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Chapter 01

Introduction

Vascular network formation

During embryonic development the cardiovascular system is one of the first structures to form and function. This is orchestrated by key morphological events, resulting in a hierarchical branched vascular network. Embryonic vasculogenesis relies on the differentiation of mesodermal precursor cells, also known as angioblasts, into endothelial cells (ECs). These differentiated cells then merge into primitive blood vessels and form the endocardium, paired aortas, primary vascular plexus in the embryo and the yolk sac vasculature in the extra-embryonic membrane (Flamme et al., 1997; Swift and Weinstein, 2009). The newly formed vessels continue to undergo expansion and remodeling via a process referred to as angiogenesis. Angiogenesis includes firstly the sprouting of the pre-existing vessels by the generation of new vascular branches. This is achieved via the migration of endothelial tip cells followed by the proliferation of endothelial stalk cells. Secondly, the newly formed neighboring vascular sprouts fuse, a process called anastomosis, through interactions between filopodia of respective tip cells. This results in the formation of the vascular network and the establishment of new cell-cell junctions. Finally, newly established networks undergo vascular stabilization and maturation by the coverage of perivascular cells, also known as mural cells (Stapor et al., 2014).

Deregulation of one of these fundamental vascular features due to defective signaling pathways can have major impacts on development, even causing embryonic lethality, or severe consequences on adult physiology that lead to a number of vascular pathologies. Many signaling pathways are known to orchestrate the development of the vascular network and one of the most important is transforming growth factor beta (TGFβ) superfamily.

TGFβ superfamily

TGFβ superfamily members are evolutionarily conserved and are involved in signal transduction pathways with key functions in a plethora of distinct cellular processes during normal development and human disease. Most notably, growth inhibition, cell differentiation, migration, invasion, apoptosis, epithelial-mesenchymal transition (EMT) and extracellular matrix (ECM) remodeling in a wide range of cell types and tissue contexts (Pardali and Dijke, 2012). Additionally, in recent years, it has also been shown that TGFβ superfamily members play a significant role in the progression of cancer, fibrosis and inflammation (Akhurst and Hata, 2012).

More than thirty secreted ligands, seven distinct type I receptors (activin receptor-like kinase or ALKs), five distinct type II receptors, seven type III receptors and eight Smad proteins have been identified as members of the TGFβ superfamily in humans and other mammals so far (Massague, 2012; Moustakas and Heldin, 2009).

All mature TGFβ ligand family members are characterized by a specific three-dimensional structure. They all have cysteine residues in their C-terminus, which form intra molecular disulphide-linked dimers. Dimerization of these proteins is important for receptor binding and activation (Dijke and Arthur, 2007). Besides the prototypic TGFβ ligands, the family also includes the bone morphogenetic proteins (BMPs), growth and differentiation factors (GDFs), activins (ACTs), inhibins (INHs), nodal growth differentiation factor (Nodal), left-right determination factor (Lefty) and Mullerian inhibiting substance (MIS). Detailed descriptions of the ligands are reviewed elsewhere (Moustakas and Heldin, 2009).

The TGFβ receptor family to which the ligands bind consists of seven type I receptors (ALK1-7) and five type II receptors, including activin receptor typeIIA (ActRIIA), activin receptor type IIB (ActRIIB), BMP type II receptor (BMPRII), TGFβ type II receptor (TβRII)

and AMH type II receptor (AMHRII). Both type I and II receptors are composed of a single transmembrane-spanning region, which is characterized by a short cysteine-rich extracellular part and a long intracellular domain. The latter is comprised of strong serine/threonine and weaker tyrosine kinase activity (Dijke and Arthur, 2007; Moustakas and Heldin, 2009). It is important to note that type I receptors consist of a short juxta-membrane, glycine-serine rich domain (GS-box), which are necessary for their activation. In addition to the type I and type II receptors, type III co-receptors, such as betaglycan (TβRIII) and endoglin (ENG) have been also identified. These accessory receptors are single transmembrane glycoproteins with short cytoplasmic tails that lack enzymatic activity. The function of these co-receptors is to facilitate ligand-receptor interactions (Dijke et al., 2008; Dijke and Arthur, 2007; Kapur et al., 2013). Finally the Smad proteins form essential parts of the downstream TGFβ signaling pathway. Smads are conventionally categorized into three groups; the receptor-regulated Smad proteins (R-Smads) 1, -2, -3, -5, and -8, common-mediator Smad protein (co-Smad) 4, and inhibitory Smad proteins (I-Smads) 6, and -7. These Smad proteins are characterized by a N-terminal Mad-homology 1 (MH1) domain, a middle linker domain and C-terminal MH2 domain. Importantly, the MH2 domain consists of a conserved Ser-Ser-X-Ser (SSXS) motif essential for binding to type I receptors (Feng and Derynck, 2005).

TGFβ signaling

TGFβ superfamily signaling involves a well-described signaling cascade that is initiated at the membrane by ligand binding and extended to the nucleus of the cell. Briefly, TGFβ ligands bind to a multi-component receptor complex at the cell surface that consists of two pairs of receptor serine/threonine kinases (type I and II). Upon ligand binding, the type II receptor, which is constitutively active, trans-phosphorylates the GS box of the type I receptor adjacent to its kinase domain. This then propagates the signal into the cell by the phosphorylation of Smad proteins. Primarily, R-Smads become phosphorylated by type I receptors on two serines within its SSXS motif of the carboxyl-terminus. Activated R-Smads are translocated into the nucleus by forming heteromeric complexes with co-Smad4, where they interact with a number of transcriptional co-activators and co-repressors to orchestrate target gene expression (Chacko et al., 2001; 2004; Inman and Hill, 2002; Kawabata et al., 1998; massague, 2012; Qin et al., 2001; Qing et al., 2004). By contrast, R-Smad translocation can be also inhibited by I-Smad6 and/or I-Smad7, which either compete with R-Smads for interaction with type I receptor or recruit Smad ubiquitin regulatory factor (Smurf) E3 ubiquitin ligases to the receptors leading to proteasomal degradation of the receptors (Ebisawa et al., 2001; Hayashi et al., 1997; Imamura et al., 1997; Murakami et al., 2003; Nakao et al., 1997). Furthermore, I-Smads can also inhibit receptor activation by dephosphorylating the receptor complex in combination with specific recruited phosphatases (Shi et al., 2004; Valdimarsdottir et al., 2006).

Interestingly, ligand binding can lead to signaling activation via more than one type II and type I receptor combination and signaling can also be triggered by many ligands that interact with the same receptor (Groppe et al., 2008). In general, TGFβ ligand superfamily members can be separated into two SMAD signaling (so-called “canonical”) categories, the TGFβ/Nodal/Activin and the BMP/GDF subfamilies (Groppe et al., 2008). Aside from the canonical SMAD activation, the TGFβ ligand superfamily can also regulate non-SMAD signaling pathways, so called the “non-canonical” pathways (Guo and Wang, 2009).

Canonical and non-canonical activation

To understand the principle pathways relevant the work in this thesis, The TGFβs and BMPs will be described as prototypes for signaling activation. TGFβs bind to the type II receptor TGFβR-II (TβR-II) and recruit and phosphorylate type I receptors TGFβR-I (ALK5, TβR-I), ActR-IB (ALK4) or ALK7 to the complex which induces phosphorylation of Smad2 and Smad3 proteins **(Fig.1)** (Bassing et al., 1994; Franzen et al., 1993; Lin et al., 1992). By contrast, BMPs typically signal via type II receptor BMPR-II and predominantly recruit with type I receptors ACVRL1 (ALK1), ACVR1 (ALK2), BMPR-IA (ALK3) or BMPR-IB (ALK6) (Dijke et al., 1994a; 1994b). Additionally, Activin and BMPs share binding for the type II receptors ActR-IIA and ActR-IIB (Yu et al., 2005). The activation of the type I receptors in this case propagates BMP signaling by phosphorylation of intracellular Smad1, Smad5 and Smad8 **(Fig.1)** (Miyazono et al., 2010). In contrast to TGFβ ligands, only BMPs are able to bind type I receptors in the absence of type II receptors but binding affinity increases remarkably when both receptor types are present (Rosenzweig et al., 1995). Besides Smad canonical pathway activation, TGFβ related ligands have been shown to activate additional downstream signaling pathways, including TRAF6 / TAK1 / p38 / JNK pathway (Tumor necrosis factor TNF receptor associated factor 6 / TGFβ associated kinase 1 / p38 / c-Jun N-terminal kinase), Ras / Erk / MAPK pathway (Ras / extracellular singal-regulated kinase / mitogen activated protein kinases), PI3K / Akt / mTOR (phosphoinosistide 3-kinase / Akt / mammalian target of rapamycin) and small GTPases RhoA, Cdc42 and Rac1 (For further reading, see review of (Mu et al., 2011).

TGFβ signaling in homeostasis and pathology

One of the most important roles of TGFβ signaling in vascular morphogenesis is that of promoting vessel maturation via EC-mural cell communication. Mural cells, include pericytes and vascular smooth muscle cells (vSMCs), elongate and wrap around small capillaries and larger vessels to regulate vessel diameter, stabilization and EC proliferation. ECs promote paracrime communication leading to the recruitment of mural cells. Platelet-derived growth factor-B/ Platelete-derived growth factor receptor-β (PDGF-B/PDGFR-β), angiopoietin 1 (Ang 1)/Tie2 and TGFβ signaling pathways have been shown to play a remarkable role in this process (Armulik et al., 2011; Gaengel et al., 2009; Stapor et al., 2014). In particular, yolk sac vasculature of endoglin deficient mice lacking TGFbRII or ALK5 exhibits EC-mural cell communication defects leading to fragile blood capillaries similar to those seen in human vascular disorders (Carvalho, 2004). Nonetheless, the protagonistic role of of TGFβ signaling in the adult vasculature is further indicated by its association with the development of human vascular diseases, including hypertension, pre-eclampsia and Hereditary haemorrhagic telangiectasia (HHT).

Hereditary hemorrhagic telangiectasia (HHT)

HHT or Osler-Weber-Rendu syndrome is an autosomal monogenic dominant disorder that affects the vascular homeostasis of approximately 1 in 5-8000 individuals (Kjeldsen et al., 2005; Marchuk et al., 1998). HTT is caused by mutations on genes involved in binding TGFβ and those associated with its signaling pathway. The disorder is characterized by the presence of a plethora of small or big arteriovenous malformations (AVMs) at specific locations of the body, which evolve with age. AVMs are distinguished by the lack of capillary beds between arteries and veins, leading to direct artery-to-vein connection **(Fig.2A).**
AVMs are found as congenital and posttraumatic forms and consist of clusters of dilated vessels. These clusters vary in size and can develop or grow over time depending on the tissue they are found in. In addition, AVMs can hemorrhagic which in most cases becomes progressively worse with age (Shovlin, 2010). Small AVMs, also called telangiectasia, are pink to red pinhead-size cutaneous lesions mainly on lips, tongue, oral cavity, fingers and

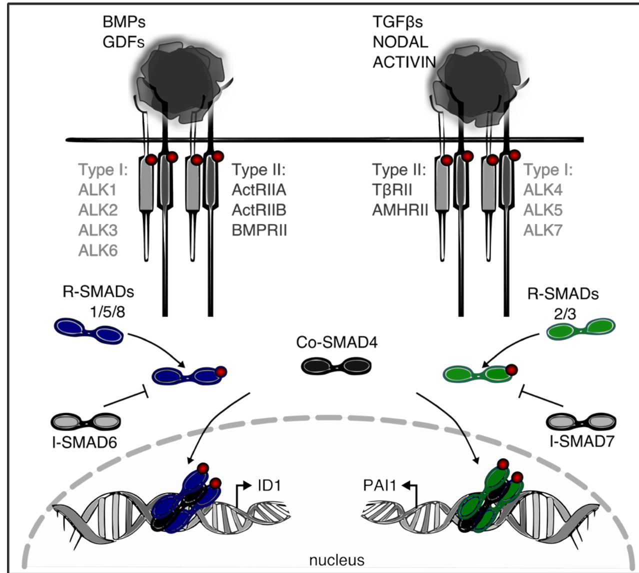

Figure1. Canonical TGFβ/BMP signaling

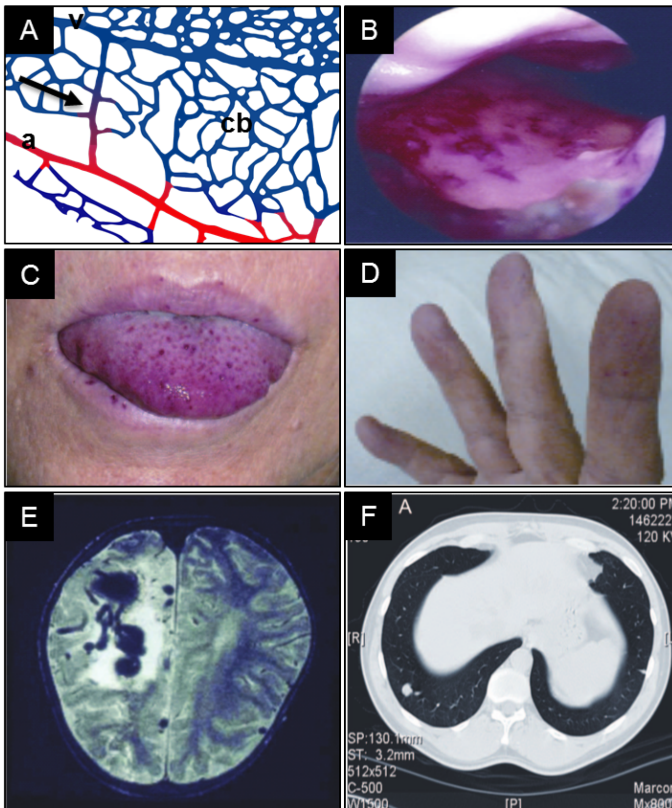

Figure 2. AVMs in HHT patients. (A) Schematic illustration of a direct connection (black arrow) between artery (a) and vein (v) missing the intervening capillary bed (cb). (B-D) Telangiectases on nasal mucosa (B), tongue/lips (C) and fingers (D). (E-F) AVMs of the brain (E) and lung (F) by MRI and CT, respectively. Modified after McDonald et al. 2011

the nasal, buccal, and gastrointestinal (GI) mucosa **(Fig.2B-D).** By later adulthood, almost all HHT patients develop telangiectasia on the hands, lips and face, usually observed only by focused examination. Periodic nasal bleedings (epistaxis) during childhood is one of the first features that bring individuals to medical attention. HHT patients with spontaneous and recurrent sever epistaxic events may develop chronic anemia and become blood transfusion dependent. Hemorrhage at the upper GI tract is usually observed in elder HHT patients and its severity increases with age. In contrast to small AVMs, large visceral AVMs or shunts, are usually present from birth and can be found in the cerebral, hepatic, pancreatic and pulmonary circulations (Shovlin, 2010) **(Fig.2E-F)**. These shunts lead to major hemodynamic changes, due to altered cardiac output, and life threatening events, particularly in younger individuals (McDonald et al., 2011).

The clinical diagnosis of adult HHT patients is based on consensus criteria referred to as the Curacao criteria. Family history of the syndrome and at least three major symptoms, including recurrent epistaxis, mucocutaneous telangiectasia in characteristic locations and visceral AVMs, are sufficient for the diagnosis (McDonald et al., 2015; Shovlin et al., 2000). In contrast to their beneficial diagnostic usage in adult patients, Curacao criteria can be misleading when applied to young asymptomatic or minimally symptomatic patients (van Gent et al., 2013). Thus, early diagnosis by molecular genetic testing is recommended for the medical management of possible at-risk family members with serious early complications.

The majority of HHT patients harbor mutations in *ENG* (chromosome 9q34) or Activin A Receptor Type I-like (ACVRL1/ALK1, chromosome 12q13) genes that cause HHT1 and HHT2, respectively (Johnson et al., 1996; McAllister et al., 1994). A minority of HHT patients are associated with mutations in Smad4 (chromosome 18q21), GDF2 (BMP9, chromosome 10q11) or RAS p21 protein activator 1 (RASA1, chromosome 5q13.3) (Gallione et al., 2004; McDonald et al., 2015; Wooderchak-Donahue et al., 2013)**.**

A myriad of different mutations including deletions, insertions, missense and splice-site mutations have been identified in HHT1 and HHT2 cases without a clear genotype-phenotype correlation emphasizing the heterogeneity of the disease (Govani and Shovlin, 2009; Paquet et al., 2001; Pece et al., 1997; Pece-Barbara et al., 1999). Reduced protein levels of ENG and ACVRL1 are found in HHT patients, supporting the idea that haploinsufficiency is the underlying cause of the disease.

Both HHT1 and HHT2 patients exhibit most of the Curaca criteria symptoms but development of specific vascular features is different between the two subtypes. In particular, pulmonary and cerebral AVMs are more common in HHT1 patients, whereas hepatic AVMs are more prevalent in HHT2 patients.

Most importantly, disease severity can be influenced by the age of onset and the locations of these vascular lesions, which differ from one individual to another and also within families. The latter suggests that other physiological or environmental factors (second hits) and/or modifier genes might be associated with the distinct phenotype of HHT. For this reason, various *in vivo* and *in vitro* models have been developed which have significantly contributed to understanding the pathogenesis of HTT and to finding novel treatment methods.

Modeling HHT1 and HHT2 *in vivo*

The majority of HHT1 or HHT2 mutations correspond to deletion and splice variant defects leading to a frameshift and hence truncated proteins that are either not expressed or retained intra-cellularly (Cymerman et al., 2000; Paquet et al., 2001; Pece et al., 1997; Pece-Barbara et al., 1999). Thus, a number of acvrl1 or endoglin loss-of-function mice have been generated in order to model HHT.

Mice deficient in acvrl1 or endoglin die in utero at E10.5-E11.5 revealing their significant role during development (Arthur et al., 2000; Bourdeau et al., 1999; Li, 1999; Oh et al., 2000). In particular, endoglin null embryos exhibit dilated and hemorrhagic vascular channels in the primitive vascular plexus of the yolk sac and the peritoneal cavity of the embryo resulting in embryonic lethality (Arthur et al., 2000; Bourdeau et al., 1999; Li, 1999). In the developing yolk sac, the vSMC marker SM22a is normally present at E9.5 around the endothelium of the capillary plexus. Loss of endoglin causes failure in vSMC development and vessel maturation due to reduced active TGFß1 protein secretion by ECs suggesting aberrant EC-pericyte interaction as contributing to the pathogenesis of HHT (Carvalho, 2004; Li, 1999).
Furthermore, acvrl1 null embryos show hyperfusion of capillary plexes and highly dilated dorsal aorta and branchial arch arteries, which is associated with impaired vSMC recruitment and differentiation (Oh et al., 2000; Urness et al., 2000). Large AVMs between dorsal aorta and cardinal veins develop and fuse by E8.5 and E9.0, respectively (Urness et al., 2000). Interestingly, the molecular program for arterial vessel identity was disrupted in the yolk sac and caudal dorsal aorta of acvrl1 null embryos, as shown by Efnb2 staining. The latter, raises the question whether the arterial-vein vessel identity is also affected in large AVMs associated with HHT.

It is intriguing to note that in both acvrl1 and endoglin null mice, development of the primitive vasculature and early vascular plexus of the yolk sac (vasculogenesis) is normal, suggesting an important role for acvrl1 and endoglin in angiogenesis rather than vasculogenesis along with vascular remodeling.

Mice heterozygous for acvrl1 (*acvrl1*$^{+/-}$) or endoglin (*endoglin*$^{+/-}$) are viable and fertile and based on their genotype, they represent murine models for HHT. Unfortunately, they exhibit some but not all vascular phenotypes of the disease, and the do not have the most severe life threatening symptoms (Bourdeau et al., 2001; 1999; Oh et al., 2000). In particular, *endoglin*$^{+/-}$ mice develop dilated, weak-walled vessels, multi-organ telangiectases or recurrent nose/mouth bleeds. Interestingly, these lesions develop with greater severity on a 129/Ola mouse genetic background than on an Ola/129-C57BL/6 intercross or C57BL/6 backcross, supporting the idea of the presence of alleles or modifier genes prone to influence severity of HHT in humans (Arthur et al., 2000; Bourdeau et al., 2001; 1999). More importantly, analysis of mouse neonatal retina from *endoglin*$^{+/-}$ mice revealed excessive angiogenesis at the vascular front, which was unexpectedly normalized by thalidomide, a vascular maturation factor that has been shown to be beneficial for reducing severity of epistaxis in HHT patients (Lebrin et al., 2010). Mural cell coverage of the vasculature due to upregulation of PDGF-B expression in endothelial tip cells was found to be the underlying mechanism of action of Thalidomide (Lebrin et al., 2010).

Acvrl1$^{+/-}$ mice on the C57BL/6 background develop multi-organ vascular lesions. These appear in the nasal mucosa, GI tract, liver, lung, spleen, skin and oral cavity. However these vascular defects exhibit age-dependent penetrance and develop at very low frequency (Srinivasan et al., 2003).

It is of note that adult endoglin$^{+/-}$ and acvrl1$^{+/-}$ mice develop spontaneous pulmonary arterial hypertension (PAH) in combination with a secondary right ventricular hypertrophy (RVH) as well as rarefaction of the peripheral lung vasculature and muscularization of small arteries (Ardelean et al., 2014; Jerkic et al., 2011; 2012; Toporsian et al., 2010). PAH was shown to be attributable to increased oxidative stress due to NO reduction and eNOS-dependent reactive oxygen species (ROS) overproduction in major internal organs including lungs, liver and colon (Jerkic et al., 2012). Recent studies have provided evidence that a distinct molecular angiogenic/angiostatic imbalance may be involved in the lungs of both HHT mouse models. In particular, endoglin$^{+/-}$ lungs exhibited augmentations in the angiostatic factor thrombospondin-1 (TSP-1) whereas, acvrl1$^{+/-}$ lungs showed normal levels of TSP-1 and instead an increase in the vascular destabilizing protein Ang-2 (Ardelean et al., 2014). Most importantly, anti-vascular endothelial growth factor (anti-VEGF) treatment using the monoclonal antibody G6-31 was able to attenuate the secondary RVH and normalize peripheral pulmonary vasculature in both models. Surprisingly, anti-VEGF treatment was also able to normalize the TSP-1 and Ang-2 levels even though initial pro-angiogenic VEGF levels in the lung tissue were indistinguishable, leading to the speculation that this is a secondary effect due to reduced pulmonary VEGF levels. Of interest is the observation of a strong tissue rather than HHT-specific anti-VEGF response in the hepatic microvessel density (MVD) of control and both *endoglin*$^{+/-}$ and *acvrl1*$^{+/-}$ mutant mice (Ardelean et al., 2014).

As explained above, development of large AVMs in solid organs is the most life threatening phenotype in HHT patients. Unfortunately, the development of those lesions in endoglin and acvrl1 heterozygous mice occurs in extremely low frequency, which makes the investigation of the underlying mechanism very limited (Torsney, 2003). The combination of (i) the vSMC defective phenotype observed in null acvrl1 and endoglin embryos and (ii) endoglin and acvrl1 expression beyond vascular ECs and including lymphatic ECs and monocytes/ macrophages, raises the question whether ECs or another vascular cell type are the major key players for the development of AVMs (Niessen et al., 2010). In order to address this, cell type-specific constitutive and conditional acvrl1 or endoglin knock out mice have been generated using the Cre-lox technology.

Constitutive-Conditional deletion of acvrl1 or endoglin

Constitutive acvrl1 deletion from all mouse arterial ECs of the brain, lung and GI tract using L1-Cre with Cre activity from late embryogenesis (E13.5) onwards, caused AVMs formation with severe hemorrhages in the brain, lungs and GI tract, leading to death by P5 (Park et al., 2009). It is interesting to note that no vascular defect was found in any other tissues of these mutant mice, including kidney, muscle, skin, heart and large intestine (Park et al., 2009).

Furthermore the same group was able to show that tamoxifen induced EC specific deletion (*Acvrl1*-iKO^{e}) of acvrl1 in the first week of postnatal life using Cdh5-Cre^{ER} gave rise to severe vascular defects in the retina undergoing angiogenesis and resulted in rapid lethality (48 hours post recombination) **(Fig.3C)**. This was due to severe pulmonary hemorrhage (Tual-Chalot et al., 2014). Interestedly, Arthur's laboratory using a similar Cre deleter, reported some overlapping vascular defects in the retina of endoglin mutant mice (Mahmoud et al., 2010) **(Fig.3B)**. In particular, both acvrl1 and endoglin mutant retina exhibited venous dilation and AVM formation, both marked by venous identity (EphB4 or Aplnr positive) and increased EC proliferation. On the other hand, endothelial deletion of acvrl1 alone affected the retinal vascular plexus between main arteries and veins, leading to hyperbranching, increased number of tip cells and reduced phosphorylation of Smad1/5/8 proteins, where in endoglin mutant mice all of the above are normal (Mahmoud et al., 2010; Tual-Chalot et al.,

2014). Furthermore and in agreement with a previous study, arteries of acvrl1 mutant retinas exhibit reduced arterial identity as indicated by Jag1 expression (Mahmoud et al., 2010; Tual-Chalot et al., 2014; Urness et al., 2000). In contrast to acvrl1 mutant retinas, endothelial deletion of endoglin results in two unique vascular defects at the periphery of the retina: (i) delayed vascular radial expansion due to endothelial migration defect and; (ii) increased capillary density followed by vascular smooth muscle cell coverage due to increased blood flow.

The importance of acvrl1 and endoglin in adult mice is highlighted by the fact that, tamoxifen induced endothelial specific (Cdh5-Cre^{ER}) or global (ROSA26-Cre^{ER}) deletion of acvrl1 results in caecal hemorrhage and mortality within 2-3 weeks upon recombination (Park et al., 2009; Tual-Chalot et al., 2014). In addition, global deletion of endoglin in adult mice using the tamoxifen inducible ROSA26-Cre^{ER} deleter give rise to signs of illness, including dehydration and diarrhea and death 4 to 10 days post recombination (Garrido-Martin et al., 2014). On the other hand, tamoxifen induced endothelial specific deletion of endoglin in adult mice using Cdh5-Cre^{ER} followed by subcutaneous Matrigel implants consisting of angiogenic cytokines (VEGF and bFGF) revealed an endothelial remodeling defect during angiogenesis, including topical venous enlargement (Mahmoud et al., 2010). It is therefore important to note, firstly, the requirement of acvrl1 and endoglin expression during angiogenesis and quiescent vasculature and secondly the requirement of physiological angiogenic stimulants for excessive AVMs formation **(Fig.3)**.

Dynamic acvrl1 expression analysis during postnatal pathophysiological conditions or "second hits", such as wound healing, leading to active angiogenesis reveals increased expression of acvrl1 in feeding arteries which was further associated with increased blood flow (Seki, 2003). In accordance with the latter, the relation between injury and the development of HHT phenotype was intensively addressed using the HHT mouse models mentioned above.

In a series of studies, *de novo* AVM development was revealed in adult mice upon global (ROSA26-Cre^{ER}) genetic ablation of acvrl1 followed by wounding. Specifically, Park et al. were able to capture the onset, progression, remodeling and hemodynamic changes of subdermal AVM at the area of the wound for 8 days in real-time, using the dorsal skinfold window chamber system (Park et al., 2009). In particular, A-V shunts were characterized by locally dilated arteries and veins consisting of areas with high-velocity, turbulent arterial blood flow and elevated levels of $Hb(O_2)$ (Park et al., 2009). Similarly, global deletion of endoglin in adult mice using a similar Cre deleter revealed dilated/tortuous vessels and formation of A-V shunts in the area close to the wounded mid-dorsum and ear (Choi et al., 2014; Garrido-Martin et al., 2014).

In a recent study from the same group, the development of AVM in the areas around the wounded ear and back skin of HHT1 and HHT2 mouse models was investigated side-by-side (Garrido-Martin et al., 2014) **(Fig.3D-F)**. Deletion of endothelial specific acvrl1 or endoglin using tamoxifen induced Scl-Cre^{ER} mice, gave rise to tortuous and enlarged vessels with multiple AV shunts at the area of the wounds similarly to those previously reported using ROSA26-Cre^{ER} (Garrido-Martin et al., 2014; Park et al., 2009). Interestedly, endoglin mutant mice survived longer than 4 weeks after recombination with no visible signs of AVMs or hemorrhages in internal organs, whereas acvrl1 mutant mice further developed AVMs in the GI tract followed by severe bleeding in the cecum and lethality after 2 weeks of Cre activation (Garrido-Martin et al., 2014).

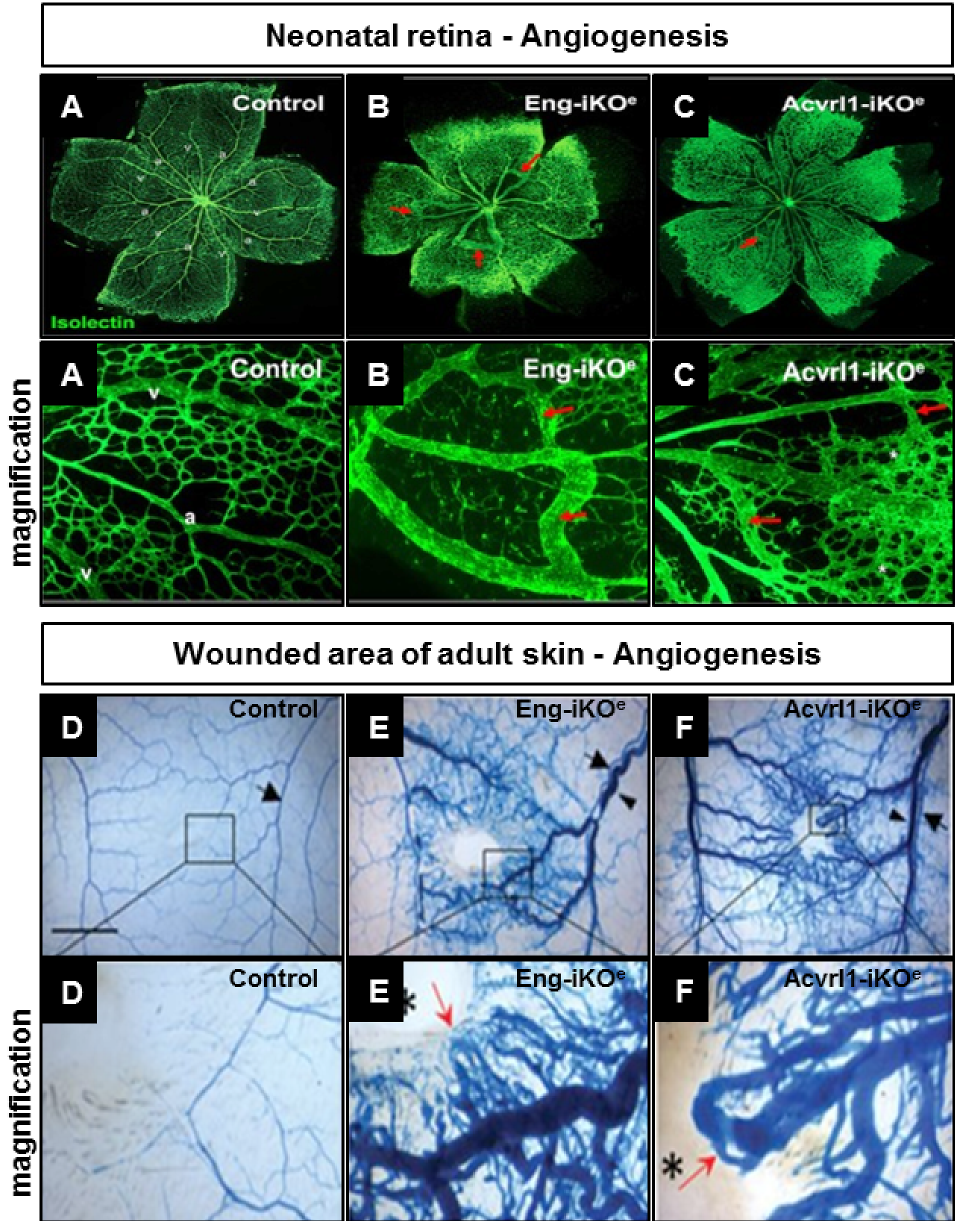

Figure 3. Development of AVMs in mouse models of HHT1 and HHT2 during angiogenic events. (A-C) Top, IsolectinB4 stained ECs in neonatal retina vessels in Control (A), Eng-iKO^e (B) and Acvrl1-iKO^e (C); at bottom are higher magnifications from the images in the top row. Red arrows indicate direct connection of arteries and veins (AVMs) in Eng-iKO^e (B) and Acvrl1-iKO^e (C). (D-F) Top, skin vasculature of control (D), Eng-iKO^e (E) and Acvrl1-iKO^e (F) back skin shown by latex dye injection after wounding (asterisk); at bottom, higher magnification of the vascular network around the wound. Red arrows indicate the development of AVMs. Modified after Tual-Chalot et al.2015 and Garrido-Martin et al. 2014.

These data showed firstly, the requirement of environmental stress, such as dermal injury, for the development of AVMs in both HHT1 and HHT2 models and secondly, the involvement of a non–EC type in the formation of AVMs in HHT1 GI tract. It is important to take in to account that hemodynamic changes, previously reported to be associated with acvrl1 ablation (Park et al., 2009; Seki, 2003; Tual-Chalot et al., 2014), may play a secondary role in the development of vascular malformations, such as AVMs, in distant tissues.

As described above, young HHT patients can also develop life threatening large AVMs in the brain, thus reproducible *in vivo* models are necessary to investigate the underlying mechanism.Brain AVMs were successfully generated in adult mice upon topical pan-cellular deletion of acvrl1 or endoglin and VEGF stimulated angiogenesis using adenoviral vectors driven by cytomegalovirus promoter (Chen et al., 2014a; 2014b; Walker et al., 2011). A more recent report from the same group also describes AVM formation in the brain, intestine, lung and around ear-tag wounds of adult acvrl1 mutant mice characterized by increased EC proliferation as indicated by $BrdU^+$ and $ki67^+$ positive staining (Chen et al., 2014a; 2014b). In contrast to their study before, vascular malformations in the angiogenic area were generated upon tamoxifen induced endothelial specific deletion of acvrl1 using Pdgfb-$iCre^{ER}$ and VEGF stimulated angiogenesis using adenoviral vector leading to lethality 6 to 13 days after recombination (Chen et al., 2014a). This demonstrates the requirement of additional angiogenic stimulus for the development of tissue specific AVMs.

Aims and chapter outline of this thesis

The aims of the research described in this thesis are three-fold: (1) to investigate a new gene targeting knock-in fusion strategy that can be used to study early steps in cardiovascular specification using human embryonic stem cells (hESCs), (2) to develop in vitro models of HHT from patient specific cells induced pluripotent stem cells (iPSCs) and (3) to identify new potential modifier genes for HHT2.

Chapter 2-3 are related to the development of a new gene targeting knock-in fusion strategy. Chapter 2 serves as a proof-of-concept study of this new knock-in fusion approach, including its biallelic applicability, whereas Chapter 3 focuses on the direct differentiation of hESC towards ECs, in which this knock-in fusion approach can be applied to study early cardiovascular commitment. Focusing on one cell type, endothelial cells, -that is a disease target and can be derived from patient hiPSC, Chapter 4 describes the derivation of iPSC lines from healthy individuals and patients diagnosed with HHT. In Chapter 5, this investigation is extended specifically to the derivation of HHT1 iPSC-ECs with view to exploring the consequences for ligand induced signaling and endothelial cell function. Chapter 6 focuses on the role of a new potential modifier gene in the formation of arteriovenous malformations (AVMs) in an HHT2 mouse model. Chapter 7 is a general discussion, which combines and discusses Chapter 2-6.

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