CHAPTER 1

General introduction and thesis outline
The endothelium and its barrier function

The endothelium is a dynamic barrier that covers the luminal side of the heart and of all the blood and lymph vessels throughout the body. It is metabolically highly active and is a central regulator of fluid homeostasis, vascular tone, coagulation, trafficking of circulating cells and transport of nutrients, of vasculogenesis and angiogenesis. Endothelial cells form a monolayer of tightly connected cells, which adhere via cell-cell junctions to neighboring cells and through Focal Adhesions (FAs) to the extracellular matrix.

Under basal conditions, plasma constituents cross the endothelium by means of transcellular transport via vesicles or paracellular exchange, i.e. via the cell-cell junctions. Large molecules (>3 nm molecular radius) are shuttled across the endothelial barrier via transcellular transport; water via small slits in the junctions between adjacent cells and via aquaporins; and small solutes (<3 nm molecular radius) via paracellular exchange. Immune cell cross paracellularly and transcellularly after interaction with specific cell-surface receptors. During inflammation the exchange of large molecules is facilitated by loss of the contact between adjacent cells and widening of the paracellular openings. Since this may eventually lead to edema and tissue damage, the size of these intercellular gaps needs to be dynamic but also carefully controlled.

Endothelial barrier dysfunction is a hallmark of a wide range of diseases and is involved in, for example, sepsis, acute respiratory distress syndrome (ARDS), edema, inflammatory disorders, cancer and diabetes. As a result of endothelial dysfunction, endothelial monolayer integrity is disrupted by the formation of inter-endothelial gaps. This occurs following myosin-based contraction of the actin cytoskeleton, which generates mechanical force on cell-cell contacts and results in partial detachment of the cells from each other and from the extracellular matrix.

As mentioned, three main systems are involved in endothelial cell shape changes and intercellular gap formation: cell-cell junctions, the actin cytoskeleton and cell-matrix interactions. Cell-cell junctions exist in three different forms: gap junctions, tight junctions and adherens junctions (AJs). Gap junctions consist of transmembrane connexin proteins which, through homotypic interactions with a connexin on a neighboring cell form a channel directly connecting the cytoplasm of two cells. Connexins are thus essential for intercellular communication. Tight junctions consist of transmembrane proteins, claudin and occludin, which link the cytoskeleton of adjacent cells and in this way, play a role in the dynamics of endothelial barrier function. Tight junctions play a dominant role in the endothelial sealing of the blood brain barrier. In most other continuous endothelia the tight junctions form mosaic structures, which allow exchange of solutes and proteins. However, in the latter endothelial beds an adhesive belt of AJs seals the endothelial monolayer and controls the endothelial permeability. AJs are the best studied cell-cell junctions in endothelial cells. AJs comprise the transmembrane protein Vascular endothelial (VE)-cadherin, which forms a calcium-dependent homodimer with VE-cadherin on an adjacent cell. Via α-catenin, β-catenin and plakoglobin, the intracellular domain of VE-cadherin is linked to the actin cytoskeleton. The actin cytoskeleton is connected to both cell-cell junctions as well as to the cell-matrix contacts.
which makes it essential for endothelial barrier function\textsuperscript{15}. Polymerized F-actin is concentrated in various cytoskeletal structures, in particular the cortical actin ring and in stress fibers. The cortical ring of F-actin which is positioned close to the cell-cell contacts promotes, under basal conditions, strong cell-cell adhesion\textsuperscript{16}. Stress fibers, which are also coated with myosin are involved in cell contraction\textsuperscript{17}.

The interaction of endothelial cells with their extracellular matrix is mediated by integrins, transmembrane proteins which, like VE-cadherin, link the extracellular environment to the intracellular actin cytoskeleton\textsuperscript{18}. The intra-cellular domain of integrins connects to the cytoskeleton via a regulatory protein complex which is called the FA complex, which is not only important for cell-matrix adhesion, but also strengthens cell-cell adhesion\textsuperscript{19}. Thus, there is a close functional and structural connection, via signaling proteins and the cytoskeleton respectively, between integrin-mediated and VE-cadherin-mediated adhesion. In concert, these adhesion complexes regulate endothelial cell shape, intercellular gaps and, consequently, barrier function and paracellular transport. Since endothelial barrier dysfunction is a hallmark of disease (e.g. diseases associated with inflammation and edema), tight regulation of these complexes is essential.

**RhoGTPases in endothelial barrier regulation**

Over the last decades it has become clear that the homotypic interaction of the VE-cadherins in AJs is controlled by the formation of molecular complexes and phosphorylation of VE-cadherin and some of its associated proteins\textsuperscript{20}. While the role of acto-myosin mediated contraction, which requires Ca\textsuperscript{2+}-dependent activation of myosin light chain kinase, has been recognized early, the complexity of F-actin interactions with, and effects on, junctional stability became appreciated only in the last years\textsuperscript{21,22}. Important regulators of endothelial acto-myosin-based contractility and junctional integrity are the Rho family of small GTPases. This family of proteins consists of approximately 20 members which are divided into classical and atypical GTPases\textsuperscript{23}. Classical RhoGTPases are regulated by the exchange of GDP for GTP, which is stimulated by guanine nucleotide exchange factors (GEFs), and GTP hydrolysis, which is stimulated by GTPase-activating proteins (GAPs). GEFs stimulate transition to the GTP-bound state, resulting in a conformational change and subsequent activation of the RhoGTPase, whereas RhoGAPs enhance otherwise low intrinsic GTP hydrolysis which reverts the RhoGTPase to the inactive, GDP-bound state\textsuperscript{24}. RhoGTPases also interact with Rho guanine dissociation inhibitors (GDIs), which keep RhoGTPases in an inactive state, preserving a cytoplasmic reservoir of activatable proteins\textsuperscript{25}. Atypical Rho family members are unable to hydrolyze GTP and are therefore constitutively GTP-bound and active. Endothelial cells contain approximately 80 GEFs, 70 GAPs and 3 GDIs. This large variation of regulatory proteins enables RhoGTPases to cooperate and antagonize each other to control different cellular tasks\textsuperscript{26}. In addition to GDP/GTP cycling, RhoGTPases can also be regulated by translocation to and from the plasma membrane and by post-translational modifications including prenylation, phosphorylation and ubiquitination\textsuperscript{27} (Figure 1).

Prenylation is the most frequent post-translational modification of RhoGTPases. During this process, a farnesyl or geranylgeranyl group is covalently linked to a C-terminal cysteine residue.
This modification is mediated by farnesyltransferases and geranylgeranyl transferases. Prenylation of RhoGTPases is important for the binding to the GDI, but is also essential to localize active RhoGTPases to membrane compartments and control their interaction with localized GEFs, GAPs and effector proteins in specific signaling pathways²⁸,²⁹ (Figure 1).

Phosphorylation of RhoGTPases, which is well described for the RhoA isoform, can alter both the activity and localization of the protein³⁰. It may regulate GTP-GDP cycling by affecting the GTP binding site, change subcellular localization and can induce conformational changes which lead to protein sequestration or degradation³¹-³³.

Ubiquitination is the third post translational modification which can regulate RhoGTPase localization, activity and degradation. In this type of modification, a ubiquitin group is covalently linked to the RhoGTPase in a three-step process. In the first step, ubiquitin is activated by ubiquitin-activating enzymes (E1). In the second step this activated ubiquitin is transferred from E1 to a ubiquitin-conjugating enzyme (E2). In the final step the ubiquitin is conjugated to the substrate by ubiquitin ligases (E3).

**Figure 1: Regulation of RhoGTPase activation**

The activity of RhoGTPases is tightly regulated. Guanine nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs) and guanine nucleotide dissociation inhibitors (GDIs) regulate the GTPase cycle. RhoGTPases can be post-translational modified by prenylation, phosphorylation and ubiquitination. As indicated in the figure, these different modifications can affect regulatory steps in the GTPase cycle, and also target the GTPases for proteasomal degradation.
The E3 ubiquitin ligases can be divided into two groups based on their structure: the homologous to the E6 AP carboxyl terminus (HECT) domain ligases, which directly transfer the ubiquitin, and the really interesting new gene (RING) finger ligases, which act as scaffolds for ubiquitin-transfer proteins. In most cases, E2 and E3 ligases form a complex to transfer ubiquitin to a target protein.

One of the best studied RING ubiquitin ligases involved in proteasomal degradation is the Skp1-Cullin1-F-box protein (SCF) complex. In this complex, Cullin-1 protein directly interacts with Skp1 and RBX1. On one side of the complex, Skp1 acts as a bridging component between Cullin-1 and an F-box protein, a receptor which recognizes substrates targeted for ubiquitination by the SCF complex. On the other side of the SCF complex, Rbx1 interacts with an E2 enzyme which transfers ubiquitin to the target protein.

**Contribution of RhoGTPases to endothelial barrier dysfunction**

The RhoGTPases that are best studied for their role in endothelial barrier regulation are RhoA, Rac1 and Cdc42. All three are essential for the regulation of permeability of the endothelial barrier through their control of dynamic cell-cell contacts. RhoA activity is associated with loss of endothelial barrier integrity, which is due to the formation of stress fibers and acto-myosin-based contraction of the endothelial cells. Although RhoA signaling generally results in increased permeability, RhoA has also been associated with barrier-protective effects. RhoA activity at membrane protrusions coincided with closure of inter-endothelial gaps. In contrast to RhoA, Rac1 and Cdc42 are well established for their role in endothelial barrier maintenance, Rac1 is important for AJ integrity and for VE-cadherin mediated adhesion. Activation of Rac1 can counteract RhoA activation and is important for junction formation and re-annealing of junctions after RhoA-induced loss of integrity. Cdc42 is also involved in junction stability but is, different from Rac1, involved in both barrier maintenance under basal conditions and barrier restoration after induction of permeability.

Inflammatory mediators and growth factors such as thrombin, vascular endothelial growth factor (VEGF) and histamine, can bind and activate receptors on the cell surface, resulting in the activation of a number of intracellular signaling pathways. Contraction of the cells requires the influx of calcium and the activation of RhoA and its downstream effector Rho kinase (ROCK) which in turn leads to phosphorylation of myosin light chain (MLC) and the formation of contractile actin fibers. Activation of tyrosine kinases among which Src and Focal Adhesion Kinase (FAK) leads to the phosphorylation of VE-cadherin and the consequent destabilization of cell-cell contacts. Similarly, inactivation of the GTPase Rac1 reduces both the stability of cell-cell contacts and the number of cell-matrix adhesions. Differential distribution of cell-matrix adhesions may also occur following activation of non-receptor tyrosine kinases, such as Abl related gene (Arg). However, the exact functional connection between cell-matrix and cell-cell adhesions in endothelial barrier dysfunction is currently unclear.
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Figure 2: The role of RhoGTPases in endothelial barrier function

A) Stable barrier: Immunofluorescent staining of in-vitro cultured HUVECs under basal conditions. Cells have a cortical actin ring (red) through which they are attached to their neighboring cells via stable adherens junctions (VE-cadherin in green). B) Disrupted barrier: Immunofluorescent staining of in-vitro cultured HUVECs stimulated with thrombin. The cells show formation of F-actin stress fibers and inter-endothelial “gaps”. Under the images the schematic representation of the role of RhoGTPases in stable and disrupted endothelial monolayers is shown.
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SCOPE AND OUTLINE OF THE THESIS

In this thesis we focus on the regulatory pathways involved in endothelial barrier dysfunction, an important pathophysiological mechanism in disease. Elucidation of these pathways will improve our understanding of barrier control in the vascular wall and may drive the development of treatments, which protects against endothelial barrier dysfunction.

We have been primarily interested in analyzing the functions of RhoGTPases since they play a pivotal role in the regulation of endothelial barrier function. RhoA is known for its barrier disruptive effects, while the function of the closely related, highly homologous GTPases RhoB and RhoC in endothelial barrier function are unknown and require further elucidation.

In addition to their activation by GTP/GDP exchange, RhoGTPases can also be modified by ubiquitination. This important post-translational modification may induce altered localization, signaling and eventually degradation of the GTPases. Within the SCF ubiquitin ligase complex substrate recognition is mediated by the F-box proteins which are part of the complex. Specific substrates and functions of many of the F-box proteins are still unknown and the role of F-box proteins in endothelial barrier regulation was not previously investigated. The role of F-box proteins in RhoGTPase function and endothelial integrity was addressed using a siRNA-mediated knockdown approach.

Since ubiquitination is a prerequisite for the degradation of Rho proteins, and initial studies have showed that inactivation of the Rho-ubiquitination pathway disrupts endothelial barrier due to cell contraction, we wondered if prolonged activation of the RhoGTPase ubiquitination pathways could enhance endothelial barrier function.

Active RhoGTPases signal towards several downstream effectors including serine/threonine and tyrosine kinases. Our laboratory previously showed that the tyrosine kinase inhibitor imatinib is protective against endothelial dysfunction. Subsequently, pharmaceutical companies have invested in designing other, new drugs which may reduce endothelial barrier dysfunction even further. However, since most of these compounds fail in clinical trials because of lack of efficacy or of severe side effects, new studies on improved kinase inhibitors for barrier protection remain warranted.

Based on the foregoing observations we aimed to study the differential effects of RhoA, RhoB and RhoC in endothelial barrier function and their regulation by degradation, in order to obtain leads for rational treatment of vascular leakage. To that end we formulated the following objectives:

1) Elucidate the differential roles of RhoA, RhoB and RhoC in endothelial monolayers under basal and stimulated conditions;
2) Screen for the effect of knockdown of F-box proteins on endothelial barrier function;
3) Test the effect of prolonged activation of ubiquitination on endothelial barrier function;
4) Screen several second and third generation bcr-abl tyrosine kinase inhibitors for their protection against thrombin-induced endothelial barrier dysfunction.
In Chapter 2 we analyzed the individual roles of RhoA, RhoB and RhoC and their mutual interactions in endothelial barrier function. The main findings of this work were that RhoB, but not RhoA or RhoC, is a negative regulator of the barrier integrity in resting endothelium. In addition, loss of endothelial integrity after thrombin stimulation is mediated by RhoA and RhoB activity, while RhoC appears to contribute mainly to the Rac1-dependent restoration of endothelial barrier function after thrombin stimulation.

In Chapter 3, we evaluated the effects of esiRNA-mediated knockdown of a selection of F-box proteins and we identified FBXW7 as an important regulator of endothelial barrier function. FBXW7 knockdown increased expression and induced differential localization of RhoB next to upregulation of the cholesterol synthesis pathway. Functionally, depletion of FBXW7 led to increased endothelial cell contractility and disruption of the endothelial barrier.

In Chapter 4, we evaluated the effect of general activation of Cullin-RING ligases and subsequent increased ubiquitination of Rho proteins on endothelial barrier function. The main findings of this work were that prolonged neddylation of the Cullin-3 ubiquitin ligase results in increased expression of RhoB in endothelial cells, which induces endothelial cell contraction and loss of barrier function. The increased expression of RhoB was in this case caused by activation of the NF-κB pathway.

In Chapter 5, we identified the tyrosine kinase inhibitor bosutinib as a novel compound that effectively protects against endothelial barrier dysfunction. Treatment with bosutinib protects against thrombin-induced loss of cell-cell contacts and endothelial barrier disruption by inhibition of the phosphorylation of Ezrin, Radixin and Moesin (ERM) and a concomitant increase in the number of FAs. These effects were linked to a combined inhibition by bosutinib of the ARG and MAP4K4 kinases in endothelial cells.

Finally, in Chapter 6, the main findings of this thesis are discussed.
REFERENCES


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