Chapter VII

Discussion and Conclusion

Discussion

In general, microvascular disease is the result of the chronic exposure of the microvasculature to adverse metabolic and hemodynamic risk factors such as hypertension, hyperglycemia or the uremic toxins associated with renal failure. Progressive loss of tissue capillaries can continue for years without clinical manifestations until tissue-ischemia and consequent fibrosis reaches a threshold after which loss of organ function is manifested, making loss of microvascular integrity a main driver of morbidity.

Microvascular disease has an unmet clinical need for new therapeutics, with few to no therapies available yet. So far, researchers have mainly focused on using conventional cell culture systems or animal models to study microvascular diseases. However, conventional cell culture systems lack sufficient complexity to assess the functionality of the microvascular system and animal models often do not accurately mimic human pathophysiology. For these reasons, there is a broad need for alternative ways to model microvascular diseases *in vitro* to study disease mechanisms and to develop interventions to modulate or stop them. This thesis is focused on the development of microfluidic cell culture devices that mimic the microarchitecture and functions of human microvessels, using so called 'Organs-on-Chips'. These devices can host and combine different human cell and tissue types to study molecular- and cellular-scale activities that underlie the human blood vessels and specific disease states. Moreover, it gives the ability to identify and study new therapeutic targets *in vitro* [1-4]. Therefore, this platform offers an alternative way to study microvascular diseases that promotes personalized medicine by cultivating human microvessels that overcome key limitations in the fields of tissue engineering, vascular biology, immunometabolism and drug discovery. It serves as a 3D cell culture system that overcomes several shortcomings of 2D cell culture such as direct contact of cells with plastic, lack of perfusion and culturing cells for a short period [5]. Organs-on-Chips are now in the forefront of biotechnology with a promising future to truly mimic the human physiology and diseases. The research question discussed in this thesis:

How can we use a microfluidic 3D cell culture platform to develop a reliable robust and physiological relevant 'microvessel-on-a-chip' platform, that may serve as an attractive and versatile replacement for a significant fraction of animal models in vascular homeostasis?

Development and applications perspective

In **Chapter II** we reviewed the recent developments in the organ-on-a-chip field. Elegant platforms have been reported that cover almost all human organs, including the lung, intestine, kidney, skin, bone marrow, brain and vasculature, among others. Organs-on-Chips have developed from an academic research concepts to commercially available products. End-Users are interested in using these devices for functional and biochemical assays in the lab. Unfortunately, none of the published organ-on-a-chip models fully satisfy end-user usability requirements including assay compatibility, ease of handling and throughput. Furthermore, the majority of available systems are single chip-based models, with external pumping and use immunohistochemical stains as primary read-out. This severely limits the sensitivity, throughput and scalability.

In order to provide a more robust system, it is of utmost importance for engineers to make sure that their products are easy to use, high-throughput, simple to operate microfluidic platform for robust screening assay and compatible with other equipment in the lab, including liquid handlers and high-content imaging systems. And this should also require the availability of proper molecular read-outs including omics such as metabolomics to answer clinical questions.

We predict that in the coming years the organ-on-a-chip industry will become a competitive market, comparable to the smartphone industry, where third parties will review the products of organs-on-chips industries based on criteria's that meets the end-user needs. Such criteria's could include design, technology and integration [6]. We look forward in seeing this new era of competition and strategy and the impact it might have on drug research.

Mimicking microvascular disease using patients' plasma

In **chapter III** we used the organ-on-a-chip technology of MIMETAS, the OrganoPlate, to create miniaturized microvessels. For this device, we designed a novel chip structure, T-junction, that enables easy generation of 96 leak-tight microvessels and we aimed to use the platform to screen vascular destabilization factors in blood. The chip also allows endothelial cells to grow on soft substrate with a modifiable physiological stiffness. More importantly, we can measure microvessel permeability in real-time that allows the phenotypic screening of compounds.

Exposure of the microvessels to permeability factors such as TNFα, thrombin and VEGF, reconstituted vascular leakage of microvascular destabilization.

Next to that, we developed a method to prepare human plasma samples from whole blood and perfuse them through the microvessels-on-chips. To prevent coagulation for the isolation and storage of plasma samples, blood was decalcified by EDTA. As this tetravalent molecule leads to a loss of intercellular contact, we performed detailed studies to inhibit complement activation, thrombin and the intrinsic pathway of the coagulation cascade. This allowed recalcification of the plasma samples while retaining the morphology of healthy microvessels. Furthermore, the microvessels perfused with human blood plasma were as leak-tight as those with culture medium. This changed when the plasma samples were spiked with factors destabilizing the microvasulature, thereby inducing vascular leakage. We also compared our results with TEER measurements carried out in the Electric Cell-substrate Impedance Sensing (ECIS). This is a real-time, label-free and impedance-based method to study the activities of cells cultured in 2D. TEER measurements with the ECIS were significantly influenced by biological and environmental factors and 2D cell culture, thereby making the endothelial cells very sensitive to cytokines and growth factors. On the contrary, our microfluidic platform showed greater resemblance to *in vivo* tissue-like physiological responses, because it is an integrated system with excellent control and standardization of prepared 3D microvessels.

Our results indicate the possibility of using the microvessels-on-chips to study the mechanism behind microvascular destabilization. Importantly, we demonstrated the resemblance of the microvascular structure *in vitro* and the diffusion of albumin, thus representing the human microvessels. The microvessels-on-chips will be a tool to discover the effect of circulatory factors in patient blood samples on microvascular destabilization and to identify endothelial factors released by the destabilizing microvessels. In our current system, the microchambers contain two phaseguide-defined lanes. These allow cell-cell contact, cell-ECM contact and diffusive transport of molecules from the microvessel to the ECM. Using collagen gel in one lane and creating monolayer of cultured endothelial cells in the other lane, we can generate series of perfusable microvessels that can be used to model certain cellular aspects of microvascular destabilization as it is occurring *in vivo*. Our system represents a unique platform to study the pathophysiology of microvascular diseases in a manner that, differently from previously reported 2D models, allows to study changes in 3D conformation of

endothelial cells and abnormalities in their function. Moreover, the strong association between functional data in the microvessels-on-chips and *in vivo* microvessels parameters indicates that this system may be used as a platform to identify new biomarkers of microvascular destabilization in response to various stimuli and to test toxicity of new drug compounds.

In drug research, there is a big need for robust human *in vitro* models as animal models can differ from humans and translation of results from animal models to humans with regards to efficacy and safety are often a challenge. In addition, by using *in vitro* models, the human variability in genotypes and phenotypes can be taken into account. In this endeavor, the evidence from our study suggest that the microvessels-on-chips platform will have a major beneficial impact. Our microvessels-on-chips platform is especially suitable for detailed parallel optical interrogation of endothelial barrier function and inflammatory responses. Since the platform integrates both perfusion and the generation of biomolecular gradients, the microvessels-on-chips can be used as a tool to study angiogenesis *in vivo* [7]. The platform may have wide applications in toxicology as, e.g. an off-target effect of a drug candidate on microvascular integrity can be measured by high-throughput image analyses of phenotypic alterations. In addition, perfusing microvessels with human plasma samples is a valuable tool to identify disease-associated circulating plasma factors that cause microvascular destabilization and help diagnosis and clinical management of patients at risk for microvascular disease related complications, such as neurodegenerative diseases [8]. These findings can then be translated to patients and back.

We acknowledge that the microvessel-on-a-chip platform has some limitations, including the fact that it does not include pericytes yet. To develop a full picture of the microvasculature, additional studies will be needed in creating a co-culture of endothelial cells and pericytes. This will for example enable us to assess the functional properties of human pericyte-EC interactions in patients with constitutive endothelial Ang-2 release and patients with haploinsufficiency for the RNA-binding protein Quaking [9].

To explore the application limits of the microvessels-on-chips for further approaches in personalized medicine, future work should focus on developing patient's microvessels by using blood outgrowth endothelial cells (BOECs) or induced pluripotent stem cells (iPSCs) derived endothelial cells and pericytes. Most importantly, the derivation of cell lines from

patients suffering from microvascular diseases guarantees representation of disease heterogeneity in real-time in a dynamic system, which is not possible for example in animal studies. As several of the target diseases, for example heart failure with preserved ejection fraction (HFpEF), are significantly more frequent in woman and therefore sex-biased, our approach can also be used to explore gender-specific mechanisms [10].

Efficacy testing and drug discovery

Chapter IV describes the use of the microvessels-on-chips to recapitulate the hemorrhagic fever in Ebola disease. This disease is a major health threat for which we lack predictive tools. The Ebola virus causes vascular integrity impairment with subsequent blood loss, leading to fatal disseminated coagulopathies and hemorrhagic shock. We showed that perfusion of the Ebola VLPs induces vascular leakage in our system. The Ebola $GP_{1,2}$ is the perpetrator in this process by perturbing the Rho/Rock pathway, causing changes in cellular mechanics. This hemorrhagic shock is treated with the Ebola disease drug candidates, melatonin and FX06.

Not all Ebola patients have signs of hemorrhagic fever with bleeding from the internal organs. Previous mouse studies have established that genetic background determines susceptibility to Ebola hemorrhagic fever [11]. Little is known about the heterogeneity in Ebola disease progression in human. This increases the death rate of vulnerable subjects and promotes Ebola virus disease to cause another global crisis. The microvessel-on-a-chip platform can serve as a chip-based diagnostic tool to quantify the severity of the Ebola disease, to diagnose or predict susceptibility at an early stage and assess the risk for healthy subjects. This can be achieved by perfusing blood plasma samples of Ebola patients to understand the dynamic in the disease progression. With this, more efficient health management strategies against Ebola can be developed.

Metabolomics as phenotypic read-out of organ-on-a-chip

The physiological read-out of endothelial function is a critical aspect in using microvessels-on-chips for disease and drug research. Metabolomics may deliver a more informative and sensitive read-out compared to relying only on morphological changes obtained with imaging analyses of phenotypic changes. It is a sensitive and robust read-out to identify metabolic markers of small molecule exposure in cells in diseased state. These properties are relevant for testing metabolic dose-response in pharmaceutical interventions and study the effect of metabolites that reflect microvascular destabilization [12].

In **Chapter V** we present an approach on using targeted liquid chromatography–tandem mass spectrometry (LC-MS/MS) metabolomics to quantify bioactive lipids that drive systemic inflammation in the microvessels, such as prostaglandins, isoprostanes, lysophosphatidic acid (LPA) classes, sphingolipids and platelet activating factor (PAF). *In vitro* studies of these bioactive lipids have been based on 2D endothelial cell cultures that, due to lack of laminar flow and the growth of the cells on plastics, often display a pro-inflammatory phenotype. Since, the microvessel-on-a-chip model resembles the physiological environment, we optimized targeted LC-MS/MS measurements of a panel of pro- and anti-inflammatory bioactive lipids to generate expression profiles both in TNFα treated 3D microvessels as well as in 2D endothelial cell cultures. Although we were dealing with very small samples and low concentration of metabolites in the microvessels-on-chips, we were able to measure bioactive lipids secreted by the endothelial cells. Furthermore, they displayed a less inflammatory response to TNFα compared to classical 2D endothelial cell cultures. This study demonstrates the possibility to use the microvessels-on-chips to study disease mechanisms, validate drug effects and drug-drug interactions, a major unmet need for research in vascular biology [13]. This allows pharmaceutical companies to reevaluate drugs, investigate on- and off-target effects of drugs that failed to meet their primary effectiveness in phase III clinical trials and repurpose them for the treatment of other diseases in an efficient and cost-effective way [14].

We have combined our microvessels-on-chips with established metabolomics platform to identify novel cellular factors that are released from destabilizing microvessels. These factors may serve as potential biomarkers *in vivo* and help to dissect cellular pathways of microvascular destabilization. We envision that in the future with metabolomics and the microvessels-on-chips we will be able to study the flux of molecules that maintain vascular

homeostasis, such as nitric oxide (NO) and glucose, in a more physiologically relevant setting. Another emerging direction promising for the microvessels-on-chips is to use single-cell metabolomics [15]. The study will give insights into the heterogeneity of cell-cell and cell-ECM interactions. These differences might have important consequences in the discovery of new biomarkers for microvascular destabilization. However, single-cell metabolomics is still in its infancy. Works still need to be done in improving the sensitivity, developing innovative cell-sampling techniques and providing robust data-processing and normalization methods.

Automated microfluidic cell culture and control of system parameters

Although a wide variety of human microvessels have been created with MIMETAS OrganoPlate, there are limited efforts so far on the integration of a pumping system and real-time oxygen sensor. A pumping system is critical for introducing controlled laminar shear stress to endothelial cells, since their response to physiological flow dynamics affects vascular health [16, 17]. Additionally, *in situ* continual measuring of oxygen is essential in precise assessment of the microenvironment and the dynamic responses of the microvessels to drugs over extended periods of time. In **Chapter VI**, we report a microfluidic pumping and oxygen sensor platform, which we developed in our lab. The platform is high-throughput and operates in an automated manner. The system was used to study the effect of static condition, bidirectional flow with the MIMETAS Rocker and unidirectional flow with our pumping system on the cytoskeleton remodeling and gene expression of the microvessels-on-chips. Our results clearly demonstrated that the laminar flow introduced with the microfluidic pumping system brings the microvessels to a more physiological state, while maintaining a stable oxygen level in the enclosed microfluidic system.

There is a growing need to automate *in vitro* experiments for improvement in accuracy, throughput and safety. Manual handling of the microvessels-on-chips greatly effects the long-term maintenance and monitoring of cells. This might become a barrier for the high-throughput and robustness of the system. In many organs-on-chips studies, the integration of laboratory automation technology has therefore been proposed [18, 19]. This system primarily consists of a liquid-handler and a robot for transposition of receptacles, allowing automated cell culture and monitor. Moreover, it accurately handles micro-scale fluid

volumes in a rapid speed, decreasing the temporal lag in extracellular response to molecules transported across cell membranes in the organs-on-chips. Therefore, the integration of robotic handling in the microvessels-on-chips will be of great value, because it will further improve the reproducibility, parallelization and longitudinal observations of the microvessels.

For further developments, the microfluidic pumping and oxygen sensor platform can be flexibly adapted to an automated cell culture platform. Together, they will be a powerful tool to enable automated cell culture, perfusion, medium addition, fluidic linking, sample collection and *in situ* monitor of cells, within a controlled temperature and oxygen environment. This should facilitate the studies of drug pharmacokinetics and pharmacodynamics (PK/PD) for an extended period of time *in vitro* [18].

Conclusion

In vitro modeling the vasculature can provide molecular and cellular insights to understand the biochemical and biophysical mechanisms of the human vascular system. The current gold standard for this are animal models and 2D cell culture. Unfortunately, these systems are significantly limited in recapitulating the human pathophysiology. Organs-on-Chips can provide a unique solution to mimic the vascular function, however, these micro-physiological systems are still in proof-of-concept stage and need further optimization to really aid in diagnosing and treating microvascular diseases. In order to utilize the full potential of the organs-on-chips for recapitulating the critical aspects of microvascular diseases, the following research question was discussed:

How can we use a microfluidic 3D cell culture platform to develop a reliable robust and physiological relevant 'microvessel-on-a-chip' platform, that may serve as an attractive and versatile replacement for a significant fraction of animal models in vascular homeostasis?

To answer this question, this thesis was focused on:

1) The assessment of the usability, compatibility and assay ability of the organ-on-a-chip in the light of end-user adoption aspects.

2) Methods to screen blood samples for the presence of inflammatory, leakage or angiogenic factors in a perfusable high-throughput microvessel-on-a-chip platform and translation to the clinic.

3) The use of the microvessel-on-a-chip platform for studying mechanisms in the vascular pathophysiology associated with Ebola virus disease and therapeutic interventions to suppress Ebola-induced vascular destabilization.

4) The application of metabolomics as a molecular read-out in the microvessels to identify biomarkers for microvascular diseases.

5) The development of a high-throughput microfluidic pump and *in situ* oxygen monitoring for the microvessel-on-a-chip platform in order to make it more physiological relevant.

The microvessel-on-a-chip platform represents a transformative system that can serve as an effective replacement and for some applications, improvement over the current animal model and 2D cell culture system for microvascular destabilization. The next step now is to further validate this system by using it to screen blood samples in a large clinical study on microvascular diseases. This will provide large number of samples from patients that can be analyzed in the microvessel-on-a-chip platform under various therapeutic conditions. The findings observed can then be translated back to patients to predict clinical outcome of treatments and at the same time evaluate the effectiveness of the system for speeding up drug research. We are convinced that the coming years the microengineered microvessels will have a major impact on society by accelerating the development of new drugs and advancing personalized medicine.

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