The endothelial compartment as a disease modifier in bleeding disorders

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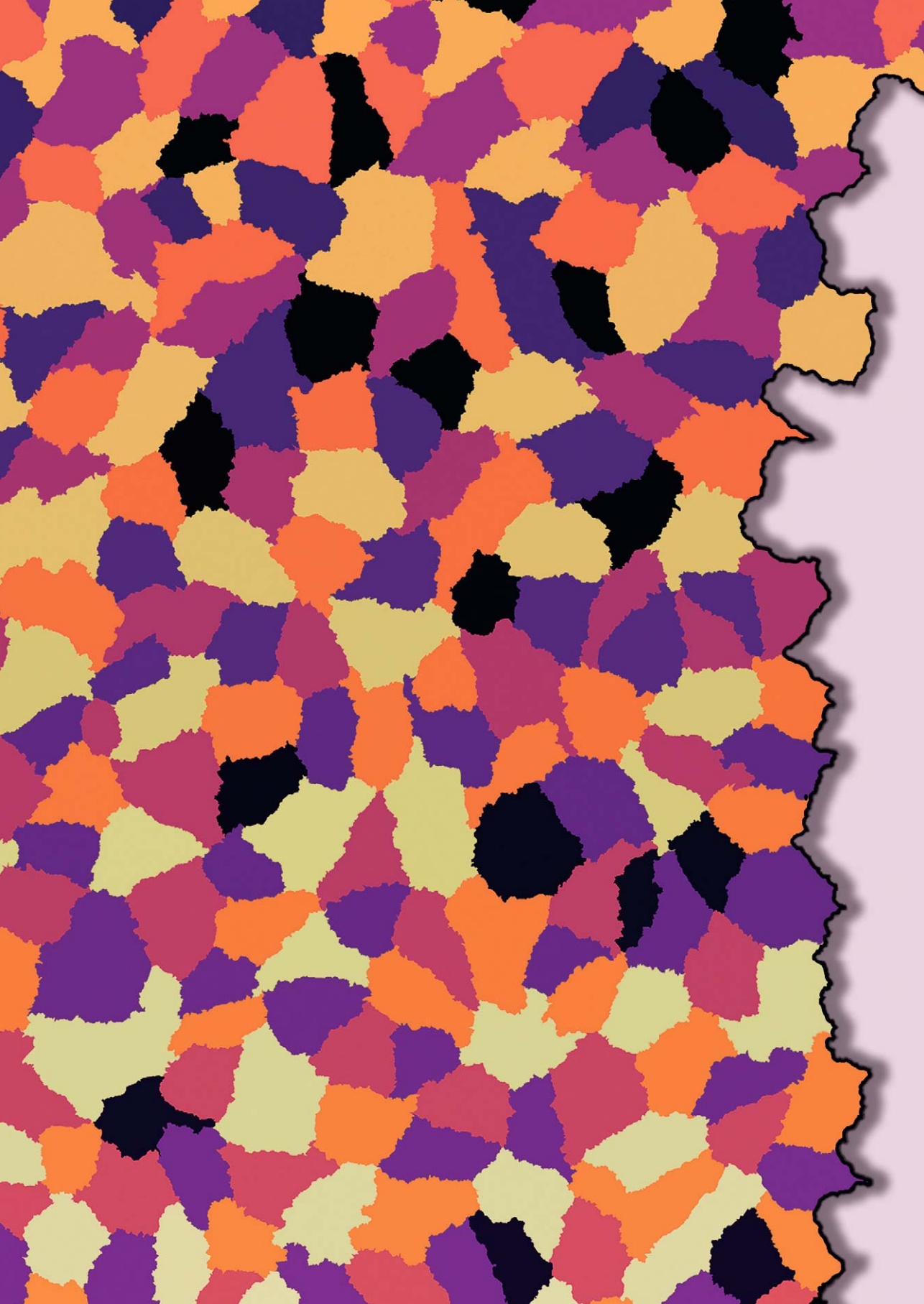
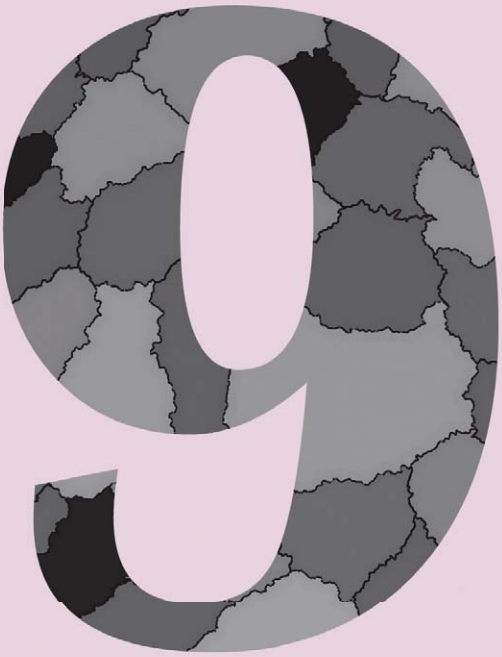


English and Dutch summary



English summary

Endothelial cells form the inner wall of vessels and play a critical role in the primary and secondary hemostasis. One of the main procoagulant roles of endothelial cells is the production of von Willebrand factor (VWF). This is a very large protein that, once activated in the blood, can bind to platelets and start the coagulation process. VWF can also bind to and protect coagulation factor VIII (FVIII). VWF is primarily produced in endothelial cells and stored in specialized secretory organelles called Weibel-Palade bodies (WPBs). These cell specific organelles are filled with dense tubules of VWF which allow for a quick secretion when stimulated. In von Willebrand disease (VWD), VWF is either defect or absent in varying degrees causing mild to severe bleeding abnormalities. This can lead to frequent nosebleeds, menorrhagia, excessive bleeding from injury or surgery, and muscle and joint bleeds. VWD can be treated by administering factor concentrates or by administration of 1-deamino-8-D-arginine vasopressin (DDAVP). This drug can stimulate endothelial cells to secrete their contents and thus quickly increases the levels of VWF and FVIII in the blood. Large variation in bleeding phenotype exist in VWD patients. This variation can be caused by different mutations but interestingly, approximately 30-50% of patients with type 1 VWD do not have pathogenic VWF gene variants. There is also a significant group of VWD patients that do not respond to treatment with DDAVP. We hypothesized that the reduced VWF levels or non-response to DDAVP may be caused by other modifiers of VWF. As primary source of VWF we focused our research on the endothelial cells of these patients. A model that can be used to study endothelial cells is named endothelial colony forming cells (ECFCs). A great advantage of ECFCs is that they carry the genetic background of the donor from which the blood for ECFC isolation was collected. In this thesis we studied various aspects of the endothelial compartment as a disease modifier in bleeding disorders using the ECFC model.

Firstly, a general background for all the studies in this thesis is presented in **Chapter 1**. There, we provide a more detailed description of endothelial cell function, VWD and its treatment options and the available endothelial research models.

In previous studies, ECFCs have already been extensively used as a model to study the pathophysiology in bleeding disorders. We reviewed the published literature in **Chapter 2** to obtain an overview of these findings. The advantages and disadvantages of using ECFCs in patient-specific research are discussed and compared with alternative models like the induced pluripotent stem cells (iPSCs) or transfected models like the human embryonic kidney 293 cells. We also summarized the findings in VWD specific research, secretory organelle exocytosis and insights into role of endothelial cells in angiogenesis.

One widely used method of analyzing cells and tissues is microscopy which yields insight into the inner workings of cells. Qualitative observations can be made by eye, but to statistically analyze the data, quantification is needed. One of the primary analysis tools we used for endothelial cell and WPB morphology was immunofluorescence microscopy. Quantification of parameters was challenging due to the high number and large heterogeneity of WPB shape within the cells. Therefore, we developed an automated quantification method for the analysis of cells and organelles using CellProfiler (**Chapter 3**). The method was specifically designed to measure the size, count and shape of the endothelial cells and to measure the count, shape, length and location of WPB within the cell. Furthermore, to show that this method could also be used by other groups for other purposes, we analyzed endosome morphology in HEK cells.

As mentioned previously, the ECFCs provide a wonderful model to study the pathophysiology in bleeding disorders. Through a relatively simple venipuncture, the ECFCs can be derived from whole blood. Once differentiated, the ECFCs endogenously produce VWF and display all general endothelial markers. Most importantly for our research is that the cells also contain the mutations of the donor which allows for patient specific research. However, there are also challenges in working with ECFCs. It has been shown that within healthy control ECFCs there is large variation in many aspects of the ECFCs like cell size and shape, proliferation speed and VWF production. One can imagine the difficulty of studying VWF production in cells of which the production varies strongly. Therefore, in **Chapter 4** we dive into this variation, the challenge it poses and offer a solution to overcome this problem. First, we explored the transcription signature of a large panel of control-derived ECFCs using RNA sequencing. This revealed that many of the differentially expressed genes were associated with inflammation and with endothelial to mesenchymal transition (EndoMT). These genes may act as potential drivers for the phenotypic heterogeneity. We used this information to create a minimal qPCR panel which can be used to easily characterize and cluster the ECFC clones. Comparing between 2 clusters of ECFCs, we show that WPB count and shape, cell count and migration speed is significantly different. This emphasizes the importance of characterizing ECFCs before using them for patient specific research. An alternative model for endothelial cell research is the iPSC-derived endothelial cell (iPSC-EC). These cells can be derived from any cell type isolated from a donor. For instance, peripheral blood mononuclear cells can be isolated from whole blood, reprogrammed to iPSCs and then differentiated into iPSC-ECs. This means that these cells also carry the genetic background of the donor and can be used for patient-specific research. However, iPSC-ECs do not seem to mature fully, showing low levels of synthesized VWF and small, round WPBs. In **Chapter 5** we performed and adjusted various differentiation

protocols in order to improve the maturation of the WPBs. We show that co-culture with pericytes, changing the concentration or timing of administration of differentiation factors, the addition of flow to the system, and the use of histone deacetylase (HDAC) inhibitors did not significantly improve maturation of iPSC-ECs. Transfection of iPSCs with transcription factor ETV2 resulted in a faster differentiation process with slightly increased VWF production and secretion. Furthermore, the WPBs were more elongated, but only after >30 days in culture.

As mentioned previously, VWD can be treated by administration of DDAVP which quickly raises VWF and FVIII levels in the blood. DDAVP is also used in other bleeding disorders. However, a large portion of patients that receive DDAVP do not or only partially respond. These patients require alternative treatments such as factor concentrates, but the reason for the non-response in most patients is not understood. For this reason, we performed a systematic review and meta-analysis on the response to DDAVP and the possible determinants of DDAVP response in patients with bleeding disorders (**Chapter 6**). In this study we included 103 articles, from which, data of 1982 patients could be extracted. Response rate varied significantly between disease types and subtypes and coagulation factor baseline levels were important determinants of response. Our findings strengthen what is already known and emphasizes the need for a standardized response definition and further research into response mechanisms.

Finally, the lessons learned in this thesis were applied in **Chapter 7**. There, we analyzed ECFCs derived from two groups of patients with VWD. First, patients with VWD without a pathogenic mutation in VWF and second, patients with VWD with a known mutation in VWF but no response to DDAVP. We hypothesized that modifiers of VWF in the endothelial cells are affected in these patients, causing the low levels of VWF or the non-response. ECFCs were analyzed and clustered using the minimal qPCR panel developed in **Chapter 4**. Patient ECFCs were matched to controls and functional aspects of the ECFCs like production of VWF, stimulated secretion, migration and morphology were compared. All morphological aspects were analyzed using the quantification pipeline developed in **Chapter 3**. We found that retention of VWF in the endoplasmic reticulum could be the cause of the DDAVP non-response in some patients. Furthermore, we correlated the extensive range of functional characteristics of endothelial cells to their proteome, uncovering processes and proteins of interest.

To summarize, this thesis adds valuable insight into the use of ECFCs and iPSC-ECs and shows how they could be used to study patient specific defects. It also shows the challenges of using these models and the importance of developing and optimizing them.