Cover Page

Universiteit Leiden

Leiden University Repository

The handle http://hdl.handle.net/1887/43155 holds various files of this Leiden University dissertation.

Author: Gkatzis, K
Title: In vitro and In vivo models for studying endothelial cell development and hereditary hemorrhagic telangiectasia
Issue Date: 2016-09-22
General discussion
PSCs, both ESCs and iPSCs are already a useful and renewable source of all cell types of the body. PSCs enable the generation of autologous cells for the in vitro investigation of molecular mechanisms underlying physiologic and pathophysiologic states characteristic of a number of diseases. In particular, with the development of robust endothelial specific differentiation protocols (Chapter 3, (Orlova et al., 2013)) and new genetic manipulation approaches (Chapter 3), hPSCs can be used to recapitulate and analyze defined steps of early human development with respect to endothelial cell fate commitment. Furthermore, the isolation and expansion of pure populations of hPSC-derived ECs (Chapter 5) enables further phenotypic and functional characterization of these cells.

Modeling embryonic development using hPSCs

In Chapter 3 and Chapter 5, we derived hESC-ECs and hiPSC-ECs using a spin-EB (embryoid body) or a monolayer-based two-dimensional (2D) differentiation approach, respectively. Both systems demonstrated controlled sequential gene expression characteristic of mesoderm formation and patterning, starting from the enrichment of key transcription factors during early embryonic mesoderm development (BRY and MESP1), via a hematovascular mesodermal precursor stage (ETV2), to ECs (CD31, ALK1, ENG), mimicking endothelial development in vivo (Chapter 3, (Orlova et al., 2013)). Therefore, both approaches provided excellent systems to characterize vascular progenitor stage-specific key signaling pathways. This research is driven not only by the practical need to find cures for the numerous human diseases caused by developmental defects, but also by the importance of identifying the critical regulators guiding commitment and diversification of endothelial progenitors, allowing further enhancement of the differentiation efficiency and the derivation of tissue-type specific ECs (Wilson et al., 2014).

In the developing mouse embryo, the VEGF signaling pathway has been implicated as the major regulator of vasculogenesis, leading to the aggregation and de-novo-formation of angioblasts into a primitive vascular plexus (Coultas et al., 2005; Dvorak et al., 1995; Klagsbrun and Soker, 1993; Poole et al., 2001). This signaling pathway family consists of five secreted VEGF ligands (VEGF-A, VEGF-B, VEGF-C, VEGF-D and PIGF), which can bind with differing specificities to three endothelial VEGF tyrosine kinase receptors (VEGFR-1, VEGFR-2 and VEGFR3) (Holmes and Zachary, 2005; Shibuya, 2013). Notably, our data showed that VEGF signaling is also required for the generation of human endothelial cells from hPSCs in vitro (Chapter 3, (Orlova et al., 2013)), as previously described by others (Patsch et al., 2015; Sahara et al., 2014; White et al., 2013). Analysis of VEGF ligands and their receptors, during mouse embryogenesis, demonstrated the interaction between VEGFR-2 and VEGF-A as essential for the development of mesoderm (Coultas et al., 2005; Rossant and Howard, 2002). In Chapter 3 we showed that treatment of differentiating hESCs with VEGF resulted in the enrichment of VEGFR2+ (KDR+) population with increased expression of ETV2 gene, a key regulator of hematopoietic lineage, suggesting a relationship between VEGFR-2 and ETV2 similar to that described in the mouse system (Kataoka et al., 2011; Rasmussen et al., 2012).

The need for a new genetic tool

As described in Chapter 3, ETV2 belongs to the transcriptional regulatory ETS protein family and is transiently expressed in the primitive streak, yolk sac blood islands, and large vessels such as dorsal aorta during early embryonic development (Lee et al., 2008). Ectopic transient expression of etv2 can significantly increase the number of ECs in differentiating mESCs (Koyano-Nakagawa et al., 2012; Lee et al., 2008) and hESCs (Elcheva et al., 2014; Lindgren et al., 2015), suggesting the protagonist role of etv2 in transcriptional complexes mediating mesodermal plasticity of early endothelial progenitor
cells. Further studies will be needed to elucidate the differentiation potential of ETV2-expressing populations in our spin-EB differentiation cultures. Mechanistically, a few Etv2 interacting regulators, such as OVOL2 (Kim et al., 2014), Jmjd1a (Knebel et al., 2006) and Gata2 (Shi et al., 2014), and DNA binding sites, including VE-cadherin (Cdh5), Fli1, Erg, Tie2 and Lmo2 (Koyano-Nakagawa et al., 2012; Liu et al., 2012), have been identified using ChIP or mass spectrometry (MS) analysis. However, major drawbacks of the latter studies include the fact that experiments were largely confined to examining interacting protein partners or DNA binding site conditions of forced ETV2 overexpression, which made it difficult to discern between functional and artifactual ChIP or MS signals. Misleading results are in line with recent, carefully controlled ChIP reports in yeast demonstrating that highly expressed genes exhibit potentially artifact-based ChIP peaks (Park et al., 2013; Teytelman et al., 2013). In Chapter 2 and Chapter 3, mouse and human ESCs were used to describe a newly developed knock-in fusion strategy based on gene targeting by HR that could circumvent these limitations by providing non-invasive physiological expression of the gene of interest and allowing further transcriptomic characterization (Figure 1). However, a major bottleneck of this knock-in fusion strategy when applied in transiently expressed genes, such as ETV2, remains the accessibility to a sufficiently large ETV2-expressing population for high-throughput analysis. Thus, it is important to optimize culture conditions that increase efficiency of and highly enrich the ETV2-expressing cell fractions.

Gene targeting by homologous recombination has been an indispensable tool in generating hESC reporter lines following a knock-in deletion strategy, in which the first exon of the gene of interest is replaced by a sequence encoding for a fluorescent protein; this results in the deletion of one wildtype allele which for important genes could alter the biological outcome or conclusions of studies using the reporter line. It is important to remember however that this knock-in strategy may not recapitulate all features of normal gene regulation. In contrast, our gene targeting knock-in fusion strategy allowed the accurate integration of the modified targeting construct into the 3’ end and in frame with the coding sequence of the gene of interest. With this approach, the C-terminus of the targeted protein can be fused either with a fluorescent protein or with a composite affinity tag protein allowing multiple protein purification to be performed using TAP technology (Rigaut et al., 1999). Using the pluripotency transcription factor Nanog as an example, in Chapter 2 we confirmed that this knock-in fusion strategy allowed the generation of functional TF fusion proteins mirroring normal protein distribution in vivo and in vitro. As seen in a subsequent study, this approach further permitted continuous non-invasive quantification of TF fusion-protein expression dynamics and its functional significance on the correlation networks of other pluripotent TFs over many generations in culture, which made this strategy critical for many clonal applications and downstream assays of single-cell studies (Filipczyk et al., 2015).

Future directions

As mentioned above, ETV2 has been one of the master regulators in the intersection of embryonic vessel and blood cell development. Using ETV2mcherry/w fusion-reporter hESCs in which the emergence of ETV2+ cells can be followed by the expression of a red fluorescent protein (mcherry), chemical compounds that induce, maintain ETV2-mcherry expression and determine their cell fate conversions could be tested and combined with extensive transcriptomic and epigenomic analysis (Figure 1). The results can lead to the identification of new signaling pathways specifying endothelial and/or hematopoietic development and generate culture conditions in which pure populations of ETV2+ cells can be expanded and stored. The latter is of particular importance in cellular transplantation therapy, as this pool of cells could be an unlimited autologous source for de novo generation of human HSCs, red blood cells, platelets, and T cells in vitro. Similarly, for the vascular system, the identification
and manipulation of critical gene networks combined with microfluidics approaches can lead to the generation of tissue specific arterial/venous/lymphatic endothelial cells that may allow one to mimic vital EC functions that are central to in vitro disease modeling and toxicology studies including microvascular permeability, vasomotor tone, coagulation and anticoagulation, inflammation and angiogenesis. Finally, single-cell continuous time-lapse imaging could reveal the clonal origin of different subtypes of endothelial and hematopoietic cells from hemangioblast in vitro.

**Figure 1. Strategies for using the ETV2^mcherry/+ hPSC fusion line.** 1. Differentiation of ETV2^mcherry/+ hPSC fusion line towards ETV2+ population. 2. Identification of cell factors required for ETV2+ cells to acquire self-renewing ability in order to expand and bank pure ETV2+ populations. 3. Identification of cell factors required for the sequential ESPCs and HSPCs commitment of ETV2+ cells. 4. Generation of arterial, venous, lymphatic endothelial cells and stable capillary networks using microfluidics technologies for in vitro tissue-specific modeling and drug toxicity studies. 5. Generation in vitro of HSPCs with primitive/definitive hematopoietic (macrophages, granulocytes, megakaryocytes, erythrocytes, dendritic cells, B and T cell lineage) and bone marrow reconstitution potential in mouse models. 6. Identification of stage-specific molecular networks (using epigenetic, transcriptomic and proteomics approaches) required for the specification and expansion of ESPCs and HSPCs derivatives from ETV2+ cells; ESPC (endothelial stem/progenitor cells), HSPC (hematopoietic stem/progenitor cells).
Modeling HHT using hiPSCs

In addition to their relevance for studying normal development, hPSCs also have potential for investigating the pathogenesis of human diseases in vitro. As described in Chapter 1, HHT is caused by mutations in genes encoding proteins involved in binding TGF-β and those associated with its signaling pathway in endothelial cells. In particular, the majority of HHT patients harbor mutations in ENG or ACVRL1 genes, which are responsible for HHT1 and HHT2, respectively. In Chapter 4 and Chapter 5, we described the derivation of iPSC lines from healthy individuals and patients diagnosed with HHT. To date, four HHT1 and three HHT2 iPSC lines harboring nonsense, missense or deletion mutations have been generated in our laboratory (Freund, Orlova, unpublished) providing a potential model system that can be used to identify mechanisms of HHT, further explain findings between in vitro/in vivo models and the human disease and screening new drug compounds.

Characterization of HHT1 iPSC-ECs

Most mutations in ACVRL1 and ENG generate a null allele, giving rise to unstable or non-functional truncated proteins in addition to the normal wildtype protein at reduced levels. This is in line with our observation that HHT1 iPSC-ECs exhibit constitutively reduced levels of ENG when compared to control iPSC-ECs (Chapter 5). However, cell density culture conditions clearly affect gene expression levels of TGFβ related genes (Chapter 5) so that care should indeed be taken to analyze control and mutant lines at the same density. Gene expression profiling of HHT1 iPSC-ECs and control iPSC-ECs revealed several differentially expressed genes that could have been the result of reduced levels of ENG. Among these, MTUS1 had not been described before in ECs. We showed that MTUS1 upregulation was directly dependent on ENG levels by using siRNA to knock-down ENG in ECs, suggesting its direct association with ENG. However, extensive experimentation will be required to determine its function in ECs and HHT1. MTUS1 encodes a number of isoforms, termed AT2 receptor-interacting proteins (ATIPs), with distinct tissue distribution and subcellular localization (Rodrigues-Ferreira and Nahmias, 2010). ATIP1, which is the principle isoform expressed in our hiPSC-ECs (Chapter 5), has mainly been associated with AT2 receptor transport from the Golgi and intracellular signaling pathways (Rodrigues-Ferreira and Nahmias, 2010). Interestingly, MTUS1 has also been found to be downregulated in a number of solid tumors, including pancreas (Seibold et al., 2003), ovary (Pils et al., 2005), head-and-neck (Ding et al., 2012; Ye et al., 2007), colon (Zuern et al., 2010), bladder (Xiao et al., 2012) and breast tumors (Rodrigues-Ferreira et al., 2009). Furthermore, ATIP1 has been shown to mimic the inhibitory effects of the AT2 receptor on bFGF and PDGF-induced ERK2 phosphorylation and cell proliferation (Nouet, 2004). An important point is that, in contrast to the canonical TGFβ/Smad pathway, TGFβ is able to signal through a number of non-Smad pathways, including the Ras / Erk / MAPK pathway. Thus, the possible involvement of ATIP1-AT2 receptor in ENG-TGFβ or bFGF-mediated Erk activation in promoting or inhibiting cell proliferation in HHT1 iPSC-ECs remains to be elucidated. In addition, one study showed that ATIP1 can also function as a Golgi membrane-associated protein, where is involved in the intercellular transport of the AT2 receptor to the plasma membrane(Wruck et al., 2005). Finally, the remaining question emerging from this study is whether intracellular crosstalk exists between ATIP1 and ENG, that might account for activation or translocation of TGFβ/BMP signaling in ECs. To address this question, yeast two-hybrid and crosslinking approaches could be applied.

HHT patients develop abnormal vascular structures, such as AVMs. The formation of AVMs is still poorly understood but several mechanisms have been proposed based on both in vitro and in vivo studies. These include increased production of VEGF (Cirulli et al., 2003; Sadick et al., 2005a; 2005b), inappropriate responses of mutated endothelial cells to TGF-β (Lebrin
et al., 2004; Xu et al., 2004), or to BMP9/10 stimulation (Kim et al., 2012; Ricard et al., 2010; Young et al., 2012), defective endothelial cell specification during the Tip cell selection (Larrivée et al., 2012) and disengagement of the endothelium from the surrounding mural cells due to reduced activation of local TGF-β (Carvalho, 2004). In all cases, the endothelium becomes more susceptible to growth factor stimulation, resulting in excessive endothelial cell proliferation and the formation of a denser and more highly branched vascular plexus (Larrivée et al., 2012; Lebrin et al., 2010; Mahmoud et al., 2010; Park et al., 2009; Ricard et al., 2012; Roman et al., 2002). The generation of control and HHT1 iPSC-ECs allowed us to study the physiological functions of disease-relevant ECs and the role of ENG in the responses of HHT1 iPSC-ECs to TGFβ or BMP9 stimulation (Chapter 5).

TGF-β signaling in control and HHT1 iPSC-ECs

A key aspect in understanding how endothelial cells respond to TGFβ related ligands is to know their receptor localization. ALK1 is expressed exclusively in ECs and ENG largely in vascular cells, pericytes and cells of the bone marrow, whereas ALK2, ALK3, ALK5, BMPRII and TGFBRII are expressed in a numerous other cell types (Leon and Arthur, 2002; Sachiko et al., 1995; Seki et al., 2005). Importantly, ALK1 and ALK5 exhibit distinct expression in blood vessels (co-expressed in ECs; ALK5 only in vSMCs), suggesting different functions in blood vessel development (Seki et al., 2005).

In ECs, TGFβ can bind and signal through both TGFBR-II/ALK5/Smad2, 3 and TGFBR-II/ACVRL1 (ALK1)/Smad1, 5 and 8 complexes (Chen and massague, 1999; Finnson et al., 2008; Goumans et al., 2003; Lux et al., 1999; Oh et al., 2000). Specifically, TGFβ1 and TGFβ3 are able to phosphorylate both Smad1/5 and Smad2/3 in a broad range of primary ECs, including MEECs, BAECs, BCECs, yolk sac (YS) and BMECs (Goumans et al., 2002). This is not consistent with a number of studies using HUVECs, in which phosphorylation of SMAD1/5/8 was not induced by TGFβ1, suggesting that the effect of TGFβ is highly dependent on the cellular context (David et al., 2007; Liu et al., 2014; Nolan-Stevaux et al., 2012; Ota et al., 2002; Seki et al., 2005; Shao et al., 2009; Wang et al., 2013).

Furthermore, these two receptor combinations in ECs have also been shown to give rise to different cellular responses. These include stimulation of cell proliferation and migration via TGFβ/ALK1 and inhibition of cell proliferation leading to vascular quiescence through the TGFβ/ALK5 complex (Goumans et al., 2002; 2003; Oh et al., 2000). It is important to note that these biphasic responses are observed only at high doses of TGFβs in primary ECs (Goumans et al., 2002). Interestingly, the TGFβ co-receptor ENG was further shown to mediate these biphasic signaling and cell functional responses in MEECs and HMEC-1 (Blanco et al., 2005; Lebrin et al., 2004). This is in line with our observation that ENG is required for TGFβ-3/ALK1-mediated activation of SMAD1/5 in hiPSC-ECs at high cell densities (Chapter 5), but further research is necessary to elucidate the biphasic functional responses in these hiPSC-ECs.

It was demonstrated previously that BMP9 can signal through BMPRII / ActRIIA / ALK1 inducing SMAD1/5/8 phosphorylation in HMVEC-d’s, BAECs and HUVECs (David et al., 2007; Scharpfenecker et al., 2007). In Chapter 5 we also showed this was the case in hiPSC-ECs. Interestingly, the BMP9 response is increased by ENG expression, which is further supported by the fact that BMP9 is the only ligand able to bind directly to ENG and ALK1 (Brown et al., 2005; David et al., 2007; Mitchell et al., 2010; Ricard et al., 2012; Scharpfenecker et al., 2007). In addition, BMP9 was also shown to inhibit proliferation and migration of ECs and to block VEGF-induced angiogenesis in vitro, as evidenced by the formation of vascular tube-like structures (David et al., 2007; Scharpfenecker et al., 2007). In contrast and in line
Discussion

with our observations, high doses of BMP9 can give rise to the activation of both, SMAD1/5 and SMAD2 and not SMAD3 in human pulmonary artery ECs (PAECs), HAECs, HMEC-1 and hPSC-ECs (Upton et al., 2009) (Chapter 5). Finally, it is important to note that treatment of HUVECs, HUAECs and HBMEC with serum leads to the phosphorylation of Smad1/5 via ALK1 similar to BMP9 (Wöltje et al., 2015). Thus, there is no doubt that downstream phosphorylation of Smad proteins is highly cellular-context dependent, where different ligand concentrations, receptor localization, serum supplementation and as we showed, cell density, resulting in conflicting outcomes of TGFβ signaling in vitro.

In Chapter 5 we provided evidence that TGFβ/BMP9 concentration and EC density conditions contribute to this “cross-over” SMAD activation, considerations that should be borne in mind when studying downstream signaling. Even though TGFβ signals through SMAD2 and BMP9 via SMAD1/5, high TGFβ and BMP9 concentrations under high cell density conditions induced phosphorylation of both SMAD1/5 and SMAD2 (Chapter 5). Importantly, the major drawback of this study includes analysis that is largely confined to examining differences in the total SMAD2/3 or SMAD1/5/8 proteins under different cell density culture conditions. Another remaining question emerging from the latter is whether these cell density studies in vitro relate to behaviour of ECs in vivo. Cell density conditions in vitro may recapitulate the three main endothelium structural types, including the continuous non-fenestrated, continuous fenestrated and discontinuous fenestrated (sinusoidal) endothelium (Atkins et al., 2011). These can be found predominantly in: continuous non-fenestrated: arteries, veins, skin, muscle, heart, lung capillaries of the brain; continuous fenestrated: capillaries of all exocrine and endocrine glands, digestive tract mucosa, kidney; discontinuous fenestrated: certain sinusoidal vascular bed in the liver and bone marrow (Atkins et al., 2011).

The exact molecular mechanisms underlying these variable SMAD activation events in ECs remain largely elusive. Mathematical models of TGFβ signaling dynamics have shown that stimulation with a given concentration of TGFβ determines signaling duration and this is dependent on the cell density: signaling persists longer when cell density is decreased (Zi and Klipp, 2007). Additionally, Clarke et al. showed that the potency of the same TGFβ concentration depended on the number of cells that are exposed to TGFβ and proposed that the “TGFβ concentration” expressed as “TGFβ molecules per cell” is a better predictor of Smad2 activation since it takes into account the number of cells in each experiment (Clarke et al., 2009). Thus, a key parameter to consider for the “cross-over” SMAD activation events in ECs maybe “ligand molecules per cell” and since individual cells are likely to express different numbers of receptors at cell surface, the “ratio of ligand to cell surface receptor number” might be more appropriate. This latter is of particular importance in hPSC field, as the number of cell surface receptors in hPSC-ECs might differ between iPSC lines and differentiation experiments, leading to heterogeneous signaling responses.

Current limitation to HHT iPSC modeling

The primary concern regarding the potential use of iPSC technology as a method to model a hereditary disease, such as HHT, is the risk that normal genetic variations or backgrounds among hiPSC cell lines derived from two different individuals may lead to non-disease related differences in phenotype. It would be impossible to determine or completely eliminate the possibility that the observed phenotypes are the direct result of causal mutations or an effect of possible genetic variation between the different hPSC lines unless very many cell lines were examined as in a population type study. A good solution to this problem is the use of isogenic-control iPSC lines in which the mutated gene has been repaired by gene targeting. This was a major limitation of the studies in this thesis although certain aspects could be examined by siRNA knockdown for example, since the underlying disease
mechanism is known to be haploinsufficiency, which reduces the level of cell surface protein available for signaling. Another limitation of the use of hPSC-ECs for HHT modeling is obtaining the subtype affected by the disease. In Chapter 6 we showed in mice that the post-arterial capillary network plays a critical role in the formation of direct AV connections in HHT2 mutants, which highlights the need of tissue-specific ECs. Thus, the development of robust hPSC-EC differentiation protocols to generate disease-tissue-specific microvascular ECs that match the in vivo counterpart as closely as possible could address this issue.

Modeling HHT using mouse models

The “HHA model” for AVM formation

Data from the most recent HHT mouse models in which Acvrl1 or Eng gene deficiency is inducible rather than ubiquitous led to the hypothesis that three events are necessary for the development of AVMs. These are: (i) Acvrl1 or Eng heterozygosity, (ii) local/somatic mutations in the remaining normal allele leading to homozygous loss and (iii) pro-angiogenic or pro-inflammatory triggers (Tual-Chalot et al., 2015). However, the concept of a local somatic mutation in HHT patients has not been experimentally proven and needs to be re-assessed using the more advanced DNA sequencing technology currently available. In Chapter 6, we provided evidence that suggests altered blood flow contributed to the development of AVM in HHT2 mice, where the flow-dependent adaptive responses in the post-arterial capillary network could in the first instance be masked by HHT phenotypes during angiogenic events. This data led us to suggest a new three event hypothesis, which we refer to as the “HHA model”, for AVM formation in HHT patients: Acvrl1 or Eng Heterozygosity representing the variable baseline condition in HHT; Hemodynamic changes leading to the formation of enlarged venous-like capillaries with abnormal flow and transient AV connections; and pro-Angiogenic triggers cause further remodeling of the transient AV shunts into large AVMs. This may also explain why new AVMs or enlargement of existing AVMs have been reported during physiological hemodynamic changes, including pregnancy (de Gussem et al., 2014; Ference et al., 1994; Shovlin et al., 2008; 1995), exercise and drug-induced systemic vasodilation (Anand and Florea, 2001; Mehta and Dubrey, 2009).

Cellular origin of AVMs

As described in Chapter 1, some HHT mouse model studies have focused on investigating the involvement of different cell types other than ECs being responsible for the formation of visceral and mucocutaneous AVMs in both HHT1 and HHT2 adult models. For that purpose, Acvrl1 and Eng were specifically deleted from smooth muscle cells, pericytes, macrophages, vSMCs and a subpopulation of ECs using Myh11-Cre<sup>ER</sup>, NG2-Cre<sup>ER</sup>, LysM-Cre and SM22a-Cre lines, respectively (Chen et al., 2014a; 2014b; Garrido-Martin et al., 2014; Milton et al., 2012).

Both, Chen et al and Garrido-Martin et al investigated whether tamoxifen induced deletion of Acvrl1 or Eng in adult pericytes (NG2-Cre<sup>ER</sup>) or smooth muscle cells (Myh11-Cre<sup>ER</sup>) is sufficient to induce AVMs in the area close to the wounded skin (Chen et al., 2014a; Garrido-Martin et al., 2014). Interestingly, Acvrl1 deletion in these cell types did not affect blood vessels undergoing active angiogenesis even after VEGF stimulation (Chen et al., 2014a; Garrido-Martin et al., 2014). Similarly, deletion of Eng in macrophages using lysosome M-positive macrophage (LysM)-Cre adult mice did not result in the development of AVMs in the brain or near the wounded-ear even after external angiogenic stimulus (Choi et al., 2014). On the other hand, conditional deletion of Acvrl1 or Eng using SM22a-Cre mice give rise to the formation of AVM in the brain and spinal cord, leading to paralysis or lethality in the first
6 to 15 weeks of life (Choi et al., 2014; Milton et al., 2012). Interestingly, a mosaic of Cre-recombined and non-recombined cells was detected in the spinal cord-AVMs, supporting the hypothesis that a mixture of affected and non-affected cells is contributing to the formation of AVMs in HHT patients. In addition, it is important to note that SM22a expression is not restricted to vSMCs but is also detected in cardiac muscle in embryos and in a wide-range of cell types in adult, confounding perhaps the experiments using SM22 as a cre-driver and suggesting that another cell type than vSMC might be responsible (Milton et al., 2012). Finally, as described in Chapter 1, the most dramatic AVM phenotype in neonatal or adult mice is only observed when Acvrl1 or Eng are primarily deleted using Scl-CreER, Cdh5-CreERT2, L1-Cre or Pdgfb-CreER (Chen et al., 2014a; Garrido-Martin et al., 2014; Mahmoud et al., 2010; Park et al., 2009; Tual-Chalot et al., 2014). These observations lead to the conclusion that the major cellular source for the development of an AVM phenotype in several organs is the endothelium. In Chapter 6 we used the Cdh5-CreERT2 mice to reveal an AVM neonatal retinal phenotype that overlapped with that of Eng-iKO and Acvrl1-iKO neonates (Mahmoud et al., 2010; Tual-Chalot et al., 2014). Together, our data suggests that endothelium is also the key cell type in our “HHA model” for AVM formation in HHT2 mice (Chapter 6). In this respect, we have also found that increased production of ROS in the arterial endothelium plays a key role in the development of AVM, further providing a molecular mechanism in the HHA model. Finally, it will not yet clear whether these increased ROS is mitochondrial, NADPH oxidase-dependent or eNOS uncoupling-derived as further work needs to be done.

HHT Modifier genes

The remarkable intra-familial variation in HHT human phenotype and the distinct disease severity in different mouse strains of HHT1 models, suggest the effect of environmental factors and/or genetic modifiers on HHT clinical manifestations. More than a decade ago, Akhurst’s laboratory mapped the genetic modifier locus Tgfbm on chromosome 1 as the major determinant of the dramatic variation in penetrance of Tgfb1 null on early prenatal mortality in mice (Bonyadi et al., 1997; Tang et al., 2003). Follow up studies have recently validated Tgfbm2 and Tgfbm3b locus as a modifiers of phenotype in Tgfb1 null mice in a plethora of different congeneric mice stains, including 129, C57 and NIH (Benzinou et al., 2012; Kawasaki et al., 2014). Using gene-centric tag-SNP technology within and flanking the TGFBM2 human locus, Banzinou et al identified two unique SNPs (rs2936018 and rs2936017) located within the non-receptor tyrosine phosphatase 14 (PTPNP14) gene showing significant genetic association of PAVM in Dutch and French HHT1 and HHT2 populations. Following a similar approach, Kawasaki et al presented evidence that functional polymorphism of two SNPs (rs10495565 and rs12474540) within the ADAM17 gene, located at TGFBM2β locus, exhibit significant correlation with the clinical outcome of PAVM in HHT1 patients. In Chapter 6 we identified GJA5 gene as new component in ALK1 signaling interplay that acts in combination with Acvrl1 in the development of the HHT phenotype. Genetic polymorphisms have been detected in both promoter regions of GJA5 and suspected to be associated with risk of cardiovascular diseases including hypertension (Molica et al., 2014). These findings emphasize the importance of knowing whether functional polymorphisms within the GJA5 gene or other genes implicated in the regulation of vascular tone can determinant different clinical risks and outcomes of HHT phenotype. Finally, since pulmonary and cerebral AVMs are more common in HHT1 patients, whereas hepatic AVMs are more prevalent in HHT2 patients, a key step leading to novel insights into the molecular mechanisms underlying HHT lesions in different tissues and possible early clinical prognosis would be the identification of tissue-specific HHT modifier genes.
Chapter 07

Future directions

With the advent of \textit{in vitro} and \textit{in vivo} HHT modeling tools, a new era to understand and explain the pathophysiology seen in HHT has begun. HHT exhibits significant phenotypic variability, in which the common, classic symptomatic feature remains the development of telangiectases and AVMs.

As we continue to improve the derivation of HHT-specific hiPSCs harboring the same disease-causing mutation and begin to produce isogenic controls that have been genetically repaired for comparison, we hope to be able to recapitulate AV shunts \textit{in vitro} and investigate to what extent these malformations are developed and maintained, by additional factors than \textit{ACVRL1} or \textit{ENG} heterozygosity, including altered blood flow, inflammation and mural cell coverage. Such an ideal platform may further allow one to identify AVM markers that could be potential drug targets for HHT therapies. In the meantime, the establishment of robust direct differentiation protocols for large-scale tissue-specific ECs will provide the opportunity to unravel why AV shunts are more common in lung and brain in HHT1 than hepatic AVMs in HHT2 patients.

Our findings in this thesis indicate that altered blood flow is necessary event for AVM development and that \textit{GJA5} can be used as potential genetic modifier for HHT2. It will be important to determine whether human polymorphic variants of \textit{GJA5} influence clinical variability and progression of HHT. Finally, yet undiscovered genetic modifiers may also be associated with the development of vascular malformations seen in HHT, leading therefore to the development of more robust animal models of HHT.
References


Chapter 07


Kim, J.Y., Lee, R.H., Kim, T.M., Kim, D.-W., Jeon, Y.-J., Huh, S.-H., Oh, S.-Y., Kyba, M., Kataoka, H., Choi, K.,
et al. (2014). OVOL2 is a critical regulator of ER71/ETV2 in generating FLK1+, hematopoietic, and endothelial cells from embryonic stem cells. Blood 124, 2948–2952.


Chapter 07

Suppresses Tumor Growth. Molecular Cancer Therapeutics 9, 379–388.


Discussion


Chapter 07


