Regulation of vascular permeability by RhoGTPases

Coelho Amado de Azevedo, J.M.

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CHAPTER 7

Summary and Discussion

SUMMARY AND DISCUSSION

Under basal conditions, the vasculature leaks solutes and small molecules to the tissues but limits extravasation of larger molecules and cells. Vascular permeability is thus an essential physiological aspect of different biological processes including embryonic vasculogenesis, angiogenesis or wound healing. However, in many pathologies including severe trauma, sepsis, cancer and acute lung injury, the endothelial barrier becomes disrupted and leakage increases. Injuries to the capillary endothelium may cause edema and swelling in organs such as the heart, brain, liver or kidneys and might ultimately lead to life threatening-conditions. Moreover, in a clinical setting, treatment options for vascular leakage are limited. Due to the lack of targeted pharmaceutical agents, focus relies on prevention of further damage and maintenance of adequate perfusion. Effective pharmacological agents that reverse endothelial dysfunction and attenuate vascular leakage are thus of the utmost importance.

With the main focus on the endothelial barrier regulation, we sought to identify new targets for the treatment of vascular leakage. In this thesis we: addressed and reviewed the importance of RhoGTPases in barrier function (**chapter 2**); studied all known GTPases and associated proteins and their contribution to barrier function under basal conditions and identified novel regulators (**chapter 3**); characterized the unknown ArhGAP45/HMHA1 (RhoGAP) as a crucial negative regulator of endothelial barrier integrity (**chapter 4**); investigated the role of mDIA1 (RhoA effector) as a contributor to the regulation of barrier function (**chapter 5**); evaluated the contribution of all known GTPases and associated genes to the regulation of endothelial barrier under inflammatory conditions (**chapter 6**).

RhoGTPases as ideal therapeutic targets in controlling vascular leakage

RhoGTPases are fundamental proteins in the biology of endothelial cells. They control many signaling pathways, including those that mediate vascular integrity (or barrier function), making them ideal targets for the control of vascular leakage. Of 22 known RhoGTPases (Etienne-Manneville & Hall 2002; Jaffe & Hall 2005; Rojas *et al.* 2012), only for a few, detailed information is available (RhoA, Rac1, Cdc42, and more recently RhoB). For other family members hardly any information is known, other than that most of them have profound effects on the EC cytoskeleton (Sorokina & Chernoff 2005). The majority of RhoGTPases cycles between an active and inactive state. Most of them are regulated in response to external or internal cues and are released from the inhibitory complex to be activated by RhoGEFs at the plasma membrane and signal to effector proteins. RhoGAPs can then inactivate RhoGTPases after which RhoGDIs sequester them in the cytoplasm in their GDP-bound form, shielding them from aberrant activation and

degradation. This balance of activation and inactivation and the interplay between these regulatory molecules is required to maintain an optimal and functional endothelial barrier. RhoGTPases are thus part of a complex, interconnected signaling network which is strictly coordinated in a spatio-temporal fashion. Classically, RhoA has been associated with the induction of acto-myosin contraction, which causes the disruption of barrier integrity upon vaso-activation. In turn, Rac1 and Cdc42 have been associated with barrier maintenance and barrier restoration. Earlier literature already indicated that RhoA might have a dual function and also act as a barrier integrity promoter, dependent of intracellular location of the protein (van Nieuw Amerongen *et al.* 2007). RhoA was later shown to be active not only during contraction and barrier disruptive conditions but as well as at membrane protrusions and correlated with the closure of small intercellular gaps (Szulcek *et al.* 2013). Similar to RhoA, Rac1 was found to have a dual role in barrier function, it can mediate both permeability (Gavard & Gutkind 2006; Garrett *et al.* 2007) and stabilization (Wójciak-Stothard *et al.* 2001; Waschke *et al.* 2004). RhoGTPases can thus exert both positive and negative effects, depending on the circumstances they need to respond to. This dualistic behavior emphasizes the complexity of this regulatory machinery but also shows that, depending on time and location, they can accommodate different cellular needs.

I – Regulation of the endothelial barrier in resting endothelial cells

Despite the immense potential that RhoGTPases have as targets to control vascular leakage, initials attempts targeting key family members *in vivo* failed. This was mainly due to the fact that these proteins play central roles in the regulation of cell shape and function, which is reflected by their relatively broad tissue expression and the fact that they are highly evolutionary conserved (from plants, to yeast to mammals). Consequently, full knockouts are often lethal in early embryogenesis (Sugihara *et al.* 1998; Chen *et al.* 2000; Heasman & Ridley 2008; Pedersen & Brakebusch 2012). Therefore, given the relevance of these proteins and the fact that there are ca. 150 regulatory elements (GAPs, GEFs, GDIs) to consider, identifying new targets related to the regulation of RhoGTPase function is a priority. An alternative experimental route to study these proteins, prior to *in vivo* experiments and the development of conditional knockouts, is to artificially interfere with or block their signaling pathways *in vitro*. Therefore, we performed a loss of function screen of 270 RhoGTPases and -associated genes in primary endothelial cells described on **chapter 3**. Considering the high numbers of regulatory proteins (c.a. 150) involved in RhoGTPase signaling cascades, we expected to detect significant redundancy between proteins. And indeed, silencing most of these regulatory proteins did not affect the basal endothelial barrier. However, knock-down of several genes did affect the endothelial barrier significantly and interestingly

did so in opposite ways. Out of 270 RhoGTPase-associated genes screened, our data showed that six regulate the endothelial barrier negatively (ArhGAP45, SYDE1, RHOB, DEF6, PLXNB2 and DIAPH1) and four positively (TIAM2, CDC42, TEM4 and FARP1). Moreover, our findings showed that among the three different groups of regulatory proteins, RhoGAPs of Cdc42 and Rac1 showed a significant negative effect on the endothelial barrier (SYDE1 and ArhGAP45 respectively), RhoGEFs of Rac1 and Cdc42 were equally involved in both barrier enhancing and -disrupting effects (namely DEF6, TIAM2, FARP1) and RhoGDIs didn't show a significant effect on barrier integrity. Considering all 22 described RhoGTPases, RhoB was the only that reached the top three of significant hits in our screen. This RhoGTPase was recently implicated in the control of vascular function and shown to be essential for the TNF-α-mediated inflammatory response (Kroon *et al.* 2013; Marcos-Ramiro *et al.* 2016). Under hypoxic conditions, RhoB's expression in pulmonary microvascular endothelial cells was associated with decreased expression of VE-cadherin (Wojciak-Stothard *et al.* 2012) and later our group also confirmed that under resting conditions, decreasing RhoB expression in HUVECs induced VE-cadherin expression and increased barrier function (Amado-Azevedo *et al.* 2017; Pronk *et al.* 2017). Unexpectedly, loss of well-established regulators like RhoA or Rac1 did not induce significant changes in basal monolayer resistance under our screen conditions. Our group (Pronk *et al.* 2017) also showed that loss of RhoA has an effect on the expression levels of other closely related RhoGTPases. Specifically, we observed that loss of RhoA increases RhoC expression and also slightly RhoB which suggests a shift in balance among RhoGTPases. Such a shift in balance of activities is important to take into consideration when concluding on the relevance of a specific RhoGTPase, based on siRNA. Interestingly, a plexin (PLXNB2) – which is a cell surface receptor with a GAP domain (Rohm *et al.* 2000; Pascoe *et al.* 2015), proved to be an important negative regulator of the basal endothelial barrier. This might be explained by defining the targets of its GAP activity. We hypothesize that if loss of function of PLXNB2 results in increased barrier function, most probably barrier enhancer GTPases such as Rac1 and Cdc42 are the target. In line with this, studies performed on macrophages have shown that PLXNB2 is also a negative regulator of basal cell motility and wound healing by inactivating Rac1 and Cdc42 (Roney *et al.* 2011).

In addition to identifying TIAM2 as a novel regulator of endothelial integrity through its activation of Rac1 and ArhGAP45 as an inactivator, we found that Cdc42 and a number of its regulatory proteins (SYDE1-GAP, FARP1-GEF and PAK7-effector) play a prominent role in the maintenance of the endothelial barrier. These findings support the model of Cdc42 playing a critical role in the regulation of the basal endothelial barrier. Classically, Cdc42 has been associated with barrier restoration rather than with maintenance, which has been mainly attributed to Rac1 (Wójciak-Stothard *et al.* 2001; Kouklis *et al.*

2003; Kouklis *et al.* 2004; Broman *et al.* 2006; Waschke *et al.* 2006). However, and in line with our findings, *in vivo* studies have shown that activated Cdc42 counteracts RhoA activity and plays a crucial role in barrier integrity (Ramchandran *et al.* 2008). Moreover, the enhancement of barrier integrity and protection by loss of Cdc42 seems to occur at the level of interendothelial junctions. In fact, it was recently described that Cdc42 deficiency *in vivo* impairs EC function and regeneration in inflammatory pulmonary vascular diseases such as ALI/ARDS (Lv *et al.* 2018). This might indeed represent a chance of developing new therapies for vascular leakage and edema formation. In conclusion, we provided additional evidence for the critical role of Cdc42 in mediating barrier integrity and protection against inflammatory agents.

The top gene identified in our screen is a rather unknown RhoGAP. Our findings show that ArhGAP45, also known as human minor histocompatibility antigen 1 (HMHA-1), is an important negative regulator of endothelial integrity in unstimulated endothelial monolayers (**chapter 4**). Until recently, it was thought that expression of this protein was restricted to hematopoietic cells (de Bueger *et al.* 1992) and solid tumors (Klein *et al.* 2002). However, it was later observed that endothelial cells and placentas also express it (Holland *et al.* 2012; van Buul *et al.* 2014; Linscheid *et al.* 2015; Amado-Azevedo *et al.* 2018). Furthermore, besides encoding a GAP domain, ArhGAP45 also encodes a N-terminal Bin/Amphiphysin/Rvs (BAR) domain (Spierings *et al.* 2004). BAR domains are highly conserved structures in proteins and associated with membrane dynamics including endocytosis and vesicle transport (Frost *et al.* 2009). de Kreuk (de Kreuk *et al.* 2013) showed that full length ArhGAP45 is auto-inhibited and that it requires unfolding to be active and exert GAP activity. We reasoned that in resting conditions, part of the cellular pool of ArhGAP45 is activated either by growth factors in the culture medium (*in vitro*) or by circulating growth or inflammatory factors (*in vivo*). In HUVECs, reducing expression of ArhGAP45 resulted in an increased monolayer electrical resistance and migration in different types of endothelial cells. Our data indicated that in resting endothelial cells, the GAP-domain of ArhGAP45 acts to inactivate Rac1. This is in line with previous *in vitro* studies using Jurkat cells where a significant increase in Rac1 activity was detected after reducing the expression of ArhGAP45 by shRNA (de Kreuk *et al.* 2013). Increased Rac1 activity is associated with increased spreading and migration and accordingly, we found that HUVECs lacking ArhGAP45, migrate larger distances and heal wounds faster than control cells. Immunostainings of HUVECs lacking ArhGAP45, revealed an increase in cellular area accompanied by an accumulation of reticular junctions (honeycomb-like structures) containing VE-cadherin which is in line with an increased barrier function. Interestingly, studies in melanoma cell lines, where the protein was found to be aberrantly up-regulated, showed that reducing expression of ArhGAP45 induced apoptosis and reduced migration and invasion although GTPase

activity was not measured (Fujii *et al.* 2002; Xu *et al.* 2017). These findings suggest that functions of this protein are cell specific and that the up-regulation observed in cancer cell lines might be associated with aberrant localization in the cell, accompanied by a switch in its function. Our data showed that in resting endothelial cells, there is a continuous, negative regulation of Rac1 signaling by ArhGAP45.

Over the past 2 decades, evidence of Rac1 playing a critical role in different cellular mechanisms has accumulated (Olson *et al.* 1995; Tapon & Hall 1997; Sugihara *et al.* 1998; Wojciak-Stothard & Ridley 2002; Tan *et al.* 2008; Ferri *et al.* 2013; Liu *et al.* 2013; Timmerman *et al.* 2015; Schiattarella *et al.* 2018). Rac1 is thus essential for proper endothelial function and vascular development. With our study, we emphasized the importance Rac1 has for barrier maintenance and integrity. Moreover, we identified and characterized a novel and unexpected regulator of Rac1 activity that upon unfolding and release of auto-inhibition inactivates Rac1, leading to a decrease in barrier function, cell spreading and migration. We show evidence for the emerging role of Rac-GAPs in the regulation of barrier function and their feasibility to become potential drug targets.

Once active, RhoGTPases signal downstream to protein effectors. The mammalian formin diaphanous 1 (mDIA1) is a known RhoA effector and a potent actin- and microtubule polymerization factor. Formins are ubiquitously expressed and regulate a number of essential cellular functions including cell elongation, movement, polarity, and microtubule dynamics. Out of 76 effector proteins analyzed, mDIA1 showed to be an important contributor to the regulation of the endothelial barrier. In **chapter 5** we show preliminary data on the effect of silencing mDIA1 on the endothelial barrier. Knocking down mDIA1 in primary endothelial cells promoted basal endothelial barrier function and induced a disorganized actin network. Given the fact that mDIA1 is one of the downstream targets of active RhoA, reducing its expression increased barrier function and influenced the organization of the actin cytoskeleton. Moreover, our data also showed that silencing mDIA1 increased endothelial wound healing ability, which is in line with previous observations in epithelial cells (Chaturvedi *et al.* 2011) and dental pulp cells (Cheng *et al.* 2017). mDIA1 and Rho-Kinase (ROCK) are the two major downstream effectors of RhoA which cooperate in actin filament formation and -stabilization - mDIA1 promotes assembly of actin stress fibers that are further strengthened by ROCK-mediated myosin-II activation. Both effector proteins are thus involved in the reorganization of the cytoskeleton and generation of intracellular tension at junctions. Recently, it was shown that mDIA1 also senses and generates mechanical forces on actin filaments (Jegou *et al.* 2013). Furthermore, besides reducing the amount of actin fibers, knocking down mDIA1 was followed by a striking increase in vinculin levels. Like mDIA1, vinculin is an actin-binding protein and both proteins are involved in cell-matrix adhesion (Watanabe *et al.* 1999). In fact, it was shown that both Vinculin and

mDIA1 compete for actin filament barbed ends (Le Clainche *et al.* 2010) and this led us to hypothesize that reducing mDIA1 expression increases vinculin binding to F-actin but due to the chaotic cytoskeletal structure post-mDIA1 silencing, vinculin accumulates at perinuclear areas. Nevertheless, this finding remains to be further investigated.

Although mDIA1 is well-known RhoA effector, our study has contributed to add additional evidence of its cumulative function in contributing to the regulation of the endothelial barrier at rest. In agreement with our findings, it was recently shown that mDIA1 is a crucial mediator of induced hyperpermeability in response to advanced glycation end products, which is known to contribute to vasculopathy associated with diabetes mellitus (Zhou *et al.* 2018). Our findings indicate that mDia1 is an important, negative regulator of endothelial integrity and migration.

The systematic loss-of-function screen which we performed in resting endothelial cells aimed to improve our understanding of the relevant mechanisms and to identify novel molecular players in the control of endothelial integrity. Once identified and fully characterized, these novel players might become potential drug targets and part of new pharmacological therapies to treat vascular leakage. By performing such a large-scale screen and analyzing all relevant proteins at once, we were able to extend the current, canonical models of endothelial barrier regulation – the RhoGTPases Rac1 is the key player for endothelial barrier maintenance and stabilization. Our findings show that Cdc42 plays an equal or even more pronounced role in the positive regulation of endothelial barrier, an idea slightly abandoned and confined to barrier restoration by the experts in the field. Moreover, based on our observations and in agreement with recent studies, RhoB is an important regulator of barrier function, being the most negative regulator among all RhoGTPases.

Our observations also emphasize the importance of regulatory proteins such as GAPs and/or GEFs as targets for the development of new therapies, based on the fact that just simply deleting these highly conserved RhoGTPases results in early lethality.

II – Regulation of the endothelial barrier under inflammatory conditions

It is well known that activation of endothelial cells by inflammatory mediators results in disassembly of intercellular junctions, acto-myosin contraction and leakage. Besides analyzing the contribution of RhoGTPases and associated genes in resting endothelial cells, we also analyzed the effect of agonist-induced loss and recovery of endothelial cell-cell contacts (**chapter 6**). To do so, we used the same loss-of-function approach described above but, in this case, stimulated the monolayers with the serine protease thrombin. This resulted in a transient activation of the endothelium, induced contraction and disassembly of cellular junctions followed by a recovery phase.

The mechanism through which thrombin induces actomyosin contraction and

permeability is well described (Essler *et al.* 1998; O'Brien *et al.* 2000; van Nieuw Amerongen *et al.* 2000). In addition to stimulating Ca^{2+} influx, thrombin also activates the RhoGTPase RhoA which signals downstream to ROCK, mediates a prolonged phosphorylation of MLC causing disassembly of interendothelial junctions. Beyond this pathway and several reported protein activation/relocalization/translocation events (Vogel *et al.* 2000; Reuther *et al.* 2001; van Nieuw Amerongen & van Hinsbergh 2001; Birukova *et al.* 2006; Beckers *et al.* 2008; Takeya *et al.* 2008; Minshall *et al.* 2010; Ritchie *et al.* 2013) knowledge about the contribution of other RhoGTPases and -associated proteins is rather limited. Exceptions are Cdc42 and Rap1 that have been widely studied for restoration of the barrier purposes (Wójciak-Stothard *et al.* 2001; Kouklis *et al.* 2004; Pannekoek *et al.* 2011; Birukova *et al.* 2013).

Our analysis identified 15 different proteins that play a role in the thrombin-induced response of endothelial cells (RTKN, TIAM2, MLC1, ARPCB1, SEPT2, SLC9A3R1, RACGAP1, RAPGEF2, RHOD, PREX1, ARHGEF7, PLXNB2, ARHGAP45, SRGAP2, ARHGEF5). Loss of these 15 genes resulted in an increased contraction or a delayed/impaired recovery post-thrombin stimulation. The distribution of these 15 hits by class of proteins was the following: 1 RhoGTPase (RhoD), five GEFs, of which four are related to Rac1 (ArhGEF5, P-REX1, ArhGEF7 and TIAM2) and one to Rap1(RapGEF2), three GAPs, all of which are related to Rac1 (ArhGAP45, RacGAP1, SRGAP2), five effector proteins (ARPCB1, MLC1, RTKN, SEPT2 and SLC9A3R1) and no RhoGDIs were identified. Interestingly, based on available literature, most of the identified hits are related to Rac1. Loss of the described 4 RacGEFs points to the crucial role active Rac1 has in counteracting permeability-increasing effects of RhoA and the same rationale can be applied to RapGEF2 regulating Rap1. Now, regarding the 3 RacGAPs identified, our data shows that reducing their expression increases basal barrier resistance which demonstrates that once they are unable to inactivate Rac1, barrier integrity improves. Interestingly, this property does not attenuate thrombin-induced decrease in electric resistance or an improved recovery when compared to the controls. The mechanistic explanation for this is unknown, although our previous work indicated that the imbalance between Rac1 vs RhoA-induced signaling, which results from GAP-mediated downregulation of GTPase activity, may impair restoration of cell-cell contacts.

Surprisingly, the atypical RhoGTpase RhoD seems to play a barrier-protective role. RhoD has been shown to be a regulator of endosome dynamics but also plays a role in actin cytoskeletal dynamics including filopodia (Aspenstrom *et al.* 2004; Blom *et al.* 2017). Studies in fibroblasts have shown that RhoD depletion altered the organization of the cytoskeleton and affected migration rates (Blom *et al.* 2017). Our findings show that reducing RhoD expression reduces basal barrier resistance and augments thrombin-induced contraction and impairs the subsequent recovery. These observations suggest

a novel concept regarding RhoGTPases and the regulation of the endothelial barrier: RhoD, more strongly than Rac1 plays an essential role in the maintenance and protection of the endothelial barrier under inflammatory conditions.

The traditional role given to Rac1 and Cdc42 as the pivotal players in endothelial stabilization and thrombin-induced barrier restoration is now challenged by our findings (Wójciak-Stothard *et al.* 2001; Vouret-Craviari *et al.* 2002; Price *et al.* 2003; Waschke *et al.* 2006; Baumer *et al.* 2008). Nevertheless, our observations do provide additional evidence to the maintenance of barrier integrity by Rac1 regulatory proteins. Our group has provided evidence that influencing the expression of a given RhoGTPase might impact the expression level of others (Pronk *et al.* 2017). This was the case with the RhoABC GTPases but we cannot exclude the same happens with Rac1 or Cdc42 related proteins. Despite not reaching statistical significance in our analysis, loss of RhoA has shown a small improvement in basal barrier resistance levels and attenuation of thrombin-induced contraction as expected. Notwithstanding, its direct and well-known regulatory proteins, namely the RhoGEFs GEF-H1 and LARG, and the $G\alpha_{12}$ (GNA12) subunit showed an exuberant response upon their knock-down, attenuating dramatically the thrombin-induced contraction and improving recovery. These findings, although not statistically significant, are biologically very relevant and show that our experimental conditions could fully recapitulate the RhoA pathway induced by thrombin stimulation (Birukova *et al.* 2006; Kitzing *et al.* 2007; Siehler 2009; Mikelis *et al.* 2013). In conclusion, our findings provide new insights on novel regulators that play a key role in thrombin-induced contraction or the subsequent junctional recovery phase. Future research may reveal if any of the identified proteins represents potentially new targets for treatments aimed at preserving vascular integrity.

Collectively, our findings elucidate novel key regulators of endothelial integrity under basal and inflammatory conditions. In agreement with the last two decades of research, our findings underline that RhoGTPases are key players in the regulation of vascular function. A novel aspect made evident by our observations is that RhoGTPases differentially regulate barrier function under basal vs. inflammatory conditions. The proteins involved in basal regulation are not necessarily the same regulating the inflammatory response. Rac1 and Cdc42 are essential for barrier integrity maintenance and RhoB is a negative regulator. Upon inflammation, Rac1 and RhoD seem to be essential for attenuation of contraction and recovery. RhoA's role as a negative regulator or enhancer of contraction was confirmed by its associated GEFs and Gα protein. Overall, our study identified known and novel regulators of endothelial barrier function. Targeting some of the described regulators might improve basal barrier maintenance and might attenuate stimulus-induced hyperpermeability. This novel information thus may contribute to the development of new therapies to treat vascular leakage and

edema. To summarize in one image the main findings of our dual screen, we grouped the two conditions we analyzed and highlighted the main findings: regulation of basal barrier integrity and endothelial response to an inflammatory mediator (Fig.1)

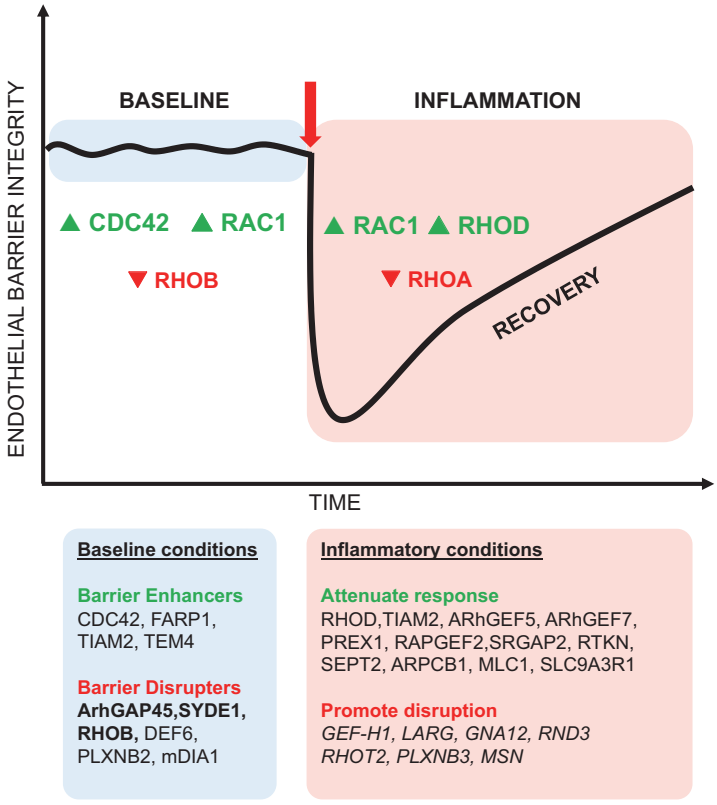

Figure 1 – Schematic overview of the main regulators identified in the siRNA screen including baseline and inflammatory conditions.

Basal endothelial barrier integrity is mainly regulated by Cdc42 and Rac1 associated proteins. RhoB was identified as a negative regulator of basal endothelial barrier integrity. In the presence of inflammatory mediators such as thrombin the majority of regulators identified that attenuate thrombin-induced contraction are related to Rac1 and RhoD.

Limitations of the study

We sought to increase the knowledge and insights on the regulation of the endothelial barrier as the main regulator of vascular leakage. To do so, we performed an extensive loss-of function screen on primary vascular endothelial cells freshly isolated from veins of umbilical cords. These cells are very well characterized (first isolated and

cultured in the 1970's (Jaffe *et al.* 1973)) and are easy to obtain. They are also easy to culture, highly proliferative and migratory and the data obtained is very reproducible, making them the major source of primary endothelial cells for many years in many labs. Nevertheless, and despite being a good model, these cells might not represent the actual *in vivo* conditions. They are derived from fetal tissue and may differ from adult vascular endothelium in terms of markers and proteins expression. Moreover, HUVECs are not fully representative of the typical endothelial cells that are most affected by vascular leakage *in vivo*, i.e. the endothelial cells in post-capillary venules (Majno *et al.* 1961; Al-Naemi & Baldwin 1999).

Using HUVECs as an *in vitro* model is currently the closest approximation to the *in vivo* situation. To use other types of endothelial cell, it is necessary to work with *ex vivo* material obtained from biopsies or autopsies, which are not readily available. Moreover, these endothelial cells generally do not grow very efficient *in vitro*. Recently, however, specialized companies offer (tissue-specific) endothelial cells that are derived from micro- and macrovasculature. The increasing availability of such cells might allow complementary, organ-specific studies.

The most common *in vitro* methods to measure barrier properties of endothelial cells are macromolecular permeability across the endothelium (with a Transwell assay) and electrical impedance of endothelial cells grown on top of gold electrodes. Both techniques, considered 'golden standards', have been widely used and despite their technical differences, both have advantages and limitations (Bischoff *et al.* 2016). In the loss-of-function screen described in this thesis, measurement of barrier function was done by electrical impedance measurements. It's a widely used method for barrier function studies and provides reliable and reproducible data which is comparable to other methods – macromolecule passage, TEER and Real Time Cell Analysis (RTCA; xCELLligence) (Aman *et al.* 2012; Szulcek *et al.* 2013; Szulcek *et al.* 2014; Beckers *et al.* 2015; Timmerman *et al.* 2015; Pronk *et al.* 2017; Hilfenhaus *et al.* 2018; Robinson *et al.* 2018). Measurement of electrical impedance in real time is a good method for screening cell properties and behavior in basal conditions or under stimulation (Mitra *et al.* 1991; Giaever & Keese 1993; Szulcek *et al.* 2014; Stolwijk *et al.* 2015). Cells are grown to confluence and kept in standard culture conditions. Due to the small alternating current used in the system, the impedance measurement is non-invasive. From one single measurement, different parameters including strength of cell-cell contacts and cell-matrix interaction can be calculated using a mathematical model. Furthermore, this system also allows proliferation profiles and evaluation of wound healing. There are, though, certain limitations that one must consider while using ECIS. Changes in pH, temperature, humidity and even changing medium have a direct effect on the impedance signal. It requires a confluent cell layer and in fact, the signal provided is

an average from cells located on the sensing electrode, thus not allowing single cell-analysis. Moreover, only passive electrical properties can be measured. As mentioned above, cellular properties such as strength of cell-cell contacts or cell-matrix interactions and membrane capacitance are the key features of this system yet no information at the molecular level can be obtained. The ECIS system is thus an excellent tool for screening purposes as well as for initial tests on cell properties and behavior and responses to stimuli.

To study the contribution of GTPases and associated genes to the regulation of endothelial barrier function we performed a systematic siRNA screen on primary endothelial cells. RNAi technology has presented researchers with an opportunity to study and gain valuable insights into functional genomics through phenotypic alterations. It has become a widely used technique for target discovery, validation and therapeutic development (Bhinder *et al.* 2014). The technique uses a synthetic RNA duplex designed to specifically target a particular mRNA molecule for degradation. It induces transient silencing or knockdown (2-4 days) of protein expression. However, as with any technology, there are some pitfalls to consider. The major one is the potential for any given siRNA to affect other genes than the intended ones (so-called off-target effects). To ensure on-target activity, several strategies have been developed including chemical modifications of the siRNA, improved design algorithms and the pooling of independent siRNAs that target an individual gene (Parsons *et al.* 2009; Bhinder *et al.* 2014; Hannus *et al.* 2014). Moreover, optimization experiments are usually carried out before the screen to ensure e.g. correct concentrations, time-points and knock-down efficiency. For such a screen, as performed for this project, or whole genome screens, knockdown efficiency of all the targets is not performed. Instead, during the optimization phase, a few siRNAs (usually the positive and negative controls, between 2 and 10 genes) are tested and evaluated. Once the conditions that promote an average knock-down efficiency of >75% for all the controls are identified, the transfection protocol is set, based on the assumption that (on average) all siRNAs in the screen will behave similarly. Of course, this is dependent on endogenous expression levels of the protein and its turnover. During the actual screen experiments, the controls used in the optimization phase are included and tested every time. Nevertheless, all these strategies cannot rule out off-target effects and therefore, validation experiments have to be performed to assure quality of the data.

We repeated our screen three times and used different pools of primary HUVECs every time, with cells from 12 different donors to reduce possible individual variations of cell phenotype and behavior. This approach allowed us to use primary cells at passage 2 instead of a using a cell line. Moreover, when analyzing the data of the 3 screens its distribution was considered normal and thus no special normalization was done. The

normalization done was intended to compared the knock-down effects observed with negative and positive controls used. The variability observed inter-screen was small and pool(of cells) dependent.

(Primary) HUVECs are still considered a hard-to-transfect cell type and for a certain period, nucleofection was the only option available to silence gene of interest. With the development of new tools and reagents for siRNA delivery, it became easier to transfect these cells. Looking back at 2012, the only other option available besides siRNA was the use of shRNAs. The delivery systems of these RNA duplexes are different and lab requirements too as for shRNA transfections access to a ML-II facility for lab work is necessary. At the time, this was not directly available and thus siRNAs technology was the chosen approach. All in all, it worked really well, the algorithms developed for siRNA design seem to be very on-target and the data obtained is very sound and reproducible.

Our initial aims and ultimate findings contribute to a better understanding of RhoGTPase signaling in endothelial barrier regulation and the roles of individual RhoGTPase (-associated) proteins in basal and inflammatory conditions. Our findings provided the scientific community with a robust list of novel and known regulators that may form a promising approach to the development of new therapies to treat patients with vascular leakage in the future.

Besides RhoGTPases there are other interesting candidates being studied in the field of vascular leakage. The tyrosine kinase Imatinib was shown to reduce vascular leakage in septic mice (Aman *et al.* 2012); the synthetic Tie2 against peptide Vasculotide was shown to also protect against vascular leakage and to reduce mortality in a sepsis murine model (Kumpers *et al.* 2011); a new type of metal complex (nonoates) was shown to participate in the regulation of vascular permeability by controlling NO release *in vitro* (Monti *et al.* 2014); the atrial natriuretic peptide (ANP) was shown to reduce endothelial leakage through a GEF-H1 dependent mechanism (Tian *et al.* 2014), targeting the B1-integrin with antibodies was shown to decrease LPS-induced vascular leakage in murine endotoxemia (Hakanpaa *et al.* 2018); and intermedin, a member of the calcitonin family, was shown to decrease vascular leakage and inflammatory responses in sepsis murine models (Xiao *et al.* 2018). Although additional work is necessary, some of these candidates hold great promise as an effective intervention for vascular leakage. Unfortunately, most of them are still confined to the lab and in between *in vitro* and murine work but the development of pharmacologic inhibitors and sequent translation to the clinic is of utmost urgency.

Future Perspectives

For the past decades, there has been a lot of enthusiasm about how genetics would change drug discovery and consequent applications in medicine. This enthusiasm, based on the assumption that the main contributor of a cellular phenotype in a given cell is its complex combination of gene expression, has slowly faded (Dugger *et al.* 2018). The array of potential targets discovered using the 'gene-to-screen' has had poor translation to clinical efficacious drugs (Hodson 2016). The quick evolution of technologies and bioinformatics tools has contributed to a new discipline that will transform the world of drug discovery and clinical use – precision medicine.

Understanding and targeting the cause of disease in individual patients is the core of precision medicine. This approach provides new insights about the basic biology and pathogenesis of the disease and the development of medicine will then target patients that most likely will benefit – a transition from the classic 'one-treatment-fits-most' to targeted treatments that fit individual patients or small groups of patients (Shin *et al.* 2017): the right patient gets the right therapy at the right dose and experiences the right response. The oncology field has been a pioneer in this area and new therapies are now being designed to more precisely target cancer cells: 1) pathway-based therapies that target essential signaling pathways necessary for cancer survival or growth and 2) immunotherapy, by artificially modulating patients' immune systems to generate a response against cancer cells (Dugger *et al.* 2018). It is now quite common to read about the programmed-cell death protein 1 (PD1) check-points inhibitors or the chimeric antigen receptor modified T-cells (CAR-T). However, there is a critical need for predictive biomarkers to help identify the patients most likely to benefit from these novel therapies, establishment of standards that report high degree of evidence and reproducibility (Kimmelman & Tannock 2018) and techniques such as next-generation sequencing and CRISPR-Cas can be extremely useful. Furthermore, other fields of medicine are in the spotlight with precision medicine including genetic diseases such as epilepsy (Epi 2015; Lindhout 2015) and also Alzheimer's disease (Isaacson *et al.* 2017), pulmonary vascular disease (Newman *et al.* 2017) or acute diseases such as sepsis (Rello *et al.* 2018). Considering how important the cardiovascular system is for health and disease, precision medicine also has an emerging role for cardiovascular disease (Leopold & Loscalzo 2018). Focusing on genetic testing and - therapeutics, the goal of this strategy is to give patients more efficient and effective care but also to reduce harm and care costs (Dainis & Ashley 2018).

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