Modeling vascular diseases using human induced pluripotent stem cells

Cao, X.

Chapter 7

Discussion and Future Perspectives

GENERAL DISCUSSION

In depth analysis of endothelial cells (ECs) and myeloid cells differentiated from human induced pluripotent stem cells (hiPSCs) were shown in this thesis to be very similar to tissue derived equivalents *in vivo*. They thus provided an essential tool to study their biology and various vascular and inflammatory diseases that are caused by their dysfunction. In this thesis, we first established an efficient protocol to derive fully functional monocytes and different subtypes of macrophages from hiPSCs in order to provide an inflammatory component for modelling of vascular diseases *in vitro*. Next, we characterized the developmental path of hiPSC-derived ECs (hiPSC-ECs) using an *ETV2*^{mCherry} reporter hiPSC line which we had generated earlier using Crispr-Cas9 gene targeting. These hiPSC-derived cells were then used to model two vascular diseases Hereditary Hemorrhagic Telangiectasia type 1 (HHT1) and Pseudomyogenic hemangioendothelioma (PHE) *in vitro*.

A 3D vasculature model in an Organ-on-Chip microfluidic format was established using hiPSC-derived cells. The cells as necessary in combination with the Organ on Chip model were then used to study two disease pathologies: HHT1, which is characterized by weak vessel walls in patients and a strong tendency to hemorrhage, and a vascular tumor, PHE, caused by translocation in the SERPNE1-FOSB genes and characterized by malignant behaviour of vascular cells for which the tumor cell of origin was unknown. Work in this thesis generated an *in vitro* model for HHT1 as well as for the vascular tumor PHE; these models provided valuable tools to investigate underlying pathologies of human vascular diseases and contributed to moving this area of research forward.

hiPSC-derived monocytes and macrophages

Human peripheral blood mononuclear cells (PBMCs) are widely used as a source of human monocytes for biomedical research. However, their availabilities are strictly limited by human tissue donation regulations and cell batches vary from different donors which restricts for their application in disease modeling *in vitro*. IPSDMs as described in this thesis represent an unlimited source of patient specific tissue resident macrophages-like cells which could be incorporated into *in vitro* disease models and organoid cultures. Previous differentiation protocols of IPSDMs mainly used a continuous harvesting strategy which lasts for several weeks with a high accumulated yield IPSDMs (Happle et al., 2018; Lachmann et al., 2015; van Wilgenburg et al., 2013). In **Chapter 2** and **3**, we developed a robust protocol for the induction of primitive erythro-myeloid progenitors (EMP)-like cells from hiPSCs, which could be further differentiated into hiPSC-derived monocytes (hiPSC-mono) and IPSDM subtypes. The differentiation process goes through several stages: lateral plate mesoderm, VEC+CD73- hemogenic endothelium, CD43+ hematopoietic progenitors (HPs), CD14+ monocytes and polarized macrophages. More than 90% purity of hiPSC-mono could be obtained

after magnetic purification in 15 days. As an advantage of the protocol, these hiPSC-mono can be either applied immediately or cryopreserved for later applications. Their post-thaw recovery rate could reach up to 50%. hiPSC-mono were still fully functional after cryopreservation proved by their high adhesion to ECs in a microfluidic flow assay previously established in our group (Halaidych et al., 2018a, 2018b). hiPSC-mono were more adhesive compared to blood monocytes (PB-mono), which correlated to their higher expression of VLA-4. This indicated a more activated state of hiPSC-mono than PB-mono, either due to their distinct development origin or different microenvironments they exposed to (*in vivo* vs *in vitro*). Thawed hiPSC-mono could be polarized to inflammatory (M1) and anti-inflammatory (M2) subtypes, manifested by their distinct gene expression and cytokines secretion profiles. IPSDMs were also characterized in various functional assays we established, where PBDMs were included for comparison. IPSDMs were fully functional regarding their high uptake of acetylated low-density lipoprotein (AcLDL), bacteria, apoptotic cells. Endocytic and efferocytotic activities were higher in IPSDMs than PBDMs, which was another indication of their primitive development origin as tissue resident macrophages (A-Gonzalez et al., 2017; Roberts et al., 2017).

The potential of macrophages in tumor immunotherapy has drawn considerable great attention and been intensively studied due to their high tumor phagocytosis activity (Chao et al., 2012; Gul and van Egmond, 2015; Weiskopf and Weissman, 2015). PBDMs were previously used to study the anti-tumor activities of blocking antibodies for the CD47-CD172a pathway. In **Chapter 2**, we described for the first time high tumor phagocytotic activity of IPSDMs in the presence of CD47 blocking antibody, which was comparable to PBDMs. This indicates potential also of IPSDMs in the development of new cancer immunotherapies and their application in candidate drugs screening *in vitro*.

The differentiation protocol and all functional assays described in **Chapter 2** were tested with three different hiPSC lines. Immune cells derived from different hiPSC lines and batches showed similar gene expression profiles and functional activities, indicating high reproducibility across lines. Compared to continuous harvesting methods, our single time harvesting protocol is shorter and more robust which is critical for the application of hiPSC-derivatives in disease modeling. The ability to be cryopreserved with preservation of function greatly facilitates downstream applications of hiPSC-mono, e.g. in disease modeling. However, this protocol is not suitable for transformation to bioreactors for mass production. Another disadvantage is that the purification step required for hiPSC-mono can be labor intensive and expensive. Besides, the extracellular matrix used for coating in this protocol including Matrigel and fetal calf serum (FCS) are animal derived and not defined, which could limit their application in terms of reproducibility in biomedical research.

Both **Chapter 2** and **3** focus on the myeloid cells derived from hiPSCs. **Chapter 2**

included detailed characterization for each stage along the differentiation. For instance, we measured the differentiation efficiency and quality of mesoderm cells, hemogenic endothelium, HPs, monocytes and IPSDMs across lines and batches. HPs on day 9 were shown to be multipotentialand give rise not only to monocytes, but also erythrocytes and granulocytes. Their lymphocytes differentiation potential was not examined in our study, although a previous study using a comparable differentiation method for HPs showed that T lymphoid could also be generated from these HPs (Uenishi et al., 2014). In **Chapter 2**, we also performed phenotypic and functional characterization of hiPSC-mono and IPSDMs, where blood-mono and PBDMs were included as controls for comparison. **Chapter 3** described mainly the detailed experiment set up and protocols for the differentiation and functional assays related to **Chapter 2**. This detailed protocol could support and enhance the reproducibility of studies using hiPSC-derived myeloid cells in the field in general.

In summary, **Chapter 2** and **3** described a robust protocol to derive hiPSC-mono which could be cryopreserved and further differentiated into IPSDMs. Pro-inflammatory M1-IPSDMs and anti-inflammatory M2-IPSDMs could be further polarized. IPSDMs were phenotypically and functionally similar to PBDMs in many aspects. However, differences were also observed between IPSDMs and PBDMs, like their endocytosis and efferocytosis activities, suggesting a primitive development origin and tissue resident macrophage-like identities of these IPSDMs. This part of work in **Chapter 2** and **3** allows us to obtain adequate fully functional patient specific monocytes and macrophages from hiPSCs, which could be added into HHT1 disease models in future works.

hiPSC-ECs

ECs are a constitutive and integral part of the cardiovascular system and their dysfunction contributes to various of cardiovascular diseases. They are also play a key role in inflammation, which are responsible for the recruitment of leukocytes into the tissue. ECs show high degree of heterogeneity *in vivo*, and can be broadly classified into arterial, venous and lymphatic based on their location and functions. ECs from different organs also show organ specific characteristics. hiPSCs provided an unlimited source of patient-specific ECs which can be potentially used for disease modeling to reveal underlying disease pathology and for drug screening. Previously our group developed an efficient monolayer differentiation protocol of CD31+ ECs from hiPSCs. These ECs were functional as evidenced by the formation of primary vascular plexus *in vitro* and their incorporation into zebrafishes' vasculature after transplantation (Orlova et al., 2014b, 2014a).

Cardiovascular cells and related diseases are one of the major research interests of our lab. Cardiomycytes and ECs are two major cell types in the heart. They interact through both physical signals and secretion of paracrine, autocrine, endocrine factors,

which contributes to maintenance of cardiac homeostasis and responses to hypertrophic stimuli (Tirziu et al., 2010). It had been shown previously that ECs and cardiomyocyteshave the same development origin, arising from *Mesp1*+ progenitors in primitive streak, which later migrate to the cardiac field of lateral plate mesoderm (cardiac mesoderm). However, it is still not clear how and when ECs and cardiomyocytes segregate from their common multipotent precursors. Previously our group developed a co-differentiation system of ECs and cardiomyocytes from hiPSCs (Giacomelli et al., 2017). ECs derived from this protocol expressed high levels of cardiac genes *MEOX2*, *GATA4*, *GATA6* and *ISL1*, indicating a cardiac endothelial identity (Giacomelli et al., 2017). In **Chapter 4** of this thesis, we took advantage of this protocol to study the development path and transcriptional control of cardiac mesoderm derived endothelial cells and myocardial cells from hiPSCs. In this chapter, single cell RNA sequencing (scRNAseq) was performed for the co-differentiation system on day 6 when both early multipotent progenitors and committed cell lineages were present. The whole population could be divided into three clusters: *MESP1*+ cardiac mesoderm (CM), *CDH5*+ endothelial progenitors (EPs) and *MYL4*+ cardiac progenitors (CPs). Unbiased pseudotime analysis showed a common development origin of EPs and CPs from CM, which correlated with previous findings *in vivo* that both ECs and cardiomyocytes in the heart developed from MESP1+ progenitors. Interestingly, we observed *ETV2* expression in both CP and EP populations. ETV2 was previously identified as a master regulator of development for the hemagiogenic lineages (Ferdous et al., 2009; Lee et al., 2008; Val et al., 2008). Its overexpression could directly reprogram human fibroblasts into EC-like cells (Morita et al., 2015). To characterize the expression and role of *ETV2* during EC and cardiomyocyte development, an ETV2^{mCherry} reporter hiPSC line was generated using CRISPR/Cas9 system. As expected, the EPs expressed the highest level of *ETV2*, although it was also expressed in a subpopulation of the CPs. We were interested in the expression and function of *ETV2* during cardiomyocytes development as no related studies had been reported to date. To understand the identity of these *ETV2*+ cells in CPs, we isolated both ETV2+ EC (double positive, DP) and ETV2+ non-EC (single positive, SP) populations on different days of differentiation and carried out bulk RNAseq analysis. DPs upregulated large number of EC-specific and NOTCH signaling pathway-related genes from day 4 to day 8, indicating a gradual maturation process of ECs. Interestingly, SPs acquired a cardiomyocyte identity during differentiation evidenced by increased expression of cardiomyocyte function-related genes. Thus, SP cells appeared to represent progenitors of cardiomyocytes. Most importantly, SP isolated on day 7 could be directly differentiated into contracting cardiomyocytes with more than 50% efficiency, while only a minor population (~5%) of ECs could be derived from these cells, providing direct evidence of the commitment of myocardial fate in SPs. Another question we were trying to answer in this chapter was whether ETV2 also plays a role

during early cardiomyocyte differentiation. ETV2+ from CPs readily acquired a cardiac identity. ETV2+ cells from EPs showed a short burst of ETV2 expression, while ETV2+ CPs expressed a steady and lower level of ETV2. A much higher percentage of ETV2+ cells were also observed in EPs than CPs. *ETV2* was also expressed in EPs for a longer period of time compared to CPs. Our results suggested that segregation of cardiac endothelial and myocardial lineages may be determined by an ETV2 expression level threshold, as well as the duration of ETV2 expression.

In summary, our work in **Chapter 4** described a time-course of RNAseq in bulk and single cell populations during the co-differentiation of EC and cardiomyocyte from hiPSCs. Transcriptional dynamics during the specification and maturation of cardiac and ECs during differentiation were characterized based on scRNAseq and bulk RNAseq results, which correlated well with each other. Moreover, we report for the first time that cardiomyocytes could also be derived from ETV2+ precursors which were previously thought to be only progenitors for hemagiogenic lineages. Our study helped to reveal more fully the *in vivo* differentiation process of cardiac mesoderm derived ECs and cardiomyocytes from multipotent CPCs in human development. Overall, this chapter helped provide more insight into the developmental biology of ECs and cardiomyocytes, which may be essential for applications in the *in vitro* modeling of cardiovascular diseases.

One limitation of our work in **Chapter 4** is that although the cardiac mesoderm derived ECs showed a heart specific signature, we did not observe more fully matured and specialized intramyocardial ECs or endocardial cells among the hiPSC-derived ECs. Recently, a scRNAseq study had been carried out on human embryonic heart which revealed two distinct EC populations, representing intramyocardial and endocardial EC identities respectively (Asp et al., 2019). We therefore hypothesized that environmental cues may play an essential role in the acquisition of tissue-specific (intramyocardial or endocardial) identities of hiPSC-derived ECs. Although work in **Chapter 4** revealed the developmengtal process of ECs and cardiomytocytes, and roles of ETV2 in this process, we nevertheless believe that a combination of this chapter with more work related to the characterization of tissue specific identities of hiPSC-derived ECs would further expand the novelty and importance of this work. In recent work not shown here, we generated ECs from both paraxial mesoderm and cardiac mesoderm precursors and made cardiac microtissues from both types of ECs using a protocol we previously published (Giacomelli et al., 2017). ECs before and after multicellular microtissue culture, as well as ECs from different mesodermal environments were compared based on their transcriptome profile. Our preliminary results indicate that both developmental origin (cardiac vs paraxial mesoderm) and cardiac specific environment (microtissue) guide and promote the further maturation and acquisition of heart tissue-specific (intramyocardial or endocardial) identities of hiPSC-derived ECs. For this part of the

work (not included in this thesis), we have completed most experiments and the data analysis is ongoing through collaborations. This will be combined and published later together with data in **Chapter 4**.

Modeling of HHT1 using hiPSC-derivatives

HHT1 is an autosomal dominant genetic disease caused by the defect of TGFβ signaling pathway due to the mutation of endoglin. Although EC is the major cell type being affected and contribute to disease phenotypes of HHT1, myeloid cells including monocytes and macrophages have been shown to be closely related to the pathologies of this disease. Both HHT1 patients and *ENG* knockout mice tend to develop more severe inflammation than heathy controls (Girod et al., 2007; Jerkic et al., 2010; Rossi et al., 2013). Functions of myeloid cells that carry *ENG* mutation were also impaired (van Laake et al., 2006; Ojeda-Fernandez et al., 2016; Post et al., 2010). Besides, it had been found that inflammation was an important trigger for the development of HHT1. Thus, we concluded that both ECs and myeloid cells need to be included in order to establish an *in vitro* model of HHT1. Although mouse models have been widely used to study HHT1, both complete knockout and conditional knockout mice fail to fully recapitulate HHT1 phenotypes in patients (Bourdeau et al., 1999, 2000; Torsney et al., 2003; Tual-Chalot et al., 2015). In comparison, hiPSC models had several advantages including patient specific genetic background, unlimited cell source, less time consuming and easier for genome editing but their use in modelling the disease in humans has not yet been described. In **Chapter 5**, we aimed to establish an *in vitro* model of HHT1 using patient specific hiPSC-derived endothelial and myeloid cells. Multiple hiPSC lines were generated from HHT1 patients that carried different *ENG* mutations. Unexpectedly, one patient was identified as mosaic with the *ENG* mutation being present only in some but not all tissues. Therefore, we could generate a pair of isogenic hiPSC lines with and without the *ENG* mutation from different somatic tissues of this patient. ECs were successfully derived from all of these hiPSC lines. ECs from disease lines showed clear reduction of ENG and unaffected expression of other EC specific makers. These mutated hiPSC-ECs responded to BMP9 and transforming growth factor-β (TGFβ) stimulation normally, behaving exactly as (isogenic) wild type ECs. In addition, cell barrier function, migration rate and ability to form 2D sprouts were also not affected in the mutated ECs. To better recapitulate the *in vivo* condition, however, we then established a 3D vascular model by coculturing of ECs and pericytes in a microfluidic chip. Using this model, we found that the ability of HHT1 ECs to form a perfusable microvascular network was severely comprised, which was partially attributable to their lower cell proliferation in 3D culture. Inflammatory responses of HHT1 ECs were enhanced when they were challenged by TNFα in the presence of BMP9, while in the same condition, their barrier function was impaired compared to WT ECs. These disease

phenotypes were only observed in the presence of the endoglin ligand BMP9. These results suggest that impairment of BMP9-ENG signaling could exacerbate inflammation, which correlated with the observation of more serious infections in HHT1 patients and mouse models (Girod et al., 2007; Jerkic et al., 2010).

To reveal the disease mechanism HHT1 using the hiPSC model, RNAseq analysis was performed for HHT1 hiPSC-ECs cultured in different conditions. *ENG*$^{+/-}$ hiPSC-ECs upregulated more inflammatory genes compared to WT ECs upon TNFα stimulation. Both WT and mutated hiPSC-ECs could respond to BMP9 stimulation, although SMAD2/3 was higher and SMAD1/5, WNT pathway genes were lower in *ENG*$^{+/-}$ hiPSC-ECs compared to WT. Interestingly, TNFα stimulation further increased these differences between HHT1 and WT hiPSC-ECs upon BMP9 treatment. Previous studies showed that WNT signaling could interplay with TGFβ/BMP pathway (Attisano and Wrana, 2013; Fuentealba et al., 2007) and was dysregulated in HHT1 patients (Tørring et al., 2015), which agreed with our RNAseq results. Notably, we found activation of WNT pathway by CHIR99021 could rescue the impaired vasculature formation of HHT1 hiPSC-ECs.

In summary, work in **Chapter 5** developed an *in vitro* HHT1 3D model using patient-derived isogenic hiPSC lines. *ENG*$^{+/-}$ hiPSC-ECs performed similarly to WT ECs in many aspects when cultured in 2D conditions without extra challenges of inflammation and BMP9. However, impaired vasculature formation by HHT1-ECs was observed in a 3D culture system. Barrier functions and TGFβ/BMP and WNT pathways were also dysregulated in mutated ECs upon inflammation and BMP9 activation. The HHT1 hiPSC model established in this chapter can be used for the study not only of hiPSC-EC biology, but also of the role of WNT signaling pathways and inflammation in HHT1 pathology. This work provides new insights into the disease mechanism underlying HHT1 which could lay the foundation for drug discovery and other pre-clinical applications in the future. HHT1 hiPSC-derived myeloid cells will be characterized in more detail in the future and incorporated into the disease model developed in this chapter. This is further discussed in **Future perspectives**. This Chapter will eventually be divided into two parts and published sperately. Both manuscripts are in preparation. One of the manuscripts is a report of the mosaic HHT1 patient and the derivation of isogenic HHT1 hiPSC lines from this patient. Characterizations of hiPSC-dervied myeloid and endothelial cells derived from these isogenic lines will also be included in this manuscript in the context of discussing when he became mosaic in development. Another manuscript is focusing on modeling HHT1 using hiPSC-derived 3D microvasculartur model and undelying disease mechanisms revealed using this model. Experimental work is almost completed and both manuscrits are in preparation for submission in the coming months after finishing of this thesis.

Modeling of PHE using hiPSC-derivatives

In **Chapter 6 and Appendix Chapter 6**, hiPSC-ECs were used for modeling of another disease called Pseudomyogenic hemangioendothelioma (PHE) which is a chromosomal translocation-driven tumor with vascular features. *SERPINE1-FOSB* translocation was successfully introduced into a control hiPSC line using CRISPR/Cas9 system. No off-target effects were observed by whole genome sequencing. ECs were successfully differentiated from the targeted hiPSC line, and showed upregulated *FOSB* expression, reflecting the notion that PHE may be a vascular specific tumor. An infiltrative pattern of hiPSC-ECs$^{SERPINE1\text{-}FOSB}$ was observed after injection into mice, resembling certain aspects the PHE phenotype observed in patients (Hornick and Fletcher, 2011). Vessels formed by hiPSC-ECs$^{SERPINE1\text{-}FOSB}$ in mice were less organized, more invasive and showed higher fibrin thrombi than hiPSC-ECs^{WT}. In additon, lower cell barrier function was also observed with mutated ECs. This correlated with aberrant thrombi formation (conglomeration of fibrin and platelets, containing red blood cells) in the mice transplanted with hiPSC-ECs$^{SERPINE1\text{-}FOSB}$. My focus in the study was the transcriptomic analysis of hiPSC-ECs with and without *SERPINE1-FOSB* translocation. Although it is already known that the FOSB overexpression caused by the translocation is the major drive of the tumorigenesis, the overview of all dysregulated signaling pathways and their correlation with disease phenotypes are still missing. Thorough comparison of the transcriptome profiles of WT and mutated hiPSC-ECs could give valuable insight about the pathology of PHE. With the whole transcriptome data obtained from control and mutated hiPSC-ECs, I first examined Differentially Expressed Genes (DEGs) using an available bioinformatic tool (edgeR), which allowed us to identify dysregulated genes affected by *SERPINE1-FOSB* translocation in ECs. Next, in order to understand functions of these dysregulated genes and signaling pathways they involved, Gene ontology (GO) and KEGG pathway enrichment analyses were performed for these DEGs identified. This showed genes related to TGF-beta signaling, adhesion, metabolism, inflammatory response, angiogenesis and endothelial cell migration were upregulated in hiPSC-ECs$^{SERPINE1\text{-}FOSB}$. In addition, I generated comprehensive gene-gene interaction networks for genes abnormally expressed in hiPSC-ECs$^{SERPINE1\text{-}FOSB}$ using Ingenuity Pathway Analysis (IPA), which revealed the causal relationship between *FOSB* overexpression and its target genes related to cancer, cellular movement and growth, and the TGF-β signaling pathway. My work also indicated direct regulation of SERPINE1 by FOSB, as well as self-regulatory effects of both genes, which further activated cellular growth and proliferation and cancer processes directly or indirectly through the activation of the TGF-β signaling pathway. My work in Appendix Chapter 6 mainly complemented the transcriptomic analysis of Chapter 6. These extra bioinformatic analyses provided more insights into the disease mechanisms of PHE and predicted two candidate drugs which can be potentially used for the treatment of this disease. Overall, my work related to the transcriptomic analysis in this project provided explanations at the molecular level

for many aberrant features of hiPSC-ECs$^{SERPINE1\text{-}FOSB}$ observed *in vitro* and *in vivo* and provided rationale for the development of targeted treatment strategies for inoperable multifocal PHE patients. In summary, the work in **Chapter 6 and Appendix Chapter 6** established an hiPSC model for the study of chromosomal translocation-induced vascular disease PHE, which gave an insight into the tumorigenesis of PHE and helped to elucidate of underlying pathologies of specific rare cancer subtypes caused by specific gene fusion. Work in **Chapter 6** has been deposited in BioRXiv and also been submitted for peer review.

Future perspectives

Modeling genetic diseases using hiPSC derivatives provides new avenues for the study of disease pathologies and screening of candidate drugs. Nowadays hiPSC lines can be routinely generated in many research labs all over the world and protocols for the induction of different functional cell types are reported continuously. Focus now though, is not just obtaining generic exemplars of different cell types but on the tissue specific variants. ECs from different organs show high heterogeneity in terms of their morphology, structure, barrier function, angiogenic potential, angiocrine/endocrine profile and metabolic rates. The complexity of EC phenotypes came from both intrinsic EC properties and programming by their surrounding environment (Reiterer and Branco, 2019). RNAseq and scRNAseq of ECs from different tissues/organs could provide in-depth profiles of tissue specific characteristics of ECs in different organs, thus help in establishing better differentiation protocols or culture conditions in the future. Recently, RNAseq analysis of brain, lung and heart endothelial cells showed specific identities of the endothelium in each organ (http://www.rehmanlab.org/ribo) (Jambusaria et al., 2020). To faithfully recapitulate disease phenotypes in tissue- or organs *in vivo*, more complex cell culture systems may be required for some diseases (eg. HHT1), where the phenotype might not be autonomous to one target cell type. Organoids mimicking different tissues or organs have already been established from hiPSCs, and include neural, gastrointestinal, liver, kidney, lung and cardiac organoids (Rowe and Daley, 2019). Most recently, a 3D human blood vessel organoid was also generated from hiPSCs through self-organizing of hiPSC-derived ECs and pericytes, which formed stable, perfusable vascular trees after transplantation into mice (Wimmer et al., 2019a, 2019b). Some of these organoids have been applied for the investigation of new therapeutic approaches for different diseases. A challenge for the application hiPSC disease models is the immature phenotype of hiPSC-derivatives compared to their primary counterparts *in vivo*. For example, hiPSC-ECs showed aberrant responses to the pro-inflammatory molecule TNF-α, as E-selectin or VCAM-1 were only upregulated in primary ECs but not in hiPSC-ECs under the same conditions *in vitro* (Rosa et al., 2019). More effort is required for the optimization of differentiation protocols or culture

conditions to obtain mature phenotypes of hiPSC-derivatives. Introduction of flow and sheer stress into the ECs culture environment is a possible solution, given the essential role of mechanotransduction on vascular development (Hahn and Schwartz, 2009). Another challenge is the reproducibility of protocols across different hiPSC lines and heterogeneity in the emerging derivatives. On the one hand, genetic heterogeneities could affect the efficiency of the differentiation protocol and quality of cells produced; On the other hand, background-related variations could be confounders of the disease-relevant phenotypic changes caused by the genetic mutation of interest. Gene correction of patient-derived hiPSC lines using advanced genome editing technologies provides a powerful control which can be applied for distinguishing pathologically relevant phenotypes from other gene background-related variations-caused phenotypes. In the groundwork for modelling HHT1 in this thesis, we mainly focused on an isogenic pair of hiPSC lines that were generated from different tissues of a mosaic HHT1 patient who carried the c.1678C>T mutation. Other HHT1 hiPSC lines mentioned in this study, including c.1084_1085del and c.689+2T>C, mutated hiPSC lines, are still need to be corrected by CRISPR/Cas9 gene editing in order to obtain their isogenic healthy controls.

Blood vessels are essential for nutrients and oxygen supply and play an important instructive role during organ development. Therefore, including an EC component and functional vasculature would be an important next step for the generation of complex and functional organoids from hiPSCs (Passier et al., 2016). Aside from ECs, mural cells (pericytes and vascular smooth muscle cells (vSMCs)) are also critical for the generation of functional vasculature *in vitro*. Dysregulated interactions between EC and mural cells was found to underlie many pathological conditions including HHT1. For instance, it had been shown that defective TGFβ/BMP signaling pathway in the HHT1 mouse model resulted in impaired vSMC development and mural cells recruitment and coverage, which lead to the fragile blood vessels and hemorrhage (Lebrin et al., 2010). In **Chapter 5**, we generated 3D vasculature for HHT1 through self-organization of hiPSC-ECs and primary human vSMCs. These self-organized vascular networks formed lumens that could be perfused with medium. In the future, this vasculature model could be further characterized in several aspects. First, the integrity and barrier function of the lumenized vasculature could be tested by connecting a microfluidic pump to the 3D microfluidic chip to introduce a steady fluid flow in the vasculature. Leakage assays could be applied to check vessel integrity and barrier function. Another interesting experiment to do would be testing the recruitment of leukocytes by these hiPSC-derived vasculatures in inflammatory conditions, which is an important aspect of blood vessels behavior *in vivo*. Specifically, monocytes could be perfused through the hiPSC-derived vasculature by connecting a microfluidic pump. Before flow through of monocytes, hiPSC-ECs in the chip should be challenged by inflammatory stimulus like TNF-α in order

to upregulate their expression of adhesion molecules. Monocytes (labeled with fluorescent dye) should be captured by ECs in the chip and their transmigration through the EC lumen could be monitored by time-lapse with a high-speed fluorescent microscope. In addition, hiPSC-mono derived using the protocol described in **Chapter 2 and 3**, could also be a source of leukocytes in this flow assay; it would be interesting to investigate their adhesion and transmigration behavior in the 3D hiPSC vasculature.

Inflammation is also closely related to the onset of many diseases such as ischemic heart disease, stroke, cancer, diabetes mellitus, chronic kidney disease, auto-immune and neurodegenerative diseases (Furman et al., 2019). We and others found that inflammatory cells, especially myeloid cells were closely related to the development of HHT1 (van Laake et al., 2006; Ojeda-Fernandez et al., 2016; Rossi et al., 2015). In these cases, it would be beneficial to include immune cells in hiPSC disease models, in order to recapitulate the inflammation component and investigate the effect of immune cells on disease phenotypes. Monocytes and macrophages play an essential role in inflammation. In **Chapter 2 and 3**, we established a robust protocol to derive monocytes and macrophages from hiPSCs and performed detailed functional characterization of these cells. This showed that the IPSDMs we derived were more similar to primitive rather than definitive macrophages. Regarding to the differentiation protocol, it would be possible to optimize it further in some respects. First, the differentiation procedure as such is relatively expensive due to the large volumes of reagents needed, most importantly the growth factor component. The requirement for and optimal concentration of growth factors could be further examined and some reagents at least replace by small molecules. For instance, Thrombopoietin (TPO) was used to induce the hematopoietic differentiation from hemogenic endothelium at a high concentration. However, it has not been tested whether it is necessary for the myeloid lineage specification. Although optimization could be time consuming and labor intensive, it may reduce the cost and time required for differentiation in the long term. Second, the differentiation efficiency of monocytes from HPCs was ~50% in our protocol, which could potentially be improved in order to avoid the purification step and intrinsic loss of cells from the protocol required for monocytes. Third, hiPSC-monocytes on differentiation day 15 already started to show an activated phenotype, as they were more adhesive and larger than blood-monocytes. Therefore, it would worth further optimizing monocyte induction and culture conditions to obtain a less activated phenotype. Last but not least, although IPSDMs were close to primitive macrophages based on their functional activities, direct evidence for their developmental trajectory is still needed. For example, the requirement of MYB expression during the differentiation process of IPSDMs should be examined, as MYB was previously found to be specifically required during definitive (third wave) differnetiation (Lieu and Reddy, 2009; Schulz et al., 2012) but not primitive (first and second wave) hematopoiesis

(Mucenski et al., 1991; Schulz et al., 2012).

As a next step of the work in this thesis, monocytes and macrophages derived from HHT1 hiPSCs could be added to the existing disease model to better recapitulate the disease phenotypes. On the one hand, myeloid cells derived from isogenic HHT1 hiPSC lines with and without *ENG* mutation could be compared in 2D culture condition to study the effect of *ENG* mutation on their characteristics and functions, like their inflammatory responses and adhesion to ECs. On the other hand, due to the close association between tissue resident macrophages and angiogenesis (**section 1.2.3** of this thesis), it would be interesting to include IPSDMs as an extra component of the 3D vasculature model established in **Chapter 5**. Hopefully, more severe and perhaps new aspects of disease phenotypes will be observed during inflammation in the presence of immune cells in the system. Bulk and single cell RNAseq could be used for the identification of dysregulated signaling pathways, multicellular interactions and other underlie disease mechanisms of HHT1. Except for HHT1, hiPSC-derived myeloid cells could also potentially be used for other disease modeling or generation of complex organoids *in vitro*. For instance, macrophages could be incorporated into the 3D cardiac microtissues which was previously established in our lab, to better mimic and study the cardiac inflammatory responses and related cardiac diseases using this system.

Regarding to the PHE studyin **Chapter 6 and Apppendix Chapter 6**, through my RNAseq analysis of ECs derived from disease hiPSCs line that carry the translocation mutation, multiple dysregulated pathways were revealed, such as altered TGF-beta signaling, adhesion, metabolism, inflammatory response, angiogenesis and endothelial cells migration. These pathways are known to be related to cancer and correlated to disease phenotypes observed in other cellular models we established. Our RNAseq analysis contributed to explaining the disease pathology of PHE at the molecular level and provided the rationale for the development of targeted treatment strategies for this vascular tumor. Notably, from our RNAseq analysis, MAPK signaling and PDGFRA and -B were found to be upregulated by the *SERPINE1-FOSB* translocation in mutated ECs. A previous study had shown that the multi-tyrosine kinase inhibitor telatinib could be a highly specific treatment for patients with multifocal unresectable PHE (van IJzendoorn et al., 2018). Thus, it would be an interesting experiment to test the effect of this drug on our PHE-hiPSC cellular models to see whether it has a positive effect on the disease phenotypes we observed *in vitro* and *in vivo*. Additionally, PI3K-Akt signaling pathway was also identified among these upregulated pathways by the chromosomal fusion in mutated ECs. This observation from our RNAseq analysis provides more rationale for the application of mTOR inhibitors as a specific treatment strategy of PHE which would be worth further investigated in the future.

In summary, this thesis explored the application of hiPSC-derived myeloid and endothelial cells in disease modeling and provided valuable insights of HHT1 and PHE

pathologies through the establishment of a hiPSC-derived vascular models. This work provided key cell sources and laid a firm foundation for the related future studies within and outside our research group.

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