G-protein and Rho GTPase signaling in endothelial barrier regulation

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CHAPTER 9

SUMMARY AND CONCLUDING REMARKS

Dynamic regulation of the endothelial barrier function is critical for proper functioning of the vasculature and human health. ECs continuously sense and respond to various stimuli that can either strengthen or disrupt the endothelium, depending on the output of large and complex signaling networks that become activated. Activation of these respective networks often include regulation via GPCRs, one of the major groups of cell surface receptors that respond to a variety of stimuli and of which ECs express many different subtypes. In general, GPCRs initiate Rho GTPase signaling cascades, thereby acting on the actin cytoskeleton and subsequently regulating EC-cell contacts. Since the latter is a direct measure of permeability and endothelial barrier function, these signaling mechanisms are indispensable for a healthy endothelium and vasculature. Despite the interest regarding GPCR-Rho GTPase-mediated signaling networks in the endothelium, critical details and molecular aspects are lacking. Therefore, this thesis aims to reveal novel molecular players in GPCR-Rho GTPase signaling networks that control the endothelial barrier function.

Use of novel FRET sensors in analysis of endothelial signaling

Charting molecular signaling networks requires a high level of spatial and temporal resolution. In the course of this work, we have used a variety of novel imaging tools to document GPCR-Rho GTPase signaling in primary human ECs. Besides performing regular protein localization microscopy, we implemented ratiometric FRET sensor measurements to study the activation of our proteins of interest. In contrast to “bulk” protein activation assays that are limited to protein “on” and “off” states, the FRET sensor technique generates dynamic and quantitative data. Furthermore, FRET sensor microscopy can be applied to measure live, single cells with high spatiotemporal resolution.

The FRET sensor approach requires a strong technical background, since distinct target proteins have unique requirements in terms of FRET sensor design. In this thesis we distinguished two groups of FRET sensors, consisting of i) G-protein FRET sensors to directly measure G-protein activation, and ii) Rho GTPase FRET sensors, which readily detect GEF and GAP activity. Chapter 5 describes the development and use of a $G_{13}$ FRET sensor, which localizes at the PM and shows responsiveness to well-accepted $G_{13}$ stimuli. Notably, a critical step in the design and functionality of this $G_{13}$ FRET sensor relied on the specific site of incorporation of the fluorescent proteins (FPs), as was also reported for the previously-developed $G_q$ and $G_i$ FRET sensors.

In chapter 2 we reviewed FRET sensor developments in the context of Rho GTPase proteins. Over the years, several unimolecular Rho GTPase FRET sensors have been developed, each consisting of essential core components; the Rho GTPase of interest, donor and acceptor light emitting FPs with a connecting linker and a Rho binding domain (RBD). While distinct Rho GTPase FRET sensor designs have shown to efficiently monitor protein activation, the arrangement of the core components are critical for biological interpretation.
of the data. Specifically, in order to retain correct localization and regulation by RhoGDIs, the Rho GTPase in the FRET sensor requires an unmodified C-terminus. Based on an existing FRET sensor for RhoA with the aforementioned design\textsuperscript{5}, we developed RhoB and RhoC FRET sensors in \textit{chapter 3}. The subcellular localization of these FRET sensors corresponded to the endogenous localization of the respective proteins, and they all showed responsiveness to GPCR agonists. To our knowledge, this is the first study that reports on RhoA/B/C activation in such high detail. The Rho GTPase FRET sensors are thus valuable to address specific biological questions in endothelial biology, which will be further highlighted in the following sections.

\textbf{Three Rho proteins: RhoA/B/C signaling in the endothelium}

By combining molecular and imaging approaches with our new FRET sensors for Rho GTPases, we tried to elucidate several questions in the field of Rho GTPase signaling and endothelial biology. \textit{Chapter 2} describes the five main Rho GTPase proteins that form the core of this thesis: Cdc42, Rac1, RhoA, RhoB and RhoC. We reviewed their structural and functional characteristics, mainly focusing on the highly homologous (> 88\%) Rho subfamily RhoA, RhoB and RhoC. This Rho subfamily has been discovered in 1985, whilst the RhoA gene emerged as the most complex one\textsuperscript{9}. This, together with the observation that RhoA/B/C show comparable effects on the actin cytoskeleton and the formation of actin stress fibers\textsuperscript{10}, has previously resulted in a focused interest in RhoA. By attributing Rho-mediated effects exclusively to RhoA, however, previous studies failed to address a critical question: “Why do humans have three different Rho proteins?” In the context of cell motility, this question has been discussed by an excellent review\textsuperscript{11}, presenting RhoA as main regulator of myosin-dependent contraction, whereas RhoB and RhoC were mainly linked to endosomal trafficking and cell locomotion, respectively. Wheeler and Ridley (2004) furthermore highlighted specific RhoA/B/C regulators and effector proteins, a critical subject in the understanding of Rho-mediated signaling events. \textit{Chapter 2} of this thesis continues on these findings and presents a more up-to-date review of distinct RhoA/B/C GEFs, GAPs and GDIs, as well as distinct regulation via downstream effectors. Furthermore, in \textit{chapter 3} we demonstrated GEF and GDI specificity towards RhoA/B/C in live, primary human ECs.

In line with the general picture of RhoA/B/C-oriented studies, also in the endothelium there has been a main interest in RhoA. In \textit{chapter 3}, we challenge this situation through comparative RhoA/B/C analyses and reported on highest expression of the RhoC GTPase in primary human ECs. Together with the observation that RhoC localized at cell-cell contacts, this resulted in our major interest in the RhoC isoform. A recent study proposed a specific function of RhoC in the restoration of reduced endothelial barrier function\textsuperscript{12}. However, our approaches to explore RhoC-mediated functions lacked specificity; we observed comparable RhoA/C responsiveness to relevant stimuli that regulate endothelial integrity (e.g. Thrombin, \textit{Figure 1A}). So far, we were therefore unable to define RhoC-specific effects in the endothelium and could not explain high RhoC expression in human ECs.
In the same RhoA/B/C comparative study of chapter 3, low RhoB expression levels suggest only limited functions of this protein in the endothelium. However, we (chapter 3) and others, have demonstrated that RhoB expression becomes significantly upregulated upon stimulation with the pro-inflammatory cytokines TNFα. Notably, this upregulation coincides with an increase in RhoB activity (Figure 1B, chapter 3), which indicates this protein as important inflammatory regulator. Strikingly, RhoB upregulation was also observed under physiological flow conditions (chapter 4), where all three RhoA/B/C proteins showed activation upon flow. Furthermore, under flow conditions, for the first time we detected spatial activation differences between RhoA and RhoC (Figure 1C). While flow-mediated RhoA activation has been reported previously, we detected Rho activation with a much higher temporal

Figure 1. Spatiotemporal activation profiles of RhoA/B/C in primary human ECs. A,B,C) Left images show schematic representations of RhoA/B/C activation in ECs of A) GPCR stimulation (e.g. Thrombin), B) stimulation with an inflammatory cytokine and C) exposure to flow. A,B,C) For RhoA and RhoC, red color indicates high activation, white color indicates low activation; for RhoB, blue color indicates high activation, white color indicates low activation (corresponding to the legends on the left upper corner in A, A,B,C) Right graphs schematically present spatial activation profiles, corresponding to images on the left. B) Dashed line in inflamed condition is speculative.
resolution and revealed novel activation profiles as compared to static endothelial measurements. Since these specific, flow-mediated signaling profiles were also observed at the level of endothelial barrier function, these findings are relevant for our understanding of endothelial barrier regulation in the human body and should be explored in greater detail.

**From S1P receptors to endothelial barrier regulation: linking G-proteins, GEFs and Rho GTPases**

While the RhoA/B/C subfamily has been linked to endothelial barrier disruption via cell contraction and the disruption of cell-cell contacts, there also exist ‘opposing’ Rho GTPases which enhance the endothelial barrier function. Both groups of Rho GTPase variants are required for the dynamic endothelium to respond to stimuli that differently affect the barrier function. Among the numerous existing cues that act on the endothelium, in chapter 6 we specifically focus on the phospholipid sphingosine-1-phosphate (S1P). S1P activates three different GPCRs, expressed in the endothelium: S1PR1, S1PR2 and S1PR3, respectively. While the S1PR1 mediates endothelial barrier enhancement, the S1PR2 and S1PR3 are mainly linked to barrier disruption. In the vasculature, the expression of different S1PRs are critical determinants for the output of S1P-mediated signaling. Notably, S1PR expression levels differ between vascular beds and even among different sections of the same blood vessel, thereby regulating differential levels in permeability. Consequently, aberrant S1PR levels have been strongly linked to vascular pathologies, such as inflammation and atherosclerosis.

**Chapter 6** of this thesis addressed several remaining questions regarding S1P signaling in the endothelium downstream of the receptors. Briefly, we report on a dynamic system that involves a barrier-promoting S1PR1-Gi-Rac1/Cdc42 - and an antagonistic, barrier-disruptive S1PR2-Gi-RhoA/B/C signaling axis. While in “normal” physiology (“resting” ECs), the endothelium relies on the S1PR1-Gi-Rac1/Cdc42 signaling, disruption of the system (in “activated” ECs) results in a shift to S1PR2-Gi-RhoA/B/C and consequently barrier disruption. While this specific example underscores the tight regulation in endothelial barrier control, it also presents molecular targets in the treatment of S1P-related vascular pathologies.

**Novel Cdc42 regulators in endothelial barrier function**

Most studies that aimed at S1P and Rho GTPase-mediated barrier regulation, predominantly focused on Rac1 and RhoA. Interestingly, in chapter 6, we demonstrate the involvement of Cdc42. While Cdc42 has been primarily linked to filopodia formation, we also connected this protein to EC spreading (chapter 6 and chapter 8). In contrast to Rac1, Cdc42 is not affected by Rho signaling (chapter 6), suggesting differential regulatory mechanisms for Cdc42 and Rac1.

**Chapter 7 and 8** explored the area of Cdc42-specific regulatory proteins at the level of GEFs and Gi-mediated activation. We showed Gβγ-dependent activation of Cdc42 and proposed the GEF pRex1 as positive regulator of S1P-mediated Cdc42 activation and cell spreading. To what extent these effects are Cdc42-specific remains to be elucidated.
Next to pRex1, chapter 7 revealed numerous GEFs with specificity towards Cdc42. Most likely, these GEFs are not involved in S1P-mediated Cdc42 activation. However, their induction of specific phenotypes and of GEF efficiency measured in primary human ECs, indicates these proteins to be important in additional signaling mechanisms in the endothelium with a central role for Cdc42. Our findings in chapter 6-9 have been summarized in Figure 2 and 3.

Figure 2. Dynamic and balanced molecular mechanisms that control S1P-mediated barrier enhancement in ECs. In “resting” ECs, S1PR1-Gi-Rac1 and S1PR1-Gbg-pRex1-Cdc42-mediated signaling is dominant over the S1PR2-G13-Rho signaling axis, resulting in cortical actin (red structures) formation, VE-cadherin (purple structure at cell borders) stabilization and subsequently endothelial barrier stabilization. Conversely, in “activated” ECs (e.g. during inflammation) the S1P2R-G13-Rho signaling axis predominantly gets activated, resulting in actin stress fiber formation, disruption of the VE-cadherin complex and consequently a switch to endothelial barrier disruption. In contrast to Cdc42, Rac1 is inhibited via RhoA signaling and vice versa. Red cross on the right demonstrates GEFs that are not involved in S1P-mediated Cdc42 activation.
Evaluation and future perspectives

This thesis has a strong technical and fundamental basis, approaching molecular aspects of endothelial signaling networks, and resolving critical mechanisms in endothelial barrier regulation. Even though our translational approach is indispensable in the field of vascular biology, it comes with certain limitations. We will discuss these limitations in the following paragraphs, in order to put our findings in perspective, but also to emphasize the need for further research.

The first issue that needs to be discussed relates to the experimental setup of our experiments. To closely mimic physiological situations, biological signaling studies are preferably based on endogenous protein levels. Most of our experiments, however, are
based on ectopic expression of FP-tagged proteins and/or FRET constructs. Recently, the technological advancement in Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-based technologies, has generated the possibility to modify proteins at a genetic level, an approach by which even endogenous proteins can be tagged with a FP and expressed at normal (regulated) levels. However, since we are working with primary human cells in in vitro culture, we are limited to a small number of passages making it nearly impossible to achieve stable incorporation of mutations into the genome. In addition, incorporation of an endogenous FRET sensor most likely also disrupts the function of the respective, endogenous protein. Thus, our specific FRET sensor approach is limited to overexpression of DNA constructs. We can optimize our strategy by using low DNA – and sensor-expression and performing side to side comparisons with biochemical assays for activation of endogenous proteins, as we have done throughout this thesis. Furthermore, the interplay between different proteins can also be approached by measuring multiple FRET sensors in one cell, a specific approach defined as FRET “multiplexing”. This advanced FRET strategy, however, shows strong requirements in terms of microscope setups and FRET sensor fluorophore characteristics. Due to limiting time, this specialized subject exceeded the focus of this thesis.

Alternatives for mimicking human vascular physiology also rely on the model system that is used. While all our experiments have been performed in 2D systems, we should aim to 3D systems for an improved representation of the physiological situation. We can think of endothelial-specific knock-in and knock-out mice to study the function of our proteins of interest in endothelial barrier regulation and vascular pathologies, e.g. atherosclerosis. Unfortunately, 3D in vivo experiments remain challenging, mainly due to the scattering of light. Consequently, future FRET-based experiments should also explore alternative 3D experiments, including endothelial sprouting assays, the use of microfluidic devices, and ex vivo models.

Our last concern exclusively focuses on the optimization of our FRET sensor experiments, and specifically on the image acquisition and data analysis we performed. In contrast to previous studies in mouse embryonic fibroblasts (MEFs)\textsuperscript{28}, we could not detect (except for flow exposure) spatial activation differences between the highly homologous proteins RhoA and RhoC. This raises the question whether we need a higher spatial resolution to understand the biological functions of our proteins of interest. This can be investigated by applying more extensive FRET data analysis as described in\textsuperscript{28,29}, but future approaches should also explore more advanced imaging techniques, e.g. total internal reflection fluorescence (TIRF) or lattice light sheet fluorescence microscopy (LSFM).

Concluding remarks
In conclusion, our research has been indispensable for the molecular understanding of endothelial biology. We revealed novel regulators and dynamic signaling networks that control the endothelial barrier function. Focusing on GPCR-Rho GTPase-controlled signaling, we challenged a specific area of endothelial barrier regulation. We specified major regulators at the level of receptors (e.g. PAR-1 and S1PRs), GEFs (e.g. pRex1) and Rho GTPases (e.g. Rac1,
Cdc42 and Rho) and furthermore highlighted the dynamics of the endothelium. Notably, these findings are perfectly underscored by our model regarding S1P-mediated barrier regulation. While in the “resting” endothelium, S1P promotes the endothelial barrier function, both barrier-promoting, as well as barrier disrupting signaling mechanisms are activated. As a result, this mechanism shows high context-specificity, prone to changes in expression levels of involved proteins and environmental factors (e.g. S1P concentration). In general, this concept of dynamic S1P-mediated barrier regulation represents the mechanism of how the endothelium continuously adapts to the environment, also referring to vascular homeostasis. For example, the dynamic behavior of the endothelium can also be found in the regulation of vascular tone, blood fluidity and nutrient transport through the vessel wall.

As deduced from the previous paragraphs, our research underscores the “healthy” endothelium as a dynamic and balanced system that continuously adapts to the environment. But does our work also contribute to the understanding, and ultimately treatment, of vascular pathologies? Although we do not reveal hands-on strategies to cure vascular diseases, we introduced novel players and mechanisms that control the dynamics in endothelial barrier regulation. In addition, aberrant endothelial barrier regulation directly translates into vascular pathologies, such as inflammation, atherosclerosis and tumor cell invasiveness. Summarizing, our research has provided new, fundamental molecular insights into the physiology of the endothelium and endothelial barrier control, and furthermore identifies novel therapeutic targets in vascular, endothelial-based pathologies.

REFERENCES