Universiteit
Leiden
The Netherlands

Modeling vascular diseases using human induced pluripotent stem cells

Cao, X.

Citation

Cao, X. (2020, September 9). *Modeling vascular diseases using human induced pluripotent stem cells*. Retrieved from https://hdl.handle.net/1887/136521

Version:	Publisher's Version
License:	Licence agreement concerning inclusion of doctoral thesis in the Institutional Repository of the University of Leiden
Downloaded from:	https://hdl.handle.net/1887/136521

Note: To cite this publication please use the final published version (if applicable).

Cover Page

Universiteit Leiden

The handle http://hdl.handle.net/1887/136521 holds various files of this Leiden University dissertation.

Author: Cao, X.
Title: Modeling vascular diseases using human induced pluripotent stem cells
Issue Date: 2020-09-09

Chapter 1

General Introduction

Abnormal vascular physiology and precipitating inflammatory pathways underlie many different diseases, including hemorrhage, stroke, vascular dementia and even cancer. Pluripotent stem cells (PSCs) can now be derived by reprogramming from any individual so that it is possible in principle to derive all somatic cells of the human body that would normally be difficult to access. In this thesis, I studied the derivation of myeloid cells from human induced pluripotent stem cells (hiPSCs) to model the inflammatory component of vascular disease and characterized the development path of hiPSC-derived endothelial cells (hiPSC-ECs) which form the vascular walls. Functional defects in either of these cell types can cause or exacerbate vascular disease. I then used these cell types to gain insight into the mechanisms underlying two genetic diseases: Hereditary Hemorrhagic Telangiectasia (HHT) which is caused by mutations in a gene called Endoglin expressed on cells of the vascular wall and inflammatory macrophages, and a vascular tumor called Pseudomyogenic hemangioendothelioma (PHE) in which endothelial cells are thought to be the tumor cell of origin but this remained to be proven. I developed new differentiation protocols to generate inflammatory cells from hiPSC, characterized these cells functionally and used Next-Generation Sequencing and bioinformatic analysis to gain insight into the molecular pathways controlling development of one particular type of endothelial cells from hiPSC and the underlying tumorigenic mechanisms of PHE.

1.1 Myeloid cells derived from hiPSCs

1.1.1 Derivation of myeloid cells from hiPSCs

Myeloid cells mainly refer to the monocytes and granulocytes in the blood but also include the derivatives of monocytes in tissue, including macrophages and dendritic cells. In this thesis, I mainly considered monocytes and macrophages within the myeloid lineage, as these cells express high levels of endoglin and thus may be most relevant to HHT type 1 (HHT1).

Macrophages in tissue have two different development origins: primitive hematopoiesis that occurs in the yolk sac of early embryo and which can give rise to primitive erythrocytes and primitive macrophages; monocytes that differentiate from hematopoietic stem cells in the bone marrow, which can differentiate into macrophages after transmigration through blood vessels during inflammation. Macrophages not only provide protection from pathogens, but also play a key role in the regulation of angiogenesis and tissue regeneration by secreting a wide range of cytokines, chemokines and growth factors. When polarized by different stimuli, macrophages can be divided functionally into several subtypes, termed M1 (LPS and IFN-γ sensitive), M2a (IL4), M2b (Fc receptors and immune-complex expression) and M2c (IL10 and glucocorticoid expression) (Mantovani et al., 2004).

Currently, tumor derived macrophage cell lines and peripheral blood monocyte-derived macrophages are commonly used as a source of macrophages for research. Both sources have limitations: tumor cell lines differ considerably from physiologically normal macrophages and primary macrophages from blood are different from those in organ tissues, have limited availability and have ethical challenges associated with informed consent and invasive methods of collection. Furthermore, studying macrophage biology in rare genetic diseases is even more difficult since there are only few patients to donate tissue. Thus, by comparison, hiPSCs differentiated to macrophages could represent an unlimited supply of human macrophages, that are either healthy or carry specific patient genotypes (Zhang and Reilly, 2017); they can thus be used for in vitro disease modelling to understand underlying disease mechanisms and consequently improve therapeutic strategies.

In 2008, Karlsson et al. reported an "embryoid body" (EB)-based protocol to differentiate hESCs into monocyte-like cells. M-CSF and IL-3 were add to induce the monocytes from EBs and floating single cells were collected continuously and then further differentiated into macrophages (Karlsson et al., 2008). Choi et al. used OP9 coculture to induce the hematopoietic differentiation from hPSCs. From the coculture system, lin-CD34+CD43+CD45+ multipotent progenitors were expanded with GM-CSF and then further differentiated into macrophages using M-CSF and IL-1β(Choi et al., 2009; 2011). In 2013, Yanagimachi and colleagues developed a monocyte differentiation protocol using monolayer culture and defined conditions. With their protocol, a large number of monocytes could be harvested continuously from day 16 to day 28, and macrophages could be derived from these monocytes after one more week (Yanagimachi et al., 2013). Meanwhile, Cowley's group also developed a serum-free protocol which was based on EB formation. They found monocytes could be continuously harvested from 14 days of differentiation onwards for a period of almost one year, although the differentiation efficiency decreased dramatically after about 6 months(van Wilgenburg et al., 2013). Lachmann and colleagues also established a EB-based differentiation protocol for continuous production of monocytes and neutrophils over a 2 month timespan using M-CSF and G-CSF respectively (Lachmann et al., 2015). Zhang et al. reported a stepwise differentiation method, in which highly pure populations of CD14+ monocytes could be derived from hiPSCs after 15 days of induction (Zhang et al., 2015). Most protocols reported so far for the differentiation of monocytes and macrophages from hiPSCs are EB-based or do not use fully defined conditions(Brault et al., 2014; Choi et al., 2011; Karlsson et al., 2008; Lachmann et al., 2015; Yanagimachi et al., 2013; Zhang et al., 2015). Recently, Takata et al. established a serum-free monolayer differentiation protocol to induce primitive macrophages from hiPSCs by Wnt inhibition from day 6 to day 10. They also showed that coculture of these macrophages with hiPSC-derived neurons could promote differentiation of microglia-

like cells (Takata et al., 2017).

One of the main aims of this thesis was to establish a more robust method for differentiation of myeloid cells from hiPSCs and show this was applicable to multiple hiPSC lines. Specifically, we aimed to use a monolayer protocol based on serum- and feeder cell-free culture conditions for the differentiation and hoped to see that the protocol was faster and more robust from line to line.

1.1.2 Characterization of hiPSC-derived macrophages

Although several differentiation methods have been established so far for monocyte and/or macrophage differentiation from hiPSCs, the question remains whether hiPSC-derived macrophages (IPSDMs) behave similarly to primary human macrophages *in vitro* and *in vivo*. A thorough characterization of IPSDMs may provide the field with a critical reference to what extent primary macrophages can be replaced by IPSDMs for disease modeling, drug screening and cell replacement therapies in the future.

Characterization of IPSDMs is usually based on their morphology, gene expression, surface marker expression, cytokine secretion, lipid uptake, cholesterol efflux, and phagocytotic activities. Cowley's group compared macrophages derived from hiPSCs using their EB-based protocol with peripheral blood-derived macrophages (PBDMs) by Scanning Electron Microscopy and Transmission Electron Microscopy, which showed similar morphologies of these two sources of cells. Their IPSDMs expressed normal levels of CD14, CD16, CD163 and CD86 compared with primary cells, whereas MHCII levels were much lower on IPSDMs than PBDMs. They also showed that IPSDMs secreted high levels of pro-inflammatory cytokines after stimulation by LPS and IFN-γ, including IL-6, TNF-α, RNATES and IL-23 (van Wilgenburg et al., 2013). With Lachmann's protocol, it was reported that IPSDMs expressed most of the macrophage specific markers, although CD11b was lower on IPSDMs compared with PBDMs. These IPSDMs could also phagocytose bacteria and secrete some of inflammatory cytokines including TNF-α, IL-6, IL-8, MCP-1 and IL-10 (Lachmann et al., 2015). Zhang et al. compared gene expression in IPSDMs and PBDMs derived from the same subject by transcriptomic analysis. They found that around 12% genes were differentially expressed between IPSDMs and PBDMs, which mainly related to the immune and defense responses (Zhang et al., 2015). They also compared the morphology, surface marker expression, LDL-uptake, bacterial phagocytosis, and cytokine secretion of M0, M1 and M2a subtypes of IPSDMs and PBDMs. Their results indicated high similarities between IPSDMs and PBDMs (Zhang et al., 2015).

However, so far only some aspects of functional activities of IPSDMs have been examined and only some subtypes of macrophages compared. Efferocytosis, a characteristic mainly of tissue resident macrophages, can remove and clear apoptotic

cells after tissue damage which is critical to maintaining tissue hemostasis. Defective efferocytosis can lead to disease including cystic fibrosis, Chronic Obstructive Pulmonary Disease and asthma. Efferocytosis activity of IPSDMs would thus greatly facilitate their application in disease modelling and studies of the pathology of these conditions.

Increasing evidence showed macrophages as one of the main effector cells of monoclonal antibody (mAb) based cancer therapy (Gul and van Egmond, 2015). Human primary macrophages phagocytose tumor cells efficiently *in vitro* through antibody-dependent cell phagocytosis and antibody-independent phagocytosis. CD47 serve as "don't eat me" signals on tumor cells by binding to SIRPα expressed on macrophages and inhibiting their phagocytotic activity. Thus CD47-SIRPα pathway can serve as a target to enhance phagocytosis of tumor cells by macrophages *in vitro* and *in vivo* (Majeti et al., 2009; Theocharides et al., 2012; Tseng et al., 2013; Willingham et al., 2012). CD47 and SIRPα blocking antibodies synergize with tumor specific mAb to promote the efficiency of cancer therapy (Chao et al., 2010; Tseng et al., 2013). Human primary macrophages have commonly been used as a tool to test CD47-SIRPα blocking antibodies and tumor specific mAb in vitro (Chao et al., 2010; Majeti et al., 2009; Willingham et al., 2012). However, the variations among different individuals and limited availability make primary macrophages less than ideal for pre-clinical drug screening. Instead, IPSDMs can be a better option due to their patient-specific genetic background and unlimited source. However, at the outset of the research in this thesis, tumor phagocytotic activity of IPSDMs still needed to be carefully checked.

To date, IPSDMs have already been used to model several genetic diseases, including Tangier disease, chronic granulomatous disease, Blau syndrome neuronal ceroid lipofuscinoses, Gaucher disease and chronic infantile neurological cutaneous, articular syndrome, mycobacterial disease (Aflaki et al., 2014; Jiang et al., 2012; Neehus et al., 2018; Panicker et al., 2012; Tanaka et al., 2012; Uusi-Rauva et al., 2017). There are no reports so far for modeling of vascular diseases using IPSDMs to the best of our knowledge.

1.2 Endothelial cells derived from hiPSCs

1.2.1 Tight-regulation of endothelial cell (EC) specification *in vivo*

During vertebrate embryo development, a functional circulatory system is a prerequisite for organ formation to meet their increasing demand for nutrients and oxygen. Together with the increasing organ size during cell specification and morphogenesis of different organs, the vasculature is also expanding, remodeling and specializing to fulfill the requirement of each organ they "serve". Blood vessels are formed through two mechanisms, termed vasculogenesis and angiogenesis. Vasculogenesis mainly refers to *de novo* EC differentiation and specification from

mesoderm precursors and occurs before the onset of blood flow. Vascular progenitors first appear in yolk sac, which come from mesodermal progenitors and form the blood islands. The outer cells of blood island differentiate into ECs and the inner cells become hematopoietic cells. Within the embryo, vascular progenitors form angioblasts which aggregate to form the ventral and dorsal aortas and the vitelline arteries and veins. Then, primary vasculature thus formed starts to expand and remodel through angiogenesis; this includes ECs sprouting, vessel branching, and intussusception from existing blood vessels (Patan, 2004). Also, further specialization of the endothelium to arterial, venous, hemogenic, and lymphatic subtypes is necessary to fulfill diverse functions of the vasculature (Marcelo et al., 2013).

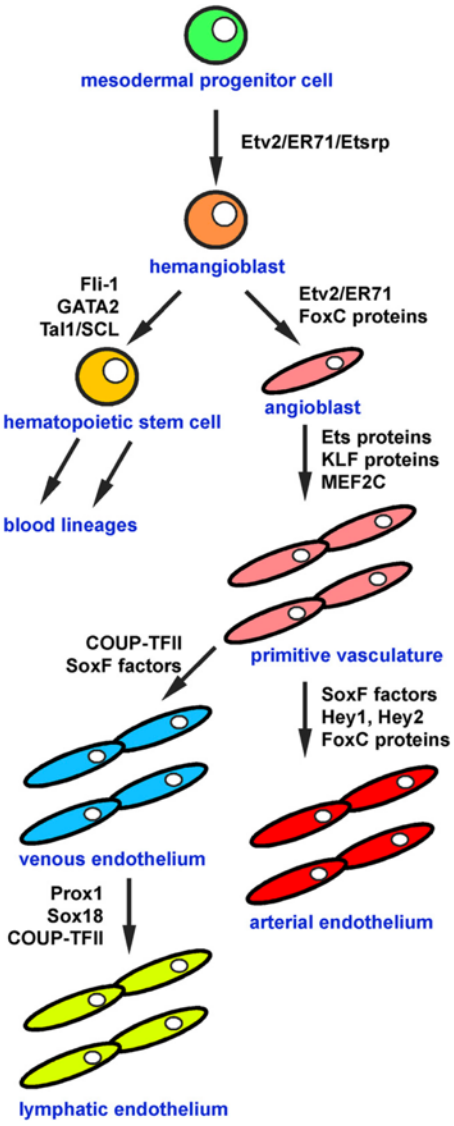
The signaling pathways that are involved in vasculogenesis and angiogenesis have been intensively studied (Figure 1). Although the hierarchy of these signals has not been clearly defined *in vivo*, numerous data have been obtained from studies using embryonic stem (ES) cell systems. Studies using mouse ES cells indicated bone morhogenic protein 4 (BMP4) as the earliest key factor that drives mesoderm differentiation and initiates fibroblast growth factor 2 (FGF2)-dependent specification of EC progenitors (Park, 2004; Pearson et al., 2008). However, with human ES cells, FGF2 is required for maintaining the undifferentiated state thus its role in the commitment of mesoderm cells to ECs is not essential. Instead, Indian Hedgehog was found as a critical factor downstream of BMP4, which promotes EC differentiation from mesoderm cells (Kelly and Hirschi, 2009). Like IHH, VEGF is another visceral endoderm-derived factor that is a key regulator of vasculogenesis. Heterozygous knockout of VEGF-A was embryonic lethal due to the failure of vascular development (Carmeliet et al., 1996; Ferrara et al., 1996). On the other hand, overexpression of VEGF-A also lead to embryonic lethality in mice due to the impaired cardiac development (Miquerol et al., 2000). These data indicate that a precise dosage of VEGF-A is required for cardiovascular development.

E-twenty six (ETS) transcription factors are also well-known as key regulators of EC development. Several members of the ETS family, including Ets1, Erg, Fli-1 and Etv2 have been shown to play essential roles in endothelial and hematopoietic differentiation (Dejana et al., 2007). Notably, all characterized enhancers and promoters of endothelial related genes so far contain multiple essential ETS binding sites, and ETS motifs are strongly associated with endothelial genes throughout the human genome (Bernat et al., 2006; Val et al., 2008). Many ETS share redundant functions, except for ETV2, which plays a specific and critical role for the differentiation of mesoderm toward an EC-fate and will be discussed specifically later.

Although it is known that ETS play a central role in the transcriptional control of EC specification, the wide spread binding sites of Ets proteins make it difficult to fully understand the regulatory network of ETS factors. Except for the canonical binding sites, ETS can also bind to noncanonical sites in conjunction of other factors (Hollenhorst et

al., 2007). Studies identified evolutionarily conserved FOX:ETS motifs which can be bound by FoxC2 and ETV2 simultaneously, and these motifs are located in the promoters and enhancers of endothelial genes including Tal1, Tie2, flk1, and VE-cadherin (Cdh5) (Val et al., 2008) .

Forkhead (Fox) Proteins also play a key role in EC specification; they include members of FoxC, FoxF, FoxH and FoxO subfamilies. FoxO1 is required for vascular development and functions as both a positive and negative regulator of transcription, suggesting that it may act as a transcriptional switch in EC specification (Paik et al., 2007).



FoxF1 is also necessary for vascular development and it may regulate the BMP signaling in splanchnic mesoderm prior to EC specification(Astorga and Carlsson, 2007). By contrast, FoxH1 overexpression impaired the vascular development and may act as an inhibitor by negatively regulating flk1 expression (Choi et al., 2007). FoxC subfamily also play an important role in vascular development. FoxC1 and FoxC2 knockout mice showed severe vascular defects and indicated that these transcription factors were required for early endothelial development (Swift and Weinstein, 2009; Val et al., 2008). Besides, FoxC1 and FoxC2 also play an important role in arterial and lymphatic endothelial cell specification and act as key downstream effectors of Notch signaling (Chu et al., 2016; Hayashi and Kume, 2008).

Figure 1. Different Stages in Endothelial Development Are Regulated by Distinct Sets of Transcription Factors. This figure is adapted from (Sarah et al., 2009).

1.2.2 Roles of *ETV2* in ECs development

The specification of ECs from mesoderm progenitors is highly depend on the activity of the *Etv2* transcription factors (van Bueren and Black, 2012; Koyano-

Nakagawa and Garry, 2017; Val, 2011). Studies in zebrafish embryos showed that ETV2 alone is necessary for vasculogenesis, while Etv2 and another ETS fator Fli1b function redundantly during early embryo angiogenesis (Craig et al., 2015). Genetic ablation of Etv2 leads to complete loss of blood and vessels, and overexpression of this factor has been shown to convert non-ECs to the endothelial lineage (Ferdous et al., 2009; Morita et al., 2015). Expression of *ETV2* starts to appear in (mouse) development in the KDR+ and PDGFRα- mesoderm cells in yolk sack and embryo. At embryonic day 7.5 (E7.5), angioblasts that migrate back to the embryo express high levels of *ETV2*. From E8.5 to E9.5, *ETV2* was observed in perivascular cells rather than differentiated ECs (Kobayashi et al., 2013). Then from E10.5, *ETV2* expression dramatically decreased except in the region of dorsal aorta (Koyano-Nakagawa et al., 2012). Postnatally, only testis still expresses *ETV2* constitutively (Haro and Janknecht, 2002). Some tissues may express *ETV2* transiently, such as hematopoietic stem cells (HSCs) (Lee et al., 2011). However, *ETV2* expression is significantly upregulated after injury which indicated its essential role in neovascularization (Park et al., 2016).

One of the key target genes that regulated by Etv2 in ECs is Flk1/VEGFR2 (Becker et al., 2016). Upregulation of VEGFR2 and neovascularization after ischemic injury in mice require the de novo expression of Etv2 in adult ECs (Park et al., 2016). Overexpression of Etv2 in vascular progenitor cells in the postnatal arterial adventitia can directed them to endothelial cell fate. Endothelial-specific genes could be upregulated in Etv2-transduced Sca1+ progenitor cells which inhibit intimal hyperplasia in wire-injured femoral arteries when grafted onto the adventitial side of the femoral artery wall (Bras et al., 2018). Many other genes were also identified as target genes that are regulated by ETV2 either using overexpression studies or identification of direct binding to promoter sequences, including Pecam1, Tie2, VE-Cadherin, Gata1, Gata2, Lmo2, Elk3, Cebpd, Scl, Mef2c, Notch4 *Spi1, Fli1, Sox7, Ece1, Nrp1*, *Nrp2*, *Flt1and Erg* (Abedin et al., 2014; Ferdous et al., 2009; Kataoka et al., 2011; Koyano-Nakagawa et al., 2012; 2015; Lee et al., 2008; Liu et al., 2012; 2015; Robinson et al., 2014). miR-130a is also a downstream target of Etv2, which promote EC differentiation from mesoderm cells prior to their patterning (Singh et al., 2015). In chapter 4 of this thesis, we generated an ETV2^{mCherry} reporter hiPSC line and used it to track and label the ETV2 expression during the derivation of ECs from hiPSCs. Roles of ETV2 played during the segregation of endothelial and myocardial lineages were also revealed using tthis reporter line in combination with bulk and scRNAseq approaches.

1.2.3 Derivation of ECs from hiPSCs

Since ECs are the major cell type affected in multiple vascular diseases, derivation of functional ECs from hiPSCs similar to patient primary ECs is the first and most essential step in modeling vascular diseases using hiPSCs. Most hiPSC-EC

differentiation protocols are based on our understanding of vascular development in the embryo. So far, most protocols for hiPSC-ECs have relied on three different approaches: coculture with stromal cells, EB formation and monolayer differentiation.

In 2009, two groups independently reported two similar methods for the differentiation of ECs from hiPSCs. Both groups found that coculture of hiPSCs with OP9 feeder cells resulted in the formation of endothelial progenitors which could be isolated and further induced into functional ECs (Choi et al., 2009; Taura et al., 2009). Later, several aggregation or EB-based differentiation methods were reported for the induction of ECs from hiPSCs (Adams et al., 2013; James et al., 2010; Kane et al., 2011; Nourse et al., 2010; Park et al., 2014; White et al., 2012). With EB-based methods, EBs are first cultured in pro-endothelial growth media (EGM) supplemented with VEGFA and BMP4 to induce mesodermal lineages. These mesoderm progenitors eventually give rise to ECs. The study also found that a Transforming Growth Factor beta (TGFβ) signaling inhibitor improved differentiation efficiency and maintained of endothelial phenotype over several passages (James et al., 2010). With these culture conditions, CD144 and CD31 positive cells were derived and purified for further expansion and differentiation. However, the majority of EB based protocols had low differentiation efficiencies, high heterogeneity of differentiated cells and were also time-consuming.

An alternative strategy now widely used is to culture hiPSC on a matrix-coated culture plate in monolayer and induce the stepwise differentiation from hiPSCs to mesoderm and finally ECs by timed addition of specific molecules and growth factors. Monolayer differentiation methods for EC differentiation from hPSCs were first described in 2010 although details were missing (Kane et al., 2010). Orlova et al. developed highly efficient monolayer differentiation methods for simultaneously producing CD31+ ECs and CD31- pericytes from hiPSCs using defined medum conditions (Orlova et al., 2014). With this protocol, a large number of ECs could be derived from hiPSCs in ~2 weeks. Notably, this protocol was tested using multiple hiPSC lines that were derived from different somatic cell types using different reprogramming methods. The functionalities of these hiPSC-ECs were evidenced by their ability to form primary vascular plexus in vitro and be incorporated into the vasculature of zebrafish (Orlova et al., 2014a). Meanwhile, others found that addition of retinoic acid could promote the expression of the mature endothelial cell marker VE-Cadherin and enhance the barrier function of differentiated ECs using monolayer protocols (Katt et al., 2016; Lippmann et al., 2014). Later, Patsch et al. developed a protocol in which Wnt activation and BMP4 were used to induce mesoderm for 3 days, followed by VEGF and Forskolin treatment for another 2 days, and then VEGF for 4 days. With this differentiation method, the authors reported up to 90% differentiation efficiency of ECs from hiPSCs (Patsch et al., 2015). Recently, an even shorter differentiation protocol was established which could induce ECs from hiPSCs in just 8 days with an efficiency of more than 80% (Harding et

al., 2017). It was reported recently that cyclic AMP synergistically enhanced the VEGF effects for the differentiation of ECs from the mesoderm stage in a serum-free 2D monolayer culture. Almost pure populations of ECs could be derived using stage-specific addition VEGF and cyclic AMP combined with the elimination of non-responder cells at early stages (Rajasingh et al., 2017). In this thesis, I used the method described by Orlova et al (2014b) to produce hiPSC-ECs from patients with inherited vascular disease and used an ETV2 reporter hiPSC line to examine molecular characteristics of the early and intermediate stages of differentiation. This allowed detailed examination of any heterpogeneity in the cell populations.

1.2.4 Characterization of hiPSC-ECs

ECs derived from hiPSCs have been isolated mainly based on their KDR, CD31 and VEC expression but their heterogeneity has been less studied. Rufaihah et al. found that high concentrations of VEGF-A (50 ng/ml) together with 8Br-cAMP could induce arterial ECs and low VEGF-A concentrations (10 ng/ml) led to a venous like EC identity. A combination of high concentration of VEGF-A and VEGF-C, together with angiopoietin 1 could induce lymphatic ECs (Rufaihah et al., 2013). Another group also confirmed that arterial and venous identities could be obtained by using different VEGF concentrations (Ram et al., 2015).

Although hiPSC-ECs have numerous features common with human primary ECs, including surface marker expression and 2D tube formation *in vitro*, comparing their functionality requires different kinds of assays. *In vivo* vessel formation is commonly used to examine functionality of hiPSC-ECs. In this assay, ECs are first mixed with 3D matrix (collagen, Matrigel, fibrinogen etc.) and transplanted subcutaneously into mice. Transplanted EC functionality is then assessed based on the vessel formation in the 3D matrix. We used this method to examine the functionality if diseased hiPSC-EC in vivo in chapter 6. Other *in vivo* models that have also been developed to test the therapeutic potential of hiPSC-ECs, included experimental hindlimb ischemia, myocardial infarction and retinopathy (Park et al., 2014; Prasain et al., 2014; Rufaihah et al., 2011; Sahara et al., 2014; Yoo et al., 2013). However, 3D matrix transplantation is the most reproducible and straightforward functional assay for hiPSC-ECs. In addition to mouse models, zebrafish embryos have also been used to test functionality of hiPSC-ECs in vivo (Kane et al., 2010; Orlova et al., 2014a). More recently, new methods to examine functionality through 3D vessel formation *in vitro* have been developed. hiPSC-ECs are first mixed in matrix and then cultured under static or flow conditions, where ECs can self-organize into vessel-like lumenised structures (Chan et al., 2015; Kusuma et al., 2013; Palpant et al., 2016; Park et al., 2014; Zanotelli et al., 2016; Zhang et al., 2017). It had been shown that ECs cultured in this way are more similar to those isolated from the 3D matrix transplant in vivo than ECs cultured in 2D culture dishes (Zanotelli et al., 2016; Zhang et

al., 2017).

Patient-specific hiPSC-ECs can be used as a model to study disease mechanisms and screen of candidate drugs. hiPSC-ECs had been used to model a number of genetic (cartdio)vascular diseases to date, including Moyamoya disease, Pulmonary arterial hypertension and Calcified aortic valve disease. (Lin et al., 2017).

1.2.5 Whole transcriptome analysis of hiPSC-ECs in development and disease

Along with the development of Next-generation sequencing (NGS) technologies, the emergence of RNA sequencing (RNAseq) brings new opportunities for diagnosis and treatment of human disease and to gain insights into how different cell types form in differentiation and development. RNAseq provides an in-depth view of the whole transcriptome within a cell sample and has a number of advantages compared to most conventional RNA measurement platforms, including qPCR and microarray. This includes hogher throughput. In comparison to more conventional methods, RNAseq can detect a greater range of transcripts expression than microarray, thus thousands of differentially expressed genes (DEGs) can be identified from comparison of two or more different samples.

Droplet-based single cell RNA sequencing (scRNAseq) is a commonly used method which I also used in my thesis. It proved an excellent tool for the study of global transcriptional dynamics during EC differentiation from hiPSCs. Several scRNAseq studies had been performed prior to my study for hiPSC-ECs differentiated with different methods. In one study, scRNAseq was performed on day 8 and day 12 of EC differentiation, although ECs accounted for only less than 10% of the total population while most cells were cardiac lineage. However, the transcriptional control and dynamics during the developmental process of EC were not further investigated in this study (Paik et al., 2018). In another study, scRNAseq was done at multiple time points during hESC-derived EC differentiation using a different protocol (McCracken et al., 2019). The transcriptional dynamics during EC specification and maturation were characterized using “pseudotime analysis” (McCracken et al., 2019). They observed a mesodermal population at day 4, followed by an emergence of endothelial and mesenchymal populations. Transcriptional signatures of endothelial commitment and maturation during the differentiation process were identified based on the transcriptional changes along pseudotime trajectory. Recently, our group developed a co-differentiation method for both ECs and cardiomyocytes from hiPSCs through a cardiac mesoderm stage (Giacomelli et al., 2017). The developmental process and transcriptional dynamics during the specification, as well as the segregation of these two lineages from mesoderm progenitors in this protocol had not so far been studied. Thus, in this thesis, we have carried out both bulk and scRNAseq to study the transcriptional control and dynamics of this differentiation system. We first generated

an ETV2^{mCherry} hiPSC reporter line in order to study the ETV2 expression pattern and its role during endothelial and myocardial lineages segregation and development. Bulk RNAseq could give a more in-depth view of the transcriptome profile, while scRNseq could generate an overview of the whole cell population in the differentiation system as well as the dynamics of each individual genes along the differentiation time.

In addition, transcriptomic analyses were also performed for ECs in HHT1 and PHE hiPSC models established in this thesis. Majorly, differentially expressed gene (DEG) and gene ontology (GO) enrichment analyses were utilized to reveal the underlying disease mechanisms of HHT1 and PHE.

1.3 Modeling HHT1 with hiPSCs

1.3.1 HHT

HHT is an autosomal dominant genetic vascular disease with a prevalence of 1 in 5000-10000 (Dakeishi et al., 2002; Marchuk et al., 1998; Westermann et al., 2003). HHT is characterized by recurrent nosebleeds and telangiectasias, especially on patients' lips, finger tips and nasal mucosa. There is a high chance for HHT patients to develop large arteriovenous malformations (AVM) in several organs, including lung (PAVM, 13-35%), brain (CAVM, 5-13%) and liver (HAVM, ~32%) (Letteboer, 2005). CAVM can lead to stroke and brain abscess. Most HHT patients show normal life spans. However, disease symptoms usually get worse during aging and new symptoms, for example gastrointestinal bleeding, may arise in old age. The "Curacao criteria" have been used for the diagnosis of HHT based on patient symptoms since 2000 (Shovlin et al., 2000). In practice, family history and the presence of AVM are often sufficient for diagnosis, due to the rare occurrence of AVM in the general population. There are few drugs that have proven consistently effective for the treatment of HHT patients yet. Treatment and care for the HHT nowadays mainly focused on prophylaxis of different symptoms, such as stroke and liver AVMs. Thalidomide and Bevacizumab are the two main drugs that are considered for HHT treatment and under clinical test (www.clinicaltrials.gov). Thalidomide and bevacizumab are known anti-angiogenic drugs that are used to treat nosebleeds in HHT patients. Thalidomide has shown ability to promote vascular maturation via PDGF-B activation (Lebrin et al., 2010) . Bevacizumab is a monoclonal antibody against vascular endothelial growth factor (VEGF) and has been used to treat fragile vessels and excessive angiogenesis that could cause nosebleeds in patients with HHT (Dupuis-Girod et al., 2012; Fleagle et al., 2012). However, their functional mechanisms and possible side-effects need to be further studied and alternative drugs are still being sought. More recently, an immune modulator, tacrilomus, is also being tested clinically.

Around 85% of HHT patients in the world have mutations in either *ENG* gene (HHT

type 1) or *ACVRL1* gene (HHT type 2). At least 525 different ENG mutations and 430 ACVRL1 mutations have been reported so far in the HHT database (see http://www.hhtmut.org). These mutations are distributed across all exons of these two genes, even though several exons seem to have higher chance to carry the mutation (Figure 2 A-B). In addition, mutations in *MADH4* gene were found to contribute the development of both HHT and juvenile polysis. Notably, all genes found so far that could lead to HHT are involved in TGFβ superfamily signaling pathway. In this thesis I focused on HHT1 caused by an ENDOGLIN mutation.

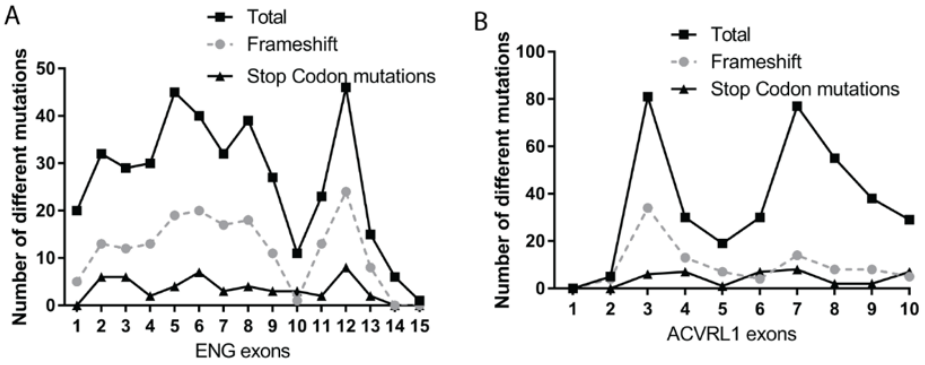


Figure 2 Distribution of all known ENG (A) and ACVRL1 (B) mutation on all exons of these two genes (all information about HHT mutations are coming from the HHT database: http://www.hhtmut.org)

1.3.2 TGFβ superfamily signaling pathway and endoglin

Endoglin is a receptor that is part of the TGFβ signaling pathway. The TGFβ superfamily includes a large number of secreted cytokines including TGFβs, activins, Bone Morphogenetic Protein (BMPs) and Growth Differentiation Factors (GDFs), which play crucial roles in different process during development and in the maintenance of hemostasis. The TGFβ family of ligands act on cells through their specific binding to transmembrane type I and type II receptors, which then can phosphorylate different R-SMADs mediators and regulate specific genes expression.

Different combinations of type I and type II receptors bind to different TGFβ family ligands. For example, TGFβ signals mainly by binding firstly to TGFβ Receptor II (TGFBR2) which recruits TGFβ Receptor I (TGFBR1, ALK5) to the receptor complex. BMP type II Receptor (BMPR2) and ALK1 type I receptor respond to different BMP ligands. Type III receptors such as betaglycan and endoglin, are essential for the regulation of TGFβ signaling specificity and affinity of ligand binding.

Human endoglin is 180 kDa disulfide-linked homodimeric transmembrane glycoprotein. It contains a large 561 amino acid extracellular domain and a serine/threonine-rich cytoplasmic region which contain 47 amino acid in the long isoform (L-endoglin) and 14 amino acid in the short isoform (S-endoglin). The extracellular domain contain an orphan domain which can mediate its interaction with TGFβ superfamily ligands, especially BMP9 and BMP10 (Castonguay et al., 2011; Saito et al., 2017). An arginine-glycine-aspartic (RGD) tripeptide is also present in the extracellular domain at Arg399-Asp401, suggesting that endoglin may also mediate cell adhesion process. Endoglin is highly expressed on vascular ECs and on activated monocytes, macrophages and smooth muscle cells. Mutations in Endoglin may thus affect the vascular cells directly or indirectly via inflammatory cells.

1.3.3 Role of endoglin in angiogenesis

Endoglin plays an important role in angiogenesis. This is evidenced by the fact that endoglin knockout mice die on gestational day 10.5 due to vascular development defects in the embryo (Arthur et al., 2000; Bourdeau et al., 1999; Li et al., 1999). Even though EC differentiation takes place, mature vascular networks do not form in the knock out mice, indicating that endoglin plays an essential role in angiogenesis rather than vasculogenesis (the differentiation of EC) (Arthur et al., 2000; Li et al., 1999). ES cells derived from endoglin knockout mice failed to undergo hematopoietic differentiation while endothelial differentiation was not affected. Proliferating Human Umbilical Vein Endothelial Cells (HUVECs) in culture showed clearly higher endoglin expression than quiescent HUVECs (Fonsatti et al., 2001). Evidence has shown that endoglin may be important for keeping the balance of TGFβ signaling through ALK1 and ALK5. Endoglin promotes ALK1 activation and indirectly inhibits ALK5 activity (Lebrin et al., 2004). Activation of TGFβ-ALK1 could induce EC proliferation and migration through Smad1/5/8 signaling pathway (in most cells only activated downstream of BMP signaling but exceptionally in ECs through TGFβ signaling), while TGFβ-ALK5 downstream activation of Smad 2 and 3 inhibits their proliferation and migration (Goumans et al., 2003). Knockout or blocking of endoglin in ECs could inhibit their proliferation and migration and enhance the effect of TGFβ1 on the inhibition of EC growth (Lebrin et al., 2004; Li et al., 2000). However, controversy still exists on the exact role of endoglin in angiogenesis. For example, both enhanced and reduced growth inhibition by TGFβ1 were observed with ECs derived from endoglin knockout mice (Lebrin et al., 2004; Pece-Barbara et al., 2005).

BMPs is another group of multifunctional growth factors in the TGFβ superfamily. Recent studies show emerging roles of BMPs played in angiogenesis. Like TGFβs, BMPs elicit their effects by binding to type I and type II receptors on the cell surface. But unlike TGFβs, BMPs showed high affinity of binding to both type I and type II receptors alone

independently. The receptor complex for BMPs also include a type III co-receptor, including betaglycan and endoglin. Endoglin can be shed from the cell surface by matrix metalloproteinase, suggesting that they can modulate the activity of BMP signaling pathway through the changing their surface expression levels (Hawinkels et al., 2010). BMP9 can bind to both ALK1 and ALK6, but with a much higher affinity to ALK1 compared to ALK6 (David et al., 2006). BMP9 triggers Smad1/5/8 phosphorylation in ECs with an EC_{50} of around 50 pg/ml. Different BMP type I receptors showed different specificities to Smad 1/5/8. ALK3 and ALK6 can activate all three Smads, whereas ALK2 can only activate Smad 1 and 5 (Miyazono et al., 2005; Reddi, 2001). Freshly-isolated mouse aorta and pulmonary endothelium showed high phosphorylation of Smad 1/5/8, indicating that ECs were physiologically activated by BMPs (Valdimarsdottir et al., 2002). Studies showed that BMP signaling through ALK1 is mainly implicated in the maturation phase of angiogenesis. BMP9 could inhibit the proliferation and migration of dermal Human Mammary Epithelial Cells (HMECs) and Bovine Aorta Endothelial Cells (BAECs) (David et al., 2006; Scharpfenecker et al., 2007). The inhibitory effect of BMP9 on ECs was confirmed in an ex vivo metatarsal culture model (Scharpfenecker et al., 2007). Besides, BMP9 also showed inhibition of neo-angiogenesis *in vivo* (David et al., 2008). All these *in vivo* and in vitro studies demonstrated that endoglin/ALK1/BMPRII signaling pathway play important roles in angiogenesis.

Heterozygous *ENG*$^{+/-}$ mice showed mild phenotypes but using a different strain of mice, they showed different disease phenotypes, providing evidence that genetic background also plays a role in the development of the disease (Bourdeau et al., 1999). Aside from a mild disease phenotype, it also appeared at very low frequency in heterozygous mice (Bourdeau et al., 1999; Torsney et al., 2003). Reduced angiogenesis in adult *ENG*$^{+/-}$ mice has been reported in pathological conditions, like femoral artery ligation(Jerkic et al., 2006), myocardial infarction (van Laake et al., 2006), and stroke (Shen et al., 2014). Treatment of *ENG*$^{+/-}$ mice with pro-angiogenic factors could induce abnormal angiogenesis but not AVMs, indicating other stimulations were necessary for the development of AVMs in mice models (Xu et al., 2016). Lebrin and colleagues found that treatment of *ENG*$^{+/-}$ mice with Thalidomide could promote vascular maturation through increased PDGF-B expression in ECs (Lebrin et al., 2010). Cell type specific loss of *ENG* showed that the endoglin deficiency in ECs was a prerequisite for AVM formation, whereas specific knockout in pericytes, vSMCs and macrophages had no effect on the AVM formation (Choi et al., 2014; Garrido-Martin et al., 2014). However, both complete knockout and conditional knockout mice model are distinct from the situation of HHT1 patients. So far, based on the studies that had been done with heterozygous mouse models, it is still not clear how these severe symptoms developed in HHT1 patients.

1.3.4 Role of myeloid cells in HHT1

1.3.4.1 Inflammation and HHT1

Inflammation is an adaptive immune response that is triggered by various conditions including infection, tissue injury, tissue stress and malfunction. A controlled inflammation response is beneficial as it could provide protection from infection and promote tissue repair and adaption to stress. However, dysregulated inflammation may have pathological consequences. For example, uncontrolled inflammation can lead to autoimmunity, fibrosis and tumor growth.

Inflammation is an important trigger for the development of HHT1 disease phenotypes in endoglin knockout mouse models. The failure to recapitulate (heterozygous) HHT1 patient phenotypes in mice suggested that additional triggers may be needed for the development of disease symptoms, including aging, wounding and inflammation. Pro-angiogenic factors or inflammatory stimuli could successfully induce AVMs in these conditional knockout *ENG* deficient mice (Choi et al., 2014; Garrido-Martin et al., 2014). Some researchers have proposed that at least three events were needed for the development of AVMs: endoglin haploinsufficiency in ECs; loss of heterozygosity (becoming homozygous) in some tissues due to accumulated endoglin mutation or shedding during inflammation by MMPs; pro-angiogenic or inflammatory stimuli (Tual-Chalot et al., 2015).

Evidence has shown that the inflammation responses were also affected due to the endoglin deficiency in both HHT1 patients and mouse models. The prevalence of severe infection in HHT1 patients is around 22.8%, which is much higher than chance in the general healthy population. It also had been shown that HHT1 patients had much higher chance of developing cerebral infection than HHT2 and HHT3 patients (Girod et al., 2007). $ENG^{+/-}$ mice also developed more severe colon inflammation induced by DSS compared with control mice (Jerkic et al., 2010). Another study found that after injection of LPS, $ENG^{+/-}$ mice exhibit reduced myeloid cell recruitment in lung tissue compared to the control mice in an systemic inflammation model (Rossi et al., 2013). In addition, functions of myeloid cells also seemed to be impaired because of the endoglin mutation. Conditional knockout of *ENG* specifically in macrophages in mice impaired the phagocytotic activity of macrophages (Ojeda-Fernandez et al., 2016). Interestingly, compared with mononuclear cells isolated from healthy individuals, mononuclear cells from HHT1 patients showed defective repair and migratory activity to the injured heart following myocardial infarction in mice (van Laake et al., 2006). Post and colleagues found that HHT1 patient blood monocytes showed impaired response to SDF-1α (Post et al., 2010). All of these studies indicated a correlation between inflammation and HHT1. On the one hand, inflammation serves as a trigger for the development of HHT1 disease symptoms. On the other hand, endoglin mutation impairs functions of myeloid cells and leads to an abnormal inflammation response in

both patients and mouse models.

1.3.4.2 Monocytes and ECs interaction in inflammation

Monocytes play a pivotal role in both the initiation and resolution of inflammation. Recruitment of blood monocytes to the site of injury or infection by extravasation through endothelium are essential for the inflammation response. The extravasation of blood monocytes is a tightly regulated process that consists of a series of interactions between monocytes and ECs and the final transmigration step. The adhesion between monocytes and ECs is mainly via the interaction between integrins (extracellular matrix protein receptors) expressed on monocytes and the immunoglobulin superfamily that is expressed on ECs (Meerschaert and Furie, 1995) (Figure 3).

Upon stimulation, for example after infection, tissue resident macrophages can be activated and secrete proinflammatory cytokines including tumor necrosis factor-α (TNF-α) and IL-1β. These inflammatory cytokines can transiently activate ECs of blood vessels nearby and upregulate their expression of chemokines and adhesion molecules, mainly P- and E-selectin, ICAM-1 and VCAM1. First, the interaction between selectins on endothelium can bind to O-glycosylated carbohydrate ligands displayed on P-selectin glycoprotein ligand-1 (PSGL-1) that expressed on monocytes. This interaction could capture monocytes and slow them down in the blood flow and then enable them to roll on the endothelium wall. The slow rolling process was strongly depend on the expression VLA-4 ($\alpha4\beta1$ integrin) and CD44 that expressed on monocytes (Mestas and Ley, 2008). Then interaction between VCAM1 on activated endothelium and VLA-4 on monocytes mediates the transition from slow rolling to firm adhesion. Next is the directional chemotactic and mechanotactic step, in which monocytes spread, polarize, and subsequently locomote laterally to find preferred sites of extravasation. The intraluminal crawling depends on the interaction of leucocyte integrins lymphocyte function-associated antigen-1 (LFA-1) and macrophage-1-antigen (Mac-1) on monocytes and their ligands ICAM-1 and ICAM-2 on ECs. Blocking of these adhesion molecules was shown to disable crawling and the subsequent transmigration (Wong et al., 2010). Notably, ICAM-1 and VCAM-1 are expressed diffusely on endothelial cell surface in rest condition, but will be concentrated to the interface with monocytes during the adhesion and rolling (Shaw et al., 2004), which is depend on the actin cytoskeleton and remodeling events that include the Src-mediated phosphorylation of cortactin, an actin binding protein (Schnoor et al., 2011; Yang et al., 2006). Finally, to migrate out of blood vessels, monocytes must negotiate the endothelium, the lamina basalis and the embedded pericytes (Figure 3). As all adhesion and rolling processes discussed above are reversible, the majority of monocytes that initially bind to endothelium cannot finish the transmigration step. Transmigration seems to be the crucial event in the monocyte adhesion cascade, as it is the only step that is hardly ever

reversed although with some notable exceptions (Woodfin et al., 2011).

Monocytes transmigrate through vascular endothelium by two different modes: the paracellular route by which monocytes migrate through the junctions between ECs and requires junctional remodeling; the transcellular route that directly through fusing vesicles in the endothelial cell. The paracellular mode is predominant although it's still difficult to accurately quantify the ratio of these two modes (Carman et al., 2007). The transmigration process is tightly regulated by the junctional molecules on ECs, including VE-Cadherin, the junctional adhesion molecule (JAM) family, PECAM-1 PVR/DNAM-1, and CD99 and their interaction with integrin on monocytes (Gerhardt and Ley, 2015; Muller, 2015; Schnoor et al., 2015).

It is known already that the adhesion to endothelium and transmigration could change the gene expression profile of monocytes dramatically. Genes that related to chemokines, immune response and inhibition of apoptosis were upregulated after transmigration of monocytes (Thomas-Ecker et al., 2007; Williams et al., 2009). After transmigrating into the tissue, monocytes will terminally differentiate into macrophages and dendritic cells, which can phagocytose pathogens and regulate the inflammation process by secretion of pro-inflammatory and anti-inflammatory cytokines. During the resolution phase of inflammation, macrophages are responsible for the cleaning up dead cells through efferocytosis, and also promote tissue regeneration and angiogenesis through the secretion of various growth factors.

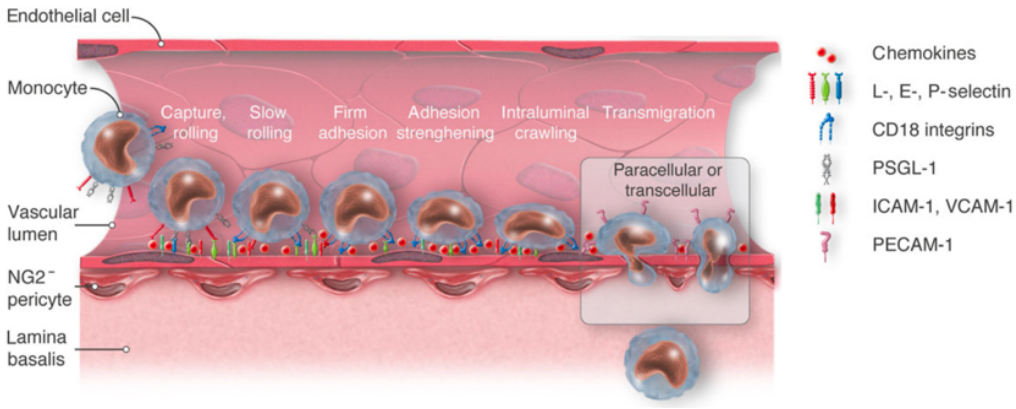


Figure 3 Schematic overview of monocyte adhesion and transmigration through vessel wall. A multistep cascade of capture, rolling, slow rolling, firm adhesion, adhesion strengthening, and intraluminal crawling precedes the transendothelial migration of monocytes. Two modes of transmigration are indicated: a paracellular and a transcellular. Activated ECs express adhesion molecules and chemokines that interact with monocytic ligands. Such interactions essentially mediate the various steps of the adhesion cascade. This figure is adapted from (Gerhardt and Ley, 2015).

1.3.4.3 Regulation of angiogenesis by macrophages

Macrophages are specialized cells that play diverse roles in human body, including immune response, tissue repair and homeostasis (Wynn et al., 2013). Macrophages derived from precursor monocytes are continuously produced from hematopoietic stem cells (HSCs), which develop through definitive hematopoiesis (Medvinsky et al., 2011) . Direct association with macrophages has been well documented in wide range of diseases (Wynn et al., 2013). Macrophages are highly heterogeneous and can change their phenotype and function depending on the stimuli or immune response induced by local tissue environment. Depending on the distinct immune function they possess, macrophages have been classified into different subsets, known as M1, M2a, M2b and M2c (Mantovani et al., 2013; Rőszer, 2015). M1 macrophages secrete various proinflammatory cytokines and chemokines to enhance the inflammation response and recruit more immune cells to the inflamed tissue, mainly neutrophils and monocytes. Dead neutrophils and tissues can then stimulate and change M1 macrophages into an M2 state (alternative activated macrophages), which can inhibit the inflammation process and promote tissue repair and angiogenesis after the injury or infection.

Macrophages can regulate angiogenesis through the secretion of variety of proangiogenic and antiangiogenic growth factors or cytokines (Corliss et al., 2016). Studies showed that VEGFA secreted by macrophages was essential for angiogenesis during wound healing (Lucas et al., 2010; Stockmann et al., 2011; Urbich et al., 2003; Willenborg et al., 2012). Also, evidence indicates that different subtypes of macrophages may exert regulatory roles in different stages of angiogenesis, according to their different secretion profiles. For example, M1 macrophages may be involved in the initiation of angiogenesis as they produce high level of VEGF-A and FGF2. M2a macrophages secrete high amounts of PDGF-BB and M2c produce high level of MMP9. M1 macrophages stimulate capillary sprouting, while M2 macrophages may aid in vessel stabilization through pericyte recruitment (Spiller et al., 2014).

Macrophages have been shown to make direct contact with ECs to regulate angiogenesis. During the development of hindbrain of mouse and zebrafish, macrophages can bridge two EC sprouts and promote tip cell fusion (Fantin et al., 2010). Studies have also shown that microglia are associated with tip cells during retina development. Knockout of microglia resulted in sparser retinal vascular network with less branching (Kubota et al., 2009). A better understanding of macrophages and their role in angiogenesis may inspire new therapeutic strategies for regenerating tissues and curing diseases that are caused by deregulated angiogenesis, such as HHT1.

1.3.5 Modeling HHT1 using hiPSCs

Until now, most of our knowledge about the disease pathology of HHT1 comes from

the study of either patient material or mutant mouse models. Using patient material is unavoidably restricted by its availability. Peripheral blood or small tissue biopsies of HHT1 patients can be obtained under certain conditions from HHT hospital centres. However, given the condition is rare, it would be difficult to draw statistically significant conclusions based on studies using cells collected from a single or low number of patients, as the disease phenotype largely depends on genetic background, patient age and general health condition. Nevertheless, some researchers have managed to obtain sufficient numbers of patient cells for gene profiling analysis, where they found nasal telangiectasia of HHT1 patients express abnormal levels of genes that involved in angiogenesis, TGFβ and Wnt signaling pathways (Tørring et al., 2015).

Although mouse models of HHT1 are most commonly used for the study of disease pathology, they do have several drawbacks. First of all, the genetic background of mice and human are quite different. Second, phenotypes showed in mouse models are different from disease phenotypes found in human patients. For example, it is still a question on how to induce AVMs in $ENG^{+/-}$ mice. Third, it may take a long time for the generation of endoglin knockout mice and the appearance of disease phenotypes, especially those related to ageing. Last but not least, mouse experiments raise ethical issues and animal protection concerns.

In 2006, iPSCs were first generated from mouse fibroblasts by overexpressing four transcription factors (known as Yamanaka factors) normally expressed in ES cells. These mouse iPSC were similar to ES cells(Takahashi and Yamanaka, 2006). Shortly thereafter, human somatic cells were also successfully reprogrammed to hiPSCs (Takahashi et al., 2007). hiPSCs can also self-renew indefinitely in culture and differentiate into many different kinds of cell types, which make them potentially an excellent tool for disease modeling since they capture the genome of the patient from who they are derived. hiPSC derived cells can be used for the study of disease pathologies and drug screening (Robinton and Daley, 2012). Recent advances in genome editing, including TALENs and CRISPR/Cas systems, now allow rapid and efficient introduction of genetic modifications in hiPSCs. Both techniques apply nuclease to cut genome DNA to get a double strand break in the specific location with the help of guiding RNA sequence. Then specific modification of the sequence could be achieved through DNA repair by non-homologous end joining (NHEJ) or homology directed recombination (HDR) when a template DNA molecule is available (Kim, 2016). These techniques further facilitate the application of hiPSCs for the modeling of different genetic diseases. For example, in order to study the effect of genetic background on the development of a genetic disease, a same mutation could be introduced into a control hiPSC line and compare it with the patient-derived hiPSC line. The mutation carried by the patient hiPSC line could also be corrected using genome editing to create an isogenic control, by which the effect of genetic variation except the mutation on the pathogenic gene could be excluded.

There are several potential advantages of using hiPSC-derived cells for modeling of HHT1. First, patient-specific hiPSCs and their derivatives carry the same genetic background as HHT1 patients. Second, unlimited numbers and different types of vascular and other cells likely similar to primary cells from patients can be derived from patient specific hiPSCs. Third, the differentiation processes of different cells types from hiPSCs are relatively short compared to the generation of mouse models, as described in section 1.1.1 and 1.2.3. Fourth, CRISPR/Cas 9 genetic modification technologies make it much easier to correct the *ENG* mutation in patient specific hiPSC lines to generate isogenic control lines, which are necessary for the study of disease related phenotypes. The potential disadvantage is that the hiPSC derivatives may not be mature enough to mimic the disease in adults.

One of the major aims of this thesis is establishing a multicellular vascular model of HHT1 using patient-derived hiPSCs. To better recapitulate the microenvironment of blood vessels *in vivo*, 3D culture combining different cellular components associated with blood vessel development and functions are required. Previous work in our group showed that in hiPSC-ECs with endoglin haploinsufficiency the TGFβ signaling pathway was unaffected and functionalities normal in 2D culture conditions (unpublished data). Here, we decided to investigate whether patient -like phenotypes would be evident in a 3D culture model combining ECs and mural cells-vascular smooth muscle cells (vSMCs) derived from isogenic HHT1 hiPSC lines. We found dysregulated interactions between EC and mural cells which had been shown to be a major underlying pathology of HHT1 in mutant mice, evidenced by impaired vSMC development and mural cell recruitment and coverage of blood vessels (Lebrin et al., 2010).

As the next step (not included in this chapter), we modeled the inflammation process with patient derivativs by thesting adhesion of monocyets on ECs. We are also working on including of inflammatory cell component – IPSDMs in our 3D vascular model. As above in **section 1.3.5** of this thesis, inflammation and myeloid cells (especially macrophages) are closely related to the onset and development of HHT1 phenotypes. Thus, by including IPSDMs in our model, we expected to observe new disease phenotypes related to inflammation condition and to identify new disease pathologies which would provide essential clues for the drug development and screening in the future.

1.4 Modeling PHE with hiPSCs

1.4.1 PHE

PHE is tumor associated with blood vessels with intermediate malignant potential which mainly affects young adults and has a male predominance. PHE has similar clinical presentation as epithelioid sarcoma. The tumor is known to express several EC markers

although with morphology distinct from vasculature and involving different anatomic planes, including dermis, subcutis, and skeletal muscle (Al-Qaderi and Mansour, 2019).

In 2014, Walther and colleagues revealed a balanced t(7;19)(q22;q13) chromosome translocation in several PHE patients which lead to a fusion of the *SERPINE1* and *FOSB* genes (Walther et al., 2014). FOSB dimerizes with Jun proteins to form AP-1 transcriptional complex which was found to drive various tumors. The overexpression of FOSB under the SERPINE1 promoter after translocation is considered as the mechanism underlying the pathology of PHE.

Local recurrence was commonly observed in PHE, while few patients develop distant metastasis (Inyang et al., 2016). Currently there is still no effective treatment for this tumor, although drugs targeted rapamycin inhibitors are under clinical test, including everolimus (Ozeki et al., 2017) and sirolimus (Gabor et al., 2018).

1.4.2 Disease Modeling of PHE

Establishing an in vitro model of PHE would greatly facilitate the study of underlying disease mechanisms and development of screening platforms for candidate drugs. However, no tumor cell line has so far been established for this disease in part because PHE is so rare. hiPSC would an alternative approach to model PHE and they could be produced by introducing the chromosomal translocation into WT hiPSCs by genome editing. Previously, chromosomal translocations have been successfully introduced into human umbilical cord-derived mesenchymal stromal cells (hMSCs), umbilical cord blood-derived CD34+ cells, and more recently human induced pluripotent stem cells (hiPSCs) using CRISPR/Cas9 (Schneidawind et al., 2018; Torres et al., 2014; Torres-Ruiz et al., 2017; Vanoli et al., 2017). Thus, we hypothesized that by introducing *SERPINE1-FOSB* translocation into hiPSCs and differentiating them into EC using established protocol (Halaidych et al., 2018; Orlova et al., 2014b; 2014a) we may establish an hiPSC disease model for PHE which could be further used for further study. This was indeed carried out in the study presented in chapter 6 where my focus was primarily on the transcriptional analysis of the mutant hiPSC-derived ECs in relationship to the isogenic wild-type cells.

1.4.3 Transcriptomic analysis of the hiPSCs model of PHE

Whole transcriptome sequencing and analysis provides a powerful tool for the revealing of dysregulated transcription networks and indicating potential underlying disease mechanisms of PHE using the PHE-hiPSC model. The study demonstrated that ECs are indeed the likely cell of origin of PHE as postulated before this study. Comparison of ECs derived from PHE and isogenic WT hiPSCs could then provide new insights into the nature of the disease. Although *FOSB* overexpression had been identified as the major driver of tumor development, it is still not fully clear what the downstream

transcriptional changes are and their correlation with observed disease phenotypes of PHE. Whole transcriptome sequencing allowed us to identify the majority of dysregulated genes in PHE-hiPSC-EC model. Gene Ontology (GO) enrichment analysis enabled us to link these identified DEGs with different cell signaling pathways and activities. Moreover, gene regulation networks could be generated using available gene-gene interaction prediction tools like Ingenuity Pathway Analysis (IPA). Novel therapeutic targets and candidate drugs could this be identified based on these RNAseq analyses.

1.5 Aims of this thesis

The overall aim of the work in this thesis was to establish vascular models in culture using hiPSC-ECs and myeloid cells for a genetic vascular diseases HHT1 and a vascular tumor PHE. To achieve this goal, several questions needed to be addressed first and each is presented in the chapters of this thesis.

Much of the research in this thesis is intended to address the underlying mechanisms of HHT1, a major focus of our research group. To model the crucial inflammatory component of HHT1 using hiPSCs, **first** we needed to establish a robust differentiation protocol for monocytes and macrophages from hiPSCs, which could be included in the vascular part of the HT-hiPSC model. The protocol for this was established (chapters 2 and 3) and the monocytes/macrophages (IPSDMs) functionally characterized. A detailed comparison of IPSDMs with PBDMs was performed in terms of gene expression and functional behavior. **Second**, the derivation and characterization of hiPSC-derived ECs, as EC is the major cell type affected in HHT1 and the major component of HHT1 hiPSC model. A reporter hiPSC line for *ETV2* was generated using CRISPR/Cas9 technology and used to initiate a study the developmental origin and maturation process of hiPSC-derived ECs (chapter 4). **Third**, derivation of an hiPSC line from HHT1 patient somatic cells and generation of its isogenic control line without endoglin mutation. Fortunately, this did not require genetic engineering since we have identified a mosaic patient who carried the endoglin mutation only in some but not all tissues. We thus managed to establish a pair of isogenic hiPSC lines from both wild type and mutated cells from this mosaic patient. **Fourth**, differentiation and characterization of ECs from isogenic HHT-hiPSC lines based on their transcriptome profile and functional analysis. **Finally**, an *in vitro* disease model for HHT1 by combining ECs and mural cells in 2D and 3D culture systems was established (chapter 5). With the disease model established the aim was to ultimately provide new insight into the HHT1 disease mechanisms and an experimental platform for screening potential drugs for disease treatment.

Finally, another disease we modeled in this thesis was PHE. This was part of a

collaborative study; my contribution was whole transcriptomic sequencing and bioinformatic analysis of PHE and WT hiPSC-ECs to identify the dysregulated transcriptional network which could contribute to revealing of the underlying pathology of PHE and identify new therapeutic targets (chapter 6). At the end, a general Discussion of the outcome of this thesis is provided in Chapter 7. A number of important issues have been addressed that would be needed to create representative human models for HHT1 and PHE in culture. The outcome has provided important cellular tools that can help move the field forward and proved the basis for better understanding of the mechanisms underlying the genetic forms of vascular disease.

References

Abedin, M., Nguyen, A., Jiang, N., Perry, C., Shelton, J., Watson, D., and Ferdous, A. (2014). Fli1 acts downstream of Etv2 to govern cell survival and vascular homeostasis via positive autoregulation. Circ Res *114*, 1690–1699.

Adams, W.J., Zhang, Y., Cloutier, J., Kuchimanchi, P., Newton, G., Sehrawat, S., Aird, W.C., Mayadas, T.N., Luscinskas, F.W., and García-Cardeña, G. (2013). Functional Vascular Endothelium Derived from Human Induced Pluripotent Stem Cells. Stem Cell Rep 1, 105–113.

Aflaki, E., Stubblefield, B., Maniwang, E., Lopez, G., Moaven, N., Goldin, E., Marugan, J., Patnaik, S., Dutra, A., Southall, N., et al. (2014). Macrophage models of Gaucher disease for evaluating disease pathogenesis and candidate drugs. Sci Transl Med *6*, 240ra73.

Al-Qaderi, A., and Mansour, A. (2019). Pseudomyogenic Hemangioendothelioma. Arch Pathol Lab Med *143*, 763–767.

Arthur, H.M., Ure, J., Smith, A.J., Renforth, G., Wilson, D.I., Torsney, E., Charlton, R., Parums, D.V., Jowett, T., Marchuk, D.A., et al. (2000). Endoglin, an Ancillary TGFβ Receptor, Is Required for Extraembryonic Angiogenesis and Plays a Key Role in Heart Development. Dev Biol *217*, 42–53.

Astorga, J., and Carlsson, P. (2007). Hedgehog induction of murine vasculogenesis is mediated by Foxf1 and Bmp4. Development *134*, 3753–3761.

Becker, P., Sacilotto, N., Nornes, S., Neal, A., Thomas, M., Liu, K., Preece, C., Ratnayaka, I., Davies, B., Bou-Gharios, G., et al. (2016). An Intronic Flk1 Enhancer Directs Arterial-Specific Expression via RBPJ-Mediated Venous Repression. Arteriosclerosis Thrombosis Vasc Biology *36*, 1209–1219.

Bernat, J., Crawford, G., Ogurtsov, A., Collins, F., Ginsburg, D., and Kondrashov, A. (2006). Distant conserved sequences flanking endothelial-specific promoters contain tissue-specific DNase-hypersensitive sites and over-represented motifs. Hum Mol Genet *15*, 2098–2105.

Bourdeau, A., Dumont, D., and Letarte, M. (1999). A murine model of hereditary hemorrhagic telangiectasia. J Clin Invest *104*, 1343–1351.

Bras, A., Yu, B., Bhaloo, S., Hong, X., Zhang, Z., Hu, Y., and Xu, Q. (2018). Adventitial Sca1+ Cells

Transduced With ETV2 Are Committed to the Endothelial Fate and Improve Vascular Remodeling After Injury. Arteriosclerosis Thrombosis Vasc Biology *38*, 232–244.

Brault, J., Goutagny, E., Telugu, N., Shao, K., Baquié, M., Satre, V., Coutton, C., Grunwald, D., Brion, J.-P., Barlogis, V., et al. (2014). Optimized Generation of Functional Neutrophils and Macrophages from Patient-Specific Induced Pluripotent Stem Cells: Ex VivoModels of X 0-Linked, AR22 0- and AR47 0- Chronic Granulomatous Diseases. Bioresearch Open Access *3*, 311–326.

van Bueren, K., and Black, B.L. (2012). Regulation of endothelial and hematopoietic development by the ETS transcription factor Etv2. Curr Opin Hematol *19*, 199–205.

Carman, C., Sage, P., Sciuto, T., de la Fuente, M., Geha, R., Ochs, H., Dvorak, H., Dvorak, A., and Springer, T. (2007). Transcellular diapedesis is initiated by invasive podosomes. Immunity *26*, 784–797.

Carmeliet, P., Ferreira, V., Breier, G., Pollefeyt, S., Kieckens, L., Gertsenstein, M., Fahrig, M., Vandenhoeck, A., Harpal, K., Eberhardt, C., et al. (1996). Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. Nature *380*, 435–439.

Castonguay, R., Werner, E., Matthews, R., Presman, E., Mulivor, A., Solban, N., Sako, D., Pearsall, R., Underwood, K., Seehra, J., et al. (2011). Soluble endoglin specifically binds bone morphogenetic proteins 9 and 10 via its orphan domain, inhibits blood vessel formation, and suppresses tumor growth. J Biol Chem *286*, 30034–30046.

Chan, X., Black, R., Dickerman, K., Federico, J., Lévesque, M., Mumm, J., and Gerecht, S. (2015). Three-Dimensional Vascular Network Assembly From Diabetic Patient-Derived Induced Pluripotent Stem Cells. Arteriosclerosis Thrombosis Vasc Biology *35*, 2677–2685.

Chao, M., Alizadeh, A., Tang, C., Myklebust, J., Varghese, B., Gill, S., Jan, M., Cha, A., Chan, C., Tan, B., et al. (2010). Anti-CD47 antibody synergizes with rituximab to promote phagocytosis and eradicate non-Hodgkin lymphoma. Cell *142*, 699–713.

Choi, E.-J., Chen, W., Jun, K., Arthur, H.M., Young, W.L., and Su, H. (2014). Novel brain arteriovenous malformation mouse models for type 1 hereditary hemorrhagic telangiectasia. Plos One *9*, e88511.

Choi, J., Dong, L., Ahn, J., Dao, D., Hammerschmidt, M., and Chen, J. (2007). FoxH1 negatively modulates flk1 gene expression and vascular formation in zebrafish. Dev Biol *304*, 735–744.

Choi, K.-D., Yu, J., Smuga-Otto, K., Salvagiotto, G., Rehrauer, W., Vodyanik, M., Thomson, J., and Slukvin, I. (2009). Hematopoietic and Endothelial Differentiation of Human Induced Pluripotent Stem Cells. Stem Cells *27*, 559–567.

Choi, K.-D., Vodyanik, M., and Slukvin, I.I. (2011). Hematopoietic differentiation and production of mature myeloid cells from human pluripotent stem cells. Nat Protoc *6*, 296–313.

Chu, M., Li, T., Shen, B., Cao, X., Zhong, H., Zhang, L., Zhou, F., Ma, W., Jiang, H., Xie, P., et al. (2016). Angiopoietin receptor Tie2 is required for vein specification and maintenance via regulating COUP-TFII. Elife *5*, 4552.

Corliss, B., Azimi, M., Munson, J., Peirce, S., and Murfee, W. (2016). Macrophages: An

Inflammatory Link Between Angiogenesis and Lymphangiogenesis. Microcirculation *23*, 95–121.

Craig, M.P., Grajevskaja, V., Liao, H.-K., Balciuniene, J., Ekker, S.C., Park, J.-S., Essner, J.J., Balciunas, D., and Sumanas, S. (2015). Etv2 and Fli1b Function Together as Key Regulators of Vasculogenesis and Angiogenesis. Arteriosclerosis Thrombosis Vasc Biology *35*, 865–876.

Dakeishi, M., Shioya, T., Wada, Y., Shindo, T., Otaka, K., Manabe, M., Nozaki, J.-I., Inoue, S., and Koizumi, A. (2002). Genetic epidemiology of hereditary hemorrhagic telangiectasia in a local community in the northern part of Japan. Hum Mutat *19*, 140–148.

David, L., Mallet, C., Mazerbourg, S., Feige, J.-J., and Bailly, S. (2006). Identification of BMP9 and BMP10 as functional activators of the orphan activin receptor-like kinase 1 (ALK1) in endothelial cells. Blood *109*, 1953–1961.

David, L., Mallet, C., Keramidas, M., Lamande, N., Gasc, J., Dupuis-Girod, S., Plauchu, H., Feige, J., and Bailly, S. (2008). Bone Morphogenetic Protein-9 Is a Circulating Vascular Quiescence Factor. Circ Res *102*, 914–922.

Dejana, E., Taddei, A., and Randi, A. (2007). Foxs and Ets in the transcriptional regulation of endothelial cell differentiation and angiogenesis. Biochimica Et Biophysica Acta Bba - Rev Cancer *1775*, 298–312.

Dupuis-Girod, S., Ginon, I., Saurin, J.-C., Marion, D., Guillot, E., Decullier, E., Roux, A., Carette, M.-F., Gilbert-Dussardier, B., Hatron, P.-Y., et al. (2012). Bevacizumab in Patients With Hereditary Hemorrhagic Telangiectasia and Severe Hepatic Vascular Malformations and High Cardiac Output. Jama *307*, 948–955.

Fantin, A., Vieira, J., Gestri, G., Denti, L., Schwarz, Q., Prykhozhij, S., Peri, F., Wilson, S., and Ruhrberg, C. (2010). Tissue macrophages act as cellular chaperones for vascular anastomosis downstream of VEGF-mediated endothelial tip cell induction. Blood *116*, 829–840.

Ferdous, A., Caprioli, A., Iacovino, M., Martin, C.M., Morris, J., Richardson, J.A., Latif, S., Hammer, R.E., Harvey, R.P., Olson, E.N., et al. (2009). Nkx2–5 transactivates the Ets-related protein 71gene and specifies an endothelial/endocardial fate in the developing embryo. Proc National Acad Sci *106*, 814–819.

Ferrara, N., Carver-Moore, K., Chen, H., Dowd, M., Lu, L., O'Shea, K., Powell-Braxton, L., Hillan, K., and Moore, M. (1996). Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. Nature *380*, 439–442.

Fleagle, J., Bobba, R., Kardinal, C., and Freter, C. (2012). Iron deficiency anemia related to hereditary hemorrhagic telangiectasia: response to treatment with bevacizumab. Am J Medical Sci *343*, 249–251.

Fonsatti, E., Vecchio, L., Altomonte, M., Sigalotti, L., Nicotra, M., Coral, S., Natali, P., and Maio, M. (2001). Endoglin: An accessory component of the TGF-?-binding receptor-complex with diagnostic, prognostic, and bioimmunotherapeutic potential in human malignancies. J Cell Physiol *188*, 1–7.

Gabor, K., Sapi, Z., Tiszlavicz, L., Fige, A., Bereczki, C., and Bartyik, K. (2018). Sirolimus therapy in the treatment of pseudomyogenic hemangioendothelioma. Pediatr Blood Cancer *65*, e26781.

Garrido-Martin, E.M., Nguyen, H.-L., Cunningham, T.A., Choe, S., Jiang, Z., Arthur, H.M., Lee, Y.-J., and Oh, P.S. (2014). Common and Distinctive Pathogenetic Features of Arteriovenous Malformations in Hereditary Hemorrhagic Telangiectasia 1 and Hereditary Hemorrhagic Telangiectasia 2 Animal Models—Brief Report. Arteriosclerosis Thrombosis Vasc Biology *34*, 2232–2236.

Gerhardt, T., and Ley, K. (2015). Monocyte trafficking across the vessel wall. Cardiovasc Res *107*, 321–330.

Giacomelli, E., Bellin, M., Sala, L., van Meer, B.J., Tertoolen, L.G., Orlova, V.V., and Mummery, C.L. (2017). Three-dimensional cardiac microtissues composed of cardiomyocytes and endothelial cells co-differentiated from human pluripotent stem cells. Development *144*, 1008–1017.

Girod, S., Giraud, S., Decullier, E., Lesca, G., Cottin, V., Faure, F., Merrot, O., Saurin, J., Cordier, J., and Plauchu, H. (2007). Hemorrhagic Hereditary Telangiectasia (Rendu-Osler Disease) and Infectious Diseases: An Underestimated Association. Clin Infect Dis *44*, 841–845.

Goumans, M., Valdimarsdottir, G., Itoh, S., Lebrin, F., Larsson, J., Mummery, C., Karlsson, S., and ten Dijke, P. (2003). Activin receptor-like kinase (ALK)1 is an antagonistic mediator of lateral TGFbeta/ALK5 signaling. Mol Cell *12*, 817–828.

Gul, N., and van Egmond, M. (2015). Antibody-Dependent Phagocytosis of Tumor Cells by Macrophages: A Potent Effector Mechanism of Monoclonal Antibody Therapy of Cancer. Cancer Res *75*, 5008–5013.

Halaidych, O., Freund, C., van den Hil, F., Salvatori, D., Riminucci, M., Mummery, C., and Orlova, V. (2018). Inflammatory Responses and Barrier Function of Endothelial Cells Derived from Human Induced Pluripotent Stem Cells. Stem Cell Rep *10*, 1642–1656.

Harding, A., Cortez-Toledo, E., Magner, N., Beegle, J., Coleal-Bergum, D., Hao, D., Wang, A., Nolta, J., and Zhou, P. (2017). Highly Efficient Differentiation of Endothelial Cells from Pluripotent Stem Cells Requires the MAPK and the PI3K Pathways. Stem Cells *35*, 909–919.

Haro, D.L., and Janknecht, R. (2002). Functional analysis of the transcription factor ER71 and its activation of the matrix metalloproteinase-1 promoter. Nucleic Acids Res *30*, 2972–2979.

Hawinkels, L., Kuiper, P., Wiercinska, E., Verspaget, H., Liu, Z., Pardali, E., Sier, C., and ten Dijke, P. (2010). Matrix Metalloproteinase-14 (MT1-MMP)-Mediated Endoglin Shedding Inhibits Tumor Angiogenesis. Cancer Res *70*, 4141–4150.

Hayashi, H., and Kume, T. (2008). Foxc transcription factors directly regulate Dll4 and Hey2 expression by interacting with the VEGF-Notch signaling pathways in endothelial cells. Plos One *3*, e2401.

Hollenhorst, P., Shah, A., Hopkins, C., and Graves, B. (2007). Genome-wide analyses reveal properties of redundant and specific promoter occupancy within the ETS gene family.

Genes Amp Dev *21*, 1882–1894.

Inyang, A., Mertens, F., Puls, F., Sumathi, V., Inwards, C., Folpe, A., Lee, C., Zhang, Y., Symmans, P., Rubin, B., et al. (2016). Primary Pseudomyogenic Hemangioendothelioma of Bone. *40*, 587–598.

James, D., Nam, H., Seandel, M., Nolan, D., Janovitz, T., Tomishima, M., Studer, L., Lee, G., Lyden, D., Benezra, R., et al. (2010). Expansion and maintenance of human embryonic stem cell–derived endothelial cells by TGFβ inhibition is Id1 dependent. Nat Biotechnol *28*, 161–166.

Jerkic, M., RODRIGUEZRBERO, A., PRIETO, M., Toporsian, M., Pericacho, M., RIVASELENA, J., OBREO, J., WANG, A., PEREZRRIOCANAL, F., and AREVALO, M. (2006). Reduced angiogenic responses in adult endoglin heterozygous mice. Cardiovasc Res *69*, 845–854.

Jerkic, M., Peter, M., Ardelean, D., Fine, M., Konerding, M., and Letarte, M. (2010). Dextran sulfate sodium leads to chronic colitis and pathological angiogenesis in Endoglin heterozygous mice. Inflamm Bowel Dis *16*, 1859–1870.

Jiang, Y., Cowley, S.A., Siler, U., Melguizo, D., Tilgner, K., Browne, C., Dewilton, A., Przyborski, S., Saretzki, G., James, W.S., et al. (2012). Derivation and Functional Analysis of Patient-Specific Induced Pluripotent Stem Cells as an In Vitro Model of Chronic Granulomatous Disease. Stem Cells *30*, 599–611.

Kane, N.M., Meloni, M., Spencer, H.L., Craig, M.A., Strehl, R., Milligan, G., Houslay, M.D., Mountford, J.C., Emanueli, C., and Baker, A.H. (2010). Derivation of Endothelial Cells From Human Embryonic Stem Cells by Directed Differentiation. Arteriosclerosis Thrombosis Vasc Biology *30*, 1389–1397.

Kane, N.M., Xiao, Q., Baker, A.H., Luo, Z., Xu, Q., and Emanueli, C. (2011). Pluripotent stem cell differentiation into vascular cells: A novel technology with promises for vascular re(generation). Pharmacol Therapeut *129*, 29–49.

Karlsson, K., Cowley, S., Martinez, F., Shaw, M., Minger, S., and James, W. (2008). Homogeneous monocytes and macrophages from human embryonic stem cells following coculture-free differentiation in M-CSF and IL-3. Exp Hematol *36*, 1167–1175.

Kataoka, H., Hayashi, M., Nakagawa, R., Tanaka, Y., Izumi, N., Nishikawa, S., Jakt, M., Tarui, H., and Nishikawa, S.-I. (2011). Etv2/ER71 induces vascular mesoderm from Flk1+PDGFRα+ primitive mesoderm. Blood *118*, 6975–6986.

Katt, M.E., Xu, Z.S., Gerecht, S., and Searson, P.C. (2016). Human Brain Microvascular Endothelial Cells Derived from the BC1 iPS Cell Line Exhibit a Blood-Brain Barrier Phenotype. Plos One *11*, e0152105.

Kelly, M.A., and Hirschi, K.K. (2009). Signaling Hierarchy Regulating Human Endothelial Cell Development. Arteriosclerosis Thrombosis Vasc Biology *29*, 718–724.

Kim, J.-S. (2016). Genome editing comes of age. Nat Protoc *11*, 1573–1578.

Kobayashi, K., Ding, G., Nishikawa, S.-I., and Kataoka, H. (2013). Role of Etv2-positive cells in the remodeling morphogenesis during vascular development. Genes Cells *18*, 704–721.

Koyano-Nakagawa, N., and Garry, D.J. (2017). Etv2 as an essential regulator of mesodermal lineage development. Cardiovasc Res *113*, 1294–1306.

Koyano-Nakagawa, N., Kweon, J., Iacovino, M., Shi, X., Rasmussen, T.L., Borges, L., Zirbes, K.M., Li, T., Perlingeiro, R.C., Kyba, M., et al. (2012). Etv2 Is Expressed in the Yolk Sac Hematopoietic and Endothelial Progenitors and Regulates Lmo2 Gene Expression. Stem Cells *30*, 1611–1623.

Koyano-Nakagawa, N., Shi, X., Rasmussen, T.L., Das, S., Walter, C.A., and Garry, D.J. (2015). Feedback Mechanisms Regulate Ets Variant 2(Etv2) Gene Expression and Hematoendothelial Lineages. J Biol Chem *290*, 28107–28119.

Kubota, Y., Takubo, K., Shimizu, T., Ohno, H., Kishi, K., Shibuya, M., Saya, H., and Suda, T. (2009). M-CSF inhibition selectively targets pathological angiogenesis and lymphangiogenesis. J Exp Medicine *206*, 1089–1102.

Kusuma, S., Shen, Y., Hanjaya-Putra, D., Mali, P., Cheng, L., and Gerecht, S. (2013). Self-organized vascular networks from human pluripotent stem cells in a synthetic matrix. Proc National Acad Sci *110*, 12601–12606.

van Laake, L.W., van den Driesche, S., Post, S., Feijen, A., Jansen, M.A., Driessens, M.H., Mager, J.J., Snijder, R.J., Westermann, C.J., Doevendans, P.A., et al. (2006). Endoglin Has a Crucial Role in Blood Cell–Mediated Vascular Repair. Circulation *114*, 2288–2297.

Lachmann, N., Ackermann, M., Frenzel, E., Liebhaber, S., Brennig, S., Happle, C., Hoffmann, D., Klimenkova, O., Lüttge, D., Buchegger, T., et al. (2015). Large-Scale Hematopoietic Differentiation of Human Induced Pluripotent Stem Cells Provides Granulocytes or Macrophages for Cell Replacement Therapies. Stem Cell Rep *4*, 282–296.

Lebrin, F., Goumans, M.-J., Jonker, L., Carvalho, R.L., Valdimarsdottir, G., Thorikay, M., Mummery, C., Arthur, H.M., and ten Dijke, P. (2004). Endoglin promotes endothelial cell proliferation and TGF-β/ALK1 signal transduction. Embo J *23*, 4018–4028.

Lebrin, F., n, S., Raymond, K., Martin, S., van den Brink, S., Freitas, C., Bréant, C., Mathivet, T., Larrivée, B., Thomas, J.-L., et al. (2010). Thalidomide stimulates vessel maturation and reduces epistaxis in individuals with hereditary hemorrhagic telangiectasia. Nat Med *16*, 420–428.

Lee, D., Park, C., Lee, H., Lugus, J.J., Kim, S., Arentson, E., Chung, Y., Gomez, G., Kyba, M., Lin, S., et al. (2008). ER71 Acts Downstream of BMP, Notch, and Wnt Signaling in Blood and Vessel Progenitor Specification. Cell Stem Cell *2*, 497–507.

Lee, D., Kim, T., and Lim, D.-S. (2011). The Er71 Is an Important Regulator of Hematopoietic Stem Cells in Adult Mice. Stem Cells *29*, 539–548.

Letteboer, T. (2005). Genotype-phenotype relationship in hereditary haemorrhagic telangiectasia. J Med Genet *43*, 371–377.

Li, C., Hampson, I., Hampson, L., Kumar, P., Bernabeu, C., and Kumar, S. (2000). CD105 antagonizes the inhibitory signaling of transforming growth factor beta1 on human vascular endothelial cells. The FASEB Journal *14*, 55–64.

Li, D., Sorensen, L., Brooke, B., Urness, L., Davis, E., Taylor, D., Boak, B., and Wendel, D. (1999). Defective angiogenesis in mice lacking endoglin. Science *284*, 1534–1537.

Lin, Y., Gil, C.-H., and Yoder, M.C. (2017). Differentiation, Evaluation, and Application of Human Induced Pluripotent Stem Cell–Derived Endothelial Cells. Arteriosclerosis Thrombosis Vasc Biology *37*, 2014–2025.

Lippmann, E.S., Al-Ahmad, A., Azarin, S.M., Palecek, S.P., and Shusta, E.V. (2014). A retinoic acid-enhanced, multicellular human blood-brain barrier model derived from stem cell sources. Sci Rep-Uk *4*, 178.

Liu, F., Kang, I., Park, C., Chang, L.-W., Wang, W., Lee, D., Lim, D.-S., Vittet, D., Nerbonne, J.M., and Choi, K. (2012). ER71 specifies Flk-1+ hemangiogenic mesoderm by inhibiting cardiac mesoderm and Wnt signaling. Blood *119*, 3295–3305.

Liu, F., Li, D., Yu, Y., Kang, I., Cha, M.-J., Kim, J., Park, C., Watson, D.K., Wang, T., and Choi, K. (2015). Induction of hematopoietic and endothelial cell program orchestrated by ETStranscription factor ER71/ ETV2. Embo Rep *16*, 654–669.

Lucas, T., Waisman, A., Ranjan, R., Roes, J., Krieg, T., Müller, W., Roers, A., and Eming, S.A. (2010). Differential Roles of Macrophages in Diverse Phases of Skin Repair. J Immunol *184*, 3964–3977.

Majeti, R., Chao, M., Alizadeh, A., Pang, W., Jaiswal, S., Gibbs, J.K., van Rooijen, N., and Weissman, I. (2009). CD47 is an adverse prognostic factor and therapeutic antibody target on human acute myeloid leukemia stem cells. Cell *138*, 286–299.

Mantovani, A., Sica, A., Sozzani, S., Allavena, P., Vecchi, A., and Locati, M. (2004). The chemokine system in diverse forms of macrophage activation and polarization. Trends Immunol *25*, 677–686.

Mantovani, A., Biswas, S., Galdiero, M., Sica, A., and Locati, M. (2013). Macrophage plasticity and polarization in tissue repair and remodelling. J Pathology *229*, 176–185.

Marcelo, K.L., Goldie, L.C., and Hirschi, K.K. (2013). Regulation of Endothelial Cell Differentiation and Specification. Circ Res *112*, 1272–1287.

Marchuk, D., Guttmacher, A., Penner, J., and Ganguly, P. (1998). Report on the workshop on Hereditary Hemorrhagic Telangiectasia, July 10-11, 1997. American Journal of Medical Genetics *76*, 269–273.

McCracken, I.R., Taylor, R.S., Kok, F.O., de la Cuesta, F., Dobie, R., Henderson, B.E., Mountford, J.C., Caudrillier, A., Henderson, N.C., Ponting, C.P., et al. (2019). Transcriptional dynamics of pluripotent stem cell-derived endothelial cell differentiation revealed by single-cell RNA sequencing. Eur Heart J *385*, 9963117.

Medvinsky, A., Rybtsov, S., and Taoudi, S. (2011). Embryonic origin of the adult hematopoietic system: advances and questions. Development *138*, 1017–1031.

Meerschaert, J., and Furie, M. (1995). The adhesion molecules used by monocytes for migration across endothelium include CD11a/CD18, CD11b/CD18, and VLA-4 on monocytes and ICAM-1, VCAM-1, and other ligands on endothelium. The Journal of Immunology *154*,

4099–4112.

Mestas, J., and Ley, K. (2008). Monocyte-Endothelial Cell Interactions in the Development of Atherosclerosis. Trends Cardiovas Med *18*, 228–232.

Miquerol, L., Langille, B., and Nagy, A. (2000). Embryonic development is disrupted by modest increases in vascular endothelial growth factor gene expression. Development *127*, 3941–3946.

Miyazono, K., Maeda, S., and Imamura, T. (2005). BMP receptor signaling: Transcriptional targets, regulation of signals, and signaling cross-talk. Cytokine Growth F R *16*, 251–263.

Morita, R., Suzuki, M., Kasahara, H., Shimizu, N., Shichita, T., Sekiya, T., Kimura, A., Sasaki, K., Yasukawa, H., and Yoshimura, A. (2015). ETS transcription factor ETV2 directly converts human fibroblasts into functional endothelial cells. Proc National Acad Sci *112*, 160–165.

Muller, W.A. (2015). The regulation of transendothelial migration: new knowledge and new questions. Cardiovasc Res *107*, 310–320.

Neehus, A.-L., Lam, J., Haake, K., Merkert, S., Schmidt, N., Mucci, A., Ackermann, M., Schubert, M., Happle, C., Kühnel, M., et al. (2018). Impaired IFN γ -Signaling and Mycobacterial Clearance in IFN γ R1-Deficient Human iPSC-Derived Macrophages. Stem Cell Rep *10*, 7–16.

Nourse, M.B., Halpin, D.E., Scatena, M., Mortisen, D.J., Tulloch, N.L., Hauch, K.D., Torok-Storb, B., Ratner, B.D., Pabon, L., and Murry, C.E. (2010). VEGF Induces Differentiation of Functional Endothelium From Human Embryonic Stem Cells. Arteriosclerosis Thrombosis Vasc Biology *30*, 80–89.

Ojeda-Fernandez, L., Recio-Poveda, L., Aristorena, M., Lastres, P., Blanco, F.J., Sanz-Rodríguez, F., Gallardo-Vara, E., de Casas-Engel, M., Corbi, A., Arthur, H.M., et al. (2016). Mice Lacking Endoglin in Macrophages Show an Impaired Immune Response. Plos Genet *12*, e1005935.

Orlova, V., van den Hil, F., Petrus-Reurer, S., Drabsch, Y., Dijke, T.P., and Mummery, C. (2014a). Generation, expansion and functional analysis of endothelial cells and pericytes derived from human pluripotent stem cells. Nat Protoc *9*, 1514–1531.

Orlova, V., Drabsch, Y., Freund, C., Petrus-Reurer, S., van den Hil, F., Muenthaisong, S., Dijke, P., and Mummery, C. (2014b). Functionality of endothelial cells and pericytes from human pluripotent stem cells demonstrated in cultured vascular plexus and zebrafish xenografts. Arteriosclerosis Thrombosis Vasc Biology *34*, 177–186.

Ozeki, M., Nozawa, A., Kanda, K., Hori, T., Nagano, A., Shimada, A., Miyazaki, T., and Fukao, T. (2017). Everolimus for Treatment of Pseudomyogenic Hemangioendothelioma. J Pediatric Hematology Oncol *39*, e328–e331.

Paik, D.T., Tian, L., Lee, J., Sayed, N., Chen, I.Y., Rhee, S., Rhee, J.-W., Kim, Y., Wirka, R.C., Buikema, J.W., et al. (2018). Large-Scale Single-Cell RNA-Seq Reveals Molecular Signatures of Heterogeneous Populations of Human Induced Pluripotent Stem Cell-Derived Endothelial Cells. Circ Res *123*, 443–450.

Paik, J.-H., Kollipara, R., Chu, G., Ji, H., Xiao, Y., Ding, Z., Miao, L., Tothova, Z., Horner, J.W., Carrasco, D.R., et al. (2007). FoxOs Are Lineage-Restricted Redundant Tumor Suppressors

and Regulate Endothelial Cell Homeostasis. Cell *128*, 309–323.

Palpant, N.J., Pabon, L., Friedman, C.E., Roberts, M., Hadland, B., Zaunbrecher, R.J., Bernstein, I., Zheng, Y., and Murry, C.E. (2016). Generating high-purity cardiac and endothelial derivatives from patterned mesoderm using human pluripotent stem cells. Nat Protoc *12*, 15–31.

Panicker, L., Miller, D., Park, T., Patel, B., Azevedo, J., Awad, O., Masood, M., Veenstra, T., Goldin, E., Stubblefield, B., et al. (2012). Induced pluripotent stem cell model recapitulates pathologic hallmarks of Gaucher disease. Proc National Acad Sci *109*, 18054–18059.

Park, C. (2004). A hierarchical order of factors in the generation of FLK1- and SCL-expressing hematopoietic and endothelial progenitors from embryonic stem cells. Development *131*, 2749–2762.

Park, C., Lee, T.-J., Bhang, S., Liu, F., Nakamura, R., Oladipupo, S.S., Pitha-Rowe, I., Capoccia, B., Choi, H., Kim, T., et al. (2016). Injury-Mediated Vascular Regeneration Requires Endothelial ER71/ETV2. Arteriosclerosis Thrombosis Vasc Biology *36*, 86–96.

Park, T., Bhutto, I., Zimmerlin, L., Huo, J.S., Nagaria, P., Miller, D., Rufaihah, A., Talbot, C., Aguilar, J., Grebe, R., et al. (2014). Vascular Progenitors From Cord Blood–Derived Induced Pluripotent Stem Cells Possess Augmented Capacity for Regenerating Ischemic Retinal Vasculature. Circulation *129*, 359–372.

Patan, S. (2004). Vasculogenesis and angiogenesis. Cancer Treatment and Research *117*, 3–32.

Patsch, C., Challet-Meylan, L., Thoma, E.C., Urich, E., Heckel, T., O'Sullivan, J.F., Grainger, S.J., Kapp, F.G., Sun, L., Christensen, K., et al. (2015). Generation of vascular endothelial and smooth muscle cells from human pluripotent stem cells. Nat Cell Biol *17*, 994–1003.

Pearson, S., czynska, P., Lacaud, G., and Kouskoff, V. (2008). The stepwise specification of embryonic stem cells to hematopoietic fate is driven by sequential exposure to Bmp4, activin A, bFGF and VEGF. Development *135*, 1525–1535.

Pece-Barbara, N., Vera, S., Kathirkamathamby, K., Liebner, S., Guglielmo, D.G., Dejana, E., Wrana, J., and Letarte, M. (2005). Endoglin Null Endothelial Cells Proliferate Faster and Are More Responsive to Transforming Growth Factor 1 with Higher Affinity Receptors and an Activated Alk1 Pathway. J Biol Chem *280*, 27800–27808.

Post, S., Smits, A., van den Broek, A., Sluijter, J., Hoefer, I., Janssen, B., Snijder, R., Mager, J., Pasterkamp, G., Mummery, C., et al. (2010). Impaired recruitment of HHT-1 mononuclear cells to the ischaemic heart is due to an altered CXCR4/CD26 balance. Cardiovasc Res *85*, 494–502.

Prasain, N., Lee, M., Vemula, S., Meador, J., Yoshimoto, M., Ferkowicz, M.J., Fett, A., Gupta, M., Rapp, B.M., Saadatzadeh, M., et al. (2014). Differentiation of human pluripotent stem cells to cells similar to cord-blood endothelial colony–forming cells. Nat Biotechnol *32*, 1151–1157.

Rajasingh, J., Ikuno, T., Masumoto, H., Yamamizu, K., Yoshioka, M., Minakata, K., Ikeda, T., Sakata, R., and Yamashita, J.K. (2017). Efficient and robust differentiation of endothelial cells

from human induced pluripotent stem cells via lineage control with VEGF and cyclic AMP. Plos One *12*, e0173271.

ram, G., Tan, J., Islam, I., Rufaihah, A., and Cao, T. (2015). Efficient differentiation of human embryonic stem cells to arterial and venous endothelial cells under feeder- and serum-free conditions. Stem Cell Res Ther *6*, 43.

Reddi, A. (2001). Interplay between bone morphogenetic proteins and cognate binding proteins in bone and cartilage development: noggin, chordin and DAN. Arthritis Res *3*, 1–5.

Robinson, A.S., Materna, S.C., Barnes, R.M., Val, S., Xu, S.-M., and Black, B.L. (2014). An arterial-specific enhancer of the human endothelin converting enzyme 1 (ECE1) gene is synergistically activated by Sox17, FoxC2, and Etv2. Dev Biol *395*, 379–389.

Robinton, D.A., and Daley, G.Q. (2012). The promise of induced pluripotent stem cells in research and therapy. Nature *481*, 295–305.

Rossi, E., Sanz-Rodríguez, F., Eleno, N., Düwell, A., Blanco, F.J., Langa, C., Botella, L.M., Cabañas, C., Lopez-Novoa, J.M., and Bernabeu, C. (2013). Endothelial endoglin is involved in inflammation: role in leukocyte adhesion and transmigration. Blood *121*, 403–415.

Rőszer, T. (2015). Understanding the Mysterious M2 Macrophage through Activation Markers and Effector Mechanisms. Mediat Inflamm *2015*, 1–16.

Rufaihah, A., Huang, N.F., Jamé, S., Lee, J.C., Nguyen, H.N., Byers, B., De, A., Okogbaa, J., Rollins, M., Reijo-Pera, R., et al. (2011). Endothelial Cells Derived From Human iPSCS Increase Capillary Density and Improve Perfusion in a Mouse Model of Peripheral Arterial Disease. Arteriosclerosis Thrombosis Vasc Biology *31*, 1035.

Rufaihah, A., Huang, N.F., Kim, J., Herold, J., Volz, K.S., Park, T., Lee, J.C., Zambidis, E.T., Reijo-Pera, R., and Cooke, J.P. (2013). Human induced pluripotent stem cell-derived endothelial cells exhibit functional heterogeneity. American Journal of Translational Research *5*, 21–35.

Sahara, M., Hansson, E.M., Wernet, O., Lui, K.O., Später, D., and Chien, K.R. (2014). Manipulation of a VEGF-Notch signaling circuit drives formation of functional vascular endothelial progenitors from human pluripotent stem cells. Cell Res *24*, 820–841.

Sarah, D., L, B. (2009). Transcriptional Control of Endothelial Cell Development Developmental Cell 16(2), 180-195.

Saito, T., Bokhove, M., Croci, R., Zamora-Caballero, S., Han, L., Letarte, M., de Sanctis, D., and Jovine, L. (2017). Structural Basis of the Human Endoglin-BMP9 Interaction: Insights into BMP Signaling and HHT1. Cell Reports *19*, 1917–1928.

Scharpfenecker, M., van Dinther, M., Liu, Z., van Bezooijen, R., Zhao, Q., Pukac, L., Lowik, C., and ten Dijke, P. (2007). BMP-9 signals via ALK1 and inhibits bFGF-induced endothelial cell proliferation and VEGF-stimulated angiogenesis. J Cell Sci *120*, 964–972.

Schneidawind, C., Jeong, J., Schneidawind, D., Kim, I.-S., Duque-Afonso, J., Wong, S., Iwasaki, M., Breese, E.H., Zehnder, J.L., Porteus, M., et al. (2018). MLL leukemia induction by t(9;11) chromosomal translocation in human hematopoietic stem cells using genome editing. Blood Adv *2*, 832–845.

Schnoor, M., Lai, F.P., Zarbock, A., Kläver, R., Polaschegg, C., Schulte, D., Weich, H.A., Oelkers, M.J., Rottner, K., and Vestweber, D. (2011). Cortactin deficiency is associated with reduced neutrophil recruitment but increased vascular permeability in vivo. J Exp Medicine *208*, 1721–1735.

Schnoor, M., Alcaide, P., Voisin, M.-B., and van Buul, J.D. (2015). Crossing the Vascular Wall: Common and Unique Mechanisms Exploited by Different Leukocyte Subsets during Extravasation. Mediat Inflamm *2015*, 1–23.

Shaw, S.K., Ma, S., Kim, M.B., Rao, R.M., Hartman, C.U., Froio, R.M., Yang, L., Jones, T., Liu, Y., Nusrat, A., et al. (2004). Coordinated Redistribution of Leukocyte LFA-1 and Endothelial Cell ICAM-1 Accompany Neutrophil Transmigration. J Exp Medicine *200*, 1571–1580.

Shen, F., Degos, V., Chu, P.-L., Han, Z., Westbroek, E.M., Choi, E.-J., Marchuk, D., Kim, H., Lawton, M.T., Maze, M., et al. (2014). Endoglin Deficiency Impairs Stroke Recovery. Stroke *45*, 2101–2106.

Shovlin, C., Guttmacher, A., Buscarini, E., Faughnan, M., Hyland, R., Westermann, C., Kjeldsen, A., and Plauchu, H. (2000). Diagnostic criteria for hereditary hemorrhagic telangiectasia (Rendu-Osler-Weber syndrome). American Journal of Medical Genetics *91*, 66–67.

Singh, B.N., Kawakami, Y., Akiyama, R., Rasmussen, T.L., Garry, M.G., Gong, W., Das, S., Shi, X., Koyano-Nakagawa, N., and Garry, D.J. (2015). The Etv2-miR-130a Network Regulates Mesodermal Specification. Cell Reports *13*, 915–923.

Spiller, K.L., Vunjak-Novakovic, G., Ng, J., Anfang, R.R., Spiller, K.J., Nakazawa, K.R., and Daulton, J.W. (2014). The role of macrophage phenotype in vascularization of tissue engineering scaffolds. Biomaterials *35*, 4477–4488.

Stockmann, C., Kirmse, S., Helfrich, I., Weidemann, A., Takeda, N., Doedens, A., and Johnson, R.S. (2011). A Wound Size–Dependent Effect of Myeloid Cell–Derived Vascular Endothelial Growth Factor on Wound Healing. J Invest Dermatol *131*, 797–801.

Swift, M., and Weinstein, B. (2009). Arterial-Venous Specification During Development. Circ Res *104*, 576–588.

Takahashi, K., and Yamanaka, S. (2006). Induction of Pluripotent Stem Cells from Mouse Embryonic and Adult Fibroblast Cultures by Defined Factors. Cell *126*, 663–676.

Takahashi, K., Tanabe, K., Ohnuki, M., Narita, M., Ichisaka, T., Tomoda, K., and Yamanaka, S. (2007). Induction of Pluripotent Stem Cells from Adult Human Fibroblasts by Defined Factors. Cell *131*, 861–872.

Takata, K., Kozaki, T., Lee, C., Thion, and Otsuka, M. (2017). Induced-Pluripotent-Stem-Cell-Derived Primitive Macrophages Provide a Platform for Modeling Tissue-Resident Macrophage Differentiation and Function. Immunity.

Tanaka, T., Takahashi, K., Yamane, M., Tomida, S., Nakamura, S., Oshima, K., Niwa, A., Nishikomori, R., Kambe, N., Hara, H., et al. (2012). Induced pluripotent stem cells from CINCA syndrome patients as a model for dissecting somatic mosaicism and drug discovery. Blood *120*, 1299–1308.

Taura, D., Sone, M., Homma, K., Oyamada, N., Takahashi, K., Tamura, N., Yamanaka, S., and Nakao, K. (2009). Induction and Isolation of Vascular Cells From Human Induced Pluripotent Stem Cells—Brief Report. Arteriosclerosis Thrombosis Vasc Biology *29*, 1100–1103.

Theocharides, A.P., Jin, L., Cheng, P.-Y., Prasolava, T.K., Malko, A.V., Ho, J.M., Poeppl, A.G., van Rooijen, N., Minden, M.D., Danska, J.S., et al. (2012). Disruption of SIRPα signaling in macrophages eliminates human acute myeloid leukemia stem cells in xenografts. J Exp Medicine *209*, 1883–1899.

Thomas-Ecker, S., Lindecke, A., Hatzmann, W., Kaltschmidt, C., Zanker, K., and Dittmar, T. (2007). Alteration in the gene expression pattern of primary monocytes after adhesion to endothelial cells. Proc National Acad Sci *104*, 5539–5544.

Torres, R., Martin, M., Garcia, A., Cigudosa, J.C., Ramirez, J., and Rodriguez-Perales, S. (2014). Engineering human tumour-associated chromosomal translocations with the RNA-guided CRISPR–Cas9 system. Nat Commun *5*, 233.

Torres-Ruiz, R., Martinez-Lage, M., Martin, M.C., Garcia, A., Bueno, C., Castaño, J., Ramirez, J.C., Menendez, P., Cigudosa, J.C., and Rodriguez-Perales, S. (2017). Efficient Recreation of t(11;22) EWSR1-FLI1 + in Human Stem Cells Using CRISPR/Cas9. Stem Cell Rep *8*, 1408–1420.

Tørring, P.M., Larsen, M., Kjeldsen, A.D., Ousager, L., Tan, Q., and Brusgaard, K. (2015). Global gene expression profiling of telangiectasial tissue from patients with hereditary hemorrhagic telangiectasia. Microvasc Res *99*, 118–126.

Torsney, E., Charlton, R., Diamond, A.G., Burn, J., Soames, J.V., and Arthur, H.M. (2003). Mouse Model for Hereditary Hemorrhagic Telangiectasia Has a Generalized Vascular Abnormality. Circulation *107*, 1653–1657.

Tseng, D., Volkmer, J., Willingham, S., Contreras-Trujillo, H., Fathman, J., Fernhoff, N., Seita, J., Inlay, M., Weiskopf, K., Miyanishi, M., et al. (2013). Anti-CD47 antibody-mediated phagocytosis of cancer by macrophages primes an effective antitumor T-cell response. Proc National Acad Sci *110*, 11103–11108.

Urbich, C., Heeschen, C., Aicher, A., Dernbach, E., Zeiher, A.M., and Dimmeler, S. (2003). Relevance of Monocytic Features for Neovascularization Capacity of Circulating Endothelial Progenitor Cells. Circulation *108*, 2511–2516.

Uusi-Rauva, K., Blom, T., von Schantz-Fant, C., Blom, T., Jalanko, A., and Kyttälä, A. (2017). Induced Pluripotent Stem Cells Derived from a CLN5 Patient Manifest Phenotypic Characteristics of Neuronal Ceroid Lipofuscinoses. Int J Mol Sci *18*, 955.

Val, D.S. (2011). Key transcriptional regulators of early vascular development. Arteriosclerosis Thrombosis Vasc Biology *31*, 1469–1475.

Val, S., Chi, N.C., Meadows, S.M., Minovitsky, S., Anderson, J.P., Harris, I.S., Ehlers, M.L., Agarwal, P., Visel, A., Xu, S.-M., et al. (2008). Combinatorial Regulation of Endothelial Gene Expression by Ets and Forkhead Transcription Factors. Cell *135*, 1053–1064.

Valdimarsdottir, G., Goumans, M.-J., Rosendahl, A., Brugman, M., Itoh, S., Lebrin, F., Sideras, P.,

and ten Dijke, P. (2002). Stimulation of Id1 expression by bone morphogenetic protein is sufficient and necessary for bone morphogenetic protein-induced activation of endothelial cells. Circulation *106*, 2263–2270.

Vanoli, F., Tomishima, M., Feng, W., Lamribet, K., Babin, L., Brunet, E., and Jasin, M. (2017). CRISPR-Cas9–guided oncogenic chromosomal translocations with conditional fusion protein expression in human mesenchymal cells. Proc National Acad Sci *114*, 3696–3701.

Walther, C., Tayebwa, J., Lilljebjörn, H., Magnusson, L., Nilsson, J., von Steyern, F., Øra, I., Domanski, H.A., Fioretos, T., Nord, K.H., et al. (2014). A novel SERPINE1-FOSBfusion gene results in transcriptional up-regulation of FOSBin pseudomyogenic haemangioendothelioma. J Pathology *232*, 534–540.

Westermann, C.J., Rosina, A.F., Vries, V., and de Coteau, P.A. (2003). The prevalence and manifestations of hereditary hemorrhagic telangiectasia in the Afro-Caribbean population of the Netherlands Antilles: A family screening. Am J Med Genet *116A*, 324–328.

White, M.P., Rufaihah, A.J., Liu, L., Ghebremariam, Y.T., Ivey, K.N., Cooke, J.P., and vastava, D. (2012). Limited Gene Expression Variation in Human Embryonic Stem Cell and Induced Pluripotent Stem Cell-Derived Endothelial Cells. Stem Cells *31*, 92–103.

van Wilgenburg, B., Browne, C., Vowles, J., and Cowley, S.A. (2013). Efficient, Long Term Production of Monocyte-Derived Macrophages from Human Pluripotent Stem Cells under Partly-Defined and Fully-Defined Conditions. Plos One *8*, e71098.

Willenborg, S., Lucas, T., van Loo, G., Knipper, J.A., Krieg, T., Haase, I., Brachvogel, B., Hammerschmidt, M., Nagy, A., Ferrara, N., et al. (2012). CCR2 recruits an inflammatory macrophage subpopulation critical for angiogenesis in tissue repair. Blood *120*, 613–625.

Williams, M.R., Sakurai, Y., Zughaier, S.M., Eskin, S.G., and McIntire, L.V. (2009). Transmigration across activated endothelium induces transcriptional changes, inhibits apoptosis, and decreases antimicrobial protein expression in human monocytes. J Leukocyte Biol *86*, 1331–1343.

Willingham, S., Volkmer, J., Gentles, A., Sahoo, D., Dalerba, P., Mitra, S., Wang, J., Contreras-Trujillo, H., Martin, R., Cohen, J., et al. (2012). The CD47-signal regulatory protein alpha (SIRPa) interaction is a therapeutic target for human solid tumors. Proc National Acad Sci *109*, 6662–6667.

Wong, C., Heit, B., and Kubes, P. (2010). Molecular regulators of leucocyte chemotaxis during inflammation. Cardiovasc Res *86*, 183–191.

Woodfin, A., Voisin, M.-B., Beyrau, M., Colom, B., Caille, D., Diapouli, F.-M., Nash, G.B., Chavakis, T., Albelda, S.M., Rainger, E.G., et al. (2011). The junctional adhesion molecule JAM-C regulates polarized transendothelial migration of neutrophils in vivo. Nat Immunol *12*, 761–769.

Wynn, T., Chawla, A., and Pollard, J. (2013). Macrophage biology in development, homeostasis and disease. Nature *496*, 445–455.

Xu, B., Wu, Y., Huey, M., Arthur, H.M., Marchuk, D.A., Hashimoto, T., Young, W.L., and Yang, G.-Y.

(2016). Vascular Endothelial Growth Factor Induces Abnormal Microvasculature in the Endoglin Heterozygous Mouse Brain. J Cereb Blood Flow Metabolism *24*, 237–244.

Yanagimachi, M., Niwa, A., Tanaka, T., Honda-Ozaki, F., Nishimoto, S., Murata, Y., Yasumi, T., Ito, J., Tomida, S., Oshima, K., et al. (2013). Robust and highly-efficient differentiation of functional monocytic cells from human pluripotent stem cells under serum- and feeder cell-free conditions. Plos One *8*, e59243.

Yang, L., Kowalski, J.R., Zhan, X., Thomas, S.M., and Luscinskas, F.W. (2006). Endothelial Cell Cortactin Phosphorylation by Src Contributes to Polymorphonuclear Leukocyte Transmigration In Vitro. Circ Res *98*, 394–402.

Yoo, C., Na, H.-J., Lee, D.-S., Heo, S., An, Y., Cha, J., Choi, C., Kim, J., Park, J.-C., and Cho, Y. (2013). Endothelial progenitor cells from human dental pulp-derived iPS cells as a therapeutic target for ischemic vascular diseases. Biomaterials *34*, 8149–8160.

Zanotelli, M.R., Ardalani, H., Zhang, J., Hou, Z., Nguyen, E.H., Swanson, S., Nguyen, B., Bolin, J., Elwell, A., Bischel, L.L., et al. (2016). Stable engineered vascular networks from human induced pluripotent stem cell-derived endothelial cells cultured in synthetic hydrogels. Acta Biomater *35*, 32–41.

Zhang, H., Xue, C., Shah, R., Bermingham, K., Hinkle, C., Li, W., Rodrigues, A., Tabita-Martinez, J., Millar, J., Cuchel, M., et al. (2015). Functional analysis and transcriptomic profiling of iPSC-derived macrophages and their application in modeling Mendelian disease. Circ Res *117*, 17–28.

Zhang, J., Schwartz, M.P., Hou, Z., Bai, Y., Ardalani, H., Swanson, S., Steill, J., Ruotti, V., Elwell, A., Nguyen, B., et al. (2017). A Genome-wide Analysis of Human Pluripotent Stem Cell-Derived Endothelial Cells in 2D or 3D Culture. Stem Cell Rep *8*, 907–918.