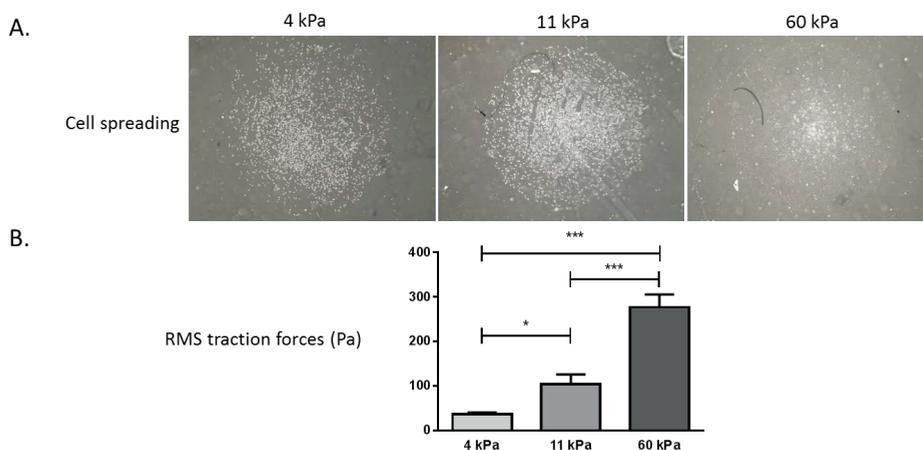
	Basal TFs	Basal force fluctuations	Thrombin-induced TFs	Basal permeability	Thrombin-induced permeability	Method
<b>Chapter 4</b>						
<b>Vehicle</b>						
Y27632 (inh. ROCK1/2)	↓↓↓	-	↓↓↓	0	↓↓	HRP
SLX2119 (inh. ROCK2)	↓↓	-	↑↑	-	-	
SLX3242 (inh. ROCK1/2)	↓↓↓	-	↓↓	-	-	
<b>MOCK</b>						
siROCK1	↑↑↑	-	↓↓	0	0	HRP
siROCK2	0	-	↓↓	0	↓↓	
siROCK1/2	↓	-	↓	↑	↓	
<b>Chapter 5</b>						
<b>Vehicle</b>						
Imatinib	↑↑↑	↓	↓↓	0	↓↓	HRP
<b>siScramble</b>						
si c-Abl	↓↓	-	↓↓↓	-	-	
siArg2	0	-	↑↑↑	↓	↓↓	ECIS
siArgBP2	0	-	↑↑↑	-	-	
<b>Chapter 6</b>						
<b>MOCK</b>						
KLF2	↓↓	↓	↑↑↑	-	-	
MOCK+shDRF2	↓	0	↑↑↑	-	-	
KLF2+shDRF2	↓	0	↓↓	-	-	
<b>Chapter 7</b>						
<b>20% O<sub>2</sub>+vehicle</b>						
1% O <sub>2</sub>	↓	↓	↓↓	↓↓↓	↓↓↓	HRP
DMOG	↑↑↑	↓	↓	↓↓	↓↓↓	

**Table 1. Overview to the reported traction force data and endothelial permeability results presented in this thesis.** One arrow indicates >10-25% change relative to the control listed on the first line, two arrows >25-50% and three arrows >50%, respectively. A zero indicate that there is not more than a 10% difference between the tested conditions, whereas a minus specifies an undetermined condition. Thrombin induced traction forces were asses at 10 min after thrombin administration and included a correction for basal values. The method indicated on the right specifies the technique used to assess endothelial permeability: Electric Cell-substrate Impedance Sensing (ECIS) or the use of horseradish peroxidase (HRP) permeability experiments.

### **Traction force microscopy is an effective tool to study endothelial monolayer behavior**

Despite the limitations of the traction force microscopy method in the context of permeability, the technique provides important biophysical information about endothelial monolayer function and the differences introduced by force-modulating proteins. This can be seen, for example, in **Chapter 5**, in which the Imatinib specific kinases Arg and c-Abl are shown to have opposing effects on traction force development in the resting monolayer. Moreover, in the DRF2 study of **Chapter 6**, we revealed a reduction of contractile force in combination with an increase in F-actin shear fibers. In general, it is believed that shear- and stress fibers are formed in response to higher stresses which enabling them to cope with these.<sup>9,10</sup> However, in this example the monolayer tension is reduced, highlighting this counterintuitive finding and the importance of studying contractile forces. Lastly, data presented here and by others revealed that substrate stiffness affects endothelial contractility. In the process of verifying our TFM set-up we performed stiffness-dependent experiments and found effects on cell spreading and the generation of traction forces (Figure 2). In these experiments, a deformable substrate of 4 kPa (Young's modulus), was used to represent normal physiology. Endothelial cells, due to their location in all tissues, encounter a diversity of physical microenvironments, ranging between 1 and 6 kPa within a healthy vessel.<sup>11-14</sup> In more pathological conditions, like diabetes, hypertension, atherosclerosis and renal disease, the normal compliant extracellular matrix as well as the associated cells stiffen significantly<sup>15-17</sup> which can progress up to 60 kPa in calcified atherosclerotic lesions.<sup>11</sup> This is in marked contrast to most of the reported experimental culture conditions, where cells are grown on plastics or glass, which have a stiffness in the gPa range. An advantage of using the TFM method is that the experiments are conducted on relevant substrate rigidities which thereby better resemble the *in vivo* situation of endothelial monolayers.

Changes in micro-environmental rigidity have a substantial impact on the functioning of different cells.<sup>18-22</sup> For example, cell migration, -differentiation and -proliferation are highly affected by substrate stiffness.<sup>18,23-25</sup> This holds also true for the endothelium, where the mechano-sensitivity was additionally shown by a more pronounced barrier-disruptive response upon the stimulation with thrombin on stiffer substrates.<sup>8,26,27</sup> In the subsequent closure of inter-endothelial gaps, the previous linear relation between gap formation and substrate rigidity was absent. Here, the fastest recovery was observed around a stiffness optimum within the normal physiological range.<sup>27</sup> In conclusion, the ability to study endothelial behavior using such detailed biophysical methods, helps to provide a more reliable view on vascular physiology within the human body.



**Figure 2. Effect of extracellular matrix stiffness on endothelial cell function.** **A.** HUVECs were seeded in a 3 mm micropattern on a polyacrylamide hydrogel with a physiological stiffness (4 kPa), an ‘inflammatory’ increased stiffness (11 kPa) and stiffness corresponding to a calcified plaque (60 kPa) and were incubated for 120 minutes before imaging. **B.** After growing the cells to confluence and serum starving them for 90 minutes, traction forces were recorded.

### Limitation of the studies

In all of the presented chapters in this thesis we made use of human umbilical vein endothelial cells (HUVECs). Although these are well characterized, freshly isolated and primary human cells, there are also some disadvantages in using this cell type. One of the downsides is that they do not fully represent the phenotype of endothelial cells which are most affected by vascular leakage. HUVECs are isolated from bigger conduit veins instead of from post-capillary venules and form part of the fetal circulation rather than of the circulation of, mostly adult, acute lung injury- and sepsis patients. However, they are one of the few options available to obtain primary human endothelial cells and by studying them at low passage numbers and on physiologically relevant substrates, HUVECs remain a valuable model system to study endothelial monolayer behavior.

Within this thesis, we primarily use the widely applied traction force microscopy method to quantify cellular contractions. A limitation of this technique in the light of endothelial barrier function, is that it reveals the exerted forces at the level of the integrin-mediated cell-matrix adhesions and not the forces within the cell monolayer (Figure 1A, blue arrows). Recently, with the use of additional modulation steps of comparable experimental input, it became possible to study forces at the height of the cell-cell junctions (Figure 1A, orange arrows).<sup>7,28</sup> These so-called intracellular stresses, account for nearly one-half of the overall forces within the endothelium<sup>8,29</sup> and are shown to relate to cell monolayer functions by which they reveal their importance.<sup>30,31</sup> In **Chapter 4** of this thesis we use this patented technique to study the effects of the two different ROCK isoforms. Despite the benefits, there are also some disadvantages in the intracellular stress approach. For instance, the spatial resolution is reduced by the necessary additional processing steps of the bead displacements. This could also be one of the reasons why the correlations we described in **Chapter 3**, of traction forces - and cell junctions,<sup>32</sup> and

F-actin fibers<sup>33</sup> - are not found in monolayer stress microscopy experiments. Assessing to what extent our data on traction force distribution relate to intracellular stresses is beyond the scope of this thesis.

A final remark is that an inflammatory reaction *in vivo* involves multiple cytokines and immune cells, which affect a variety of different cell types that reside close to an infection site. This interplay of factors and cells, altogether, determines the outcome of the inflammatory response. For our *in vitro* experiments we use a more simplistic single cell model in combination with a single barrier-disruptive factor. This enables us to study specific mechanisms in a more isolated setting, which contribute to the inflammatory reaction, but do not determine the outcome of the immune response per se. For the implementation of the knowledge obtained here it is therefore important to realize that our results need to be translated back into a more physiological condition, which includes the dynamic interaction of several circulating factors and different types of cells.

### General conclusions and future direction

To conclude, our *in vitro* and *in vivo* studies have demonstrated that the actomyosin-generated tension by the vascular endothelium itself, plays an important role in endothelial barrier disruption and inflammation as a whole. Despite the fact that there was no direct link between traction force magnitude and the formation of inter-endothelial gaps, a close correlation with cell junctions and the actin cytoskeleton was established. Also, processes like the formation of lamellipodia and filopodia, and the cellular response to substrate stiffening displayed a close relationship with traction forces. The regulation of these contractile forces as well as the control of cell-cell- and cell-matrix interactions was shown to be mediated by force-modulating proteins in combination with the Rho-GTPases. Herein, especially ROCK2 and Arg have proven to be part of one of the fundamental molecular mechanisms that can prevent vascular leakage. These proteins have the potential to form new drug targets in, for example, ALI or sepsis, for which no specific therapeutic options are currently available. Although the insights in these processes is increasing, translating our recent knowledge into effective therapies remains an enormous challenge.

Future research will aim at elucidating the exact relationship between contractile forces and agonist-induced disassembly of focal adhesion- and adherens junction complexes. To do so, high resolution traction force microscopy is required in combination with the expression of different fluorescently labeled cadherin, integrin, cytoskeletal and linker proteins. By this approach the cell-cell and cell-matrix organization and -modulation can be studied in live cells and related to the traction force dynamics in more detail. Furthermore, within these experiments intracellular stress calculations may be included, however, more effort should be invested in recently developed techniques which enable the quantification of forces at the level of individual proteins. One of these methods is the DNA-hairpin approach, which can detect the binding of integrins by the force-dependent unfolding of a DNA strand and the subsequent fluorescence emission after the separation of the conjugated fluorophore-quencher pair.<sup>34,35</sup> Another technique, more suitable to measure forces within the adherens junctions, is the fluorescence resonance energy transfer (FRET) technology.<sup>36,37</sup> With the use of this method, local tethering forces in a VE-cadherin protein can be determined by the use of light emitting molecular tension probes,<sup>38,39</sup> which could even be used in combination with traction force microscopy. Both techniques require high resolution microscopy and some more complex laboratory handling, but will provide new insights in the spatial distribution of molecular tension and their response to barrier-disruptive agents.

Another important development is that more and more emphasis is put on the physiologically relevant microenvironment of cells in *in vitro* experiments, also in the field of endothelial contractility. For the endothelium this means analyzing cells under physiological substrate stiffness<sup>8</sup> possibly in combination with relevant biological stretch of the blood vessel<sup>40</sup> and shear forces by the flowing blood.<sup>25,32,41,42</sup> Moreover, we expect that in the future this will also be combined with co-cultures of pericytes and smooth muscle cells in different cell arrays, following the development of organ-on-a-chip models. Even better would be to make the switch to *in vivo* experiments, where also the immune system is involved in inflammatory response. Despite the fact

that some studies started to quantify contractions within living tissues, additional efforts need to be invested to establish reliable, high quality techniques to measure forces in these complex settings.<sup>43,44</sup>

In summary, the work in this thesis has shown that traction force microscopy can be implemented to measure endothelial monolayer contractions in resting and activated conditions. This provided new insights into the mechanotransduction within the endothelium and highlighted the role of different force-modulating proteins. Despite the fact that our initial hypothesis on a direct correlation of traction forces with endothelial permeability was not confirmed, we identify, using the TFM method, promising new candidates for future drug development against vascular leakage.

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