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REVIEW

ALK1 signaling in development and disease: new paradigms

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Abstract Activin A receptor like type 1 (ALK1) is a transmembrane serine/threonine receptor kinase in the transforming growth factor-beta receptor family that is expressed on endothelial cells. Defects in ALK1 signaling cause the autosomal dominant vascular disorder, hereditary hemorrhagic telangiectasia (HHT), which is characterized by development of direct connections between arteries and veins, or arteriovenous malformations (AVMs). Although previous studies have implicated ALK1 in various aspects of sprouting angiogenesis, including tip/stalk cell selection, migration, and proliferation, recent work suggests an intriguing role for ALK1 in transducing a flow-based signal that governs directed endothelial cell migration within patent, perfused vessels. In this review, we present an updated view of the mechanism of ALK1 signaling, put forth a unified hypothesis to explain the cellular missteps that lead to AVMs associated with ALK1 deficiency, and discuss emerging roles for ALK1 signaling in diseases beyond HHT.

Keywords Activin A receptor like type 1 · Hereditary hemorrhagic telangiectasia · Angiogenesis · Arteriovenous malformation · Bone morphogenetic protein · Endoglin

Abbreviations

ActRII	Activin receptor type II
ACVRL1	Activin A receptor like type I (gene)
ALK1	Activin A receptor like type I (protein)
AMHRII	Anti-mullerian hormone receptor type 2
ANGPT2	Angiopoietin 2
AV	Arterial/venous
AVM	Arteriovenous malformation
BMP	Bone morphogenetic protein
BMPR	Bone morphogenetic protein receptor (BMPR)
CX40	Connexin 40 (also known as GJA5)
CXCR4	C-X-C motif chemokine receptor type 4
CXCL12	C-X-C motif chemokine ligand type 12
DLL4	Delta-like 4
E	Embryonic day
EC	Endothelial cell
EC50	Effective concentration-50
EDN1	Endothelin 1
EFNB2	Ephrin B2
ENG	Endoglin
ESM1	Endothelial cell specific molecule 1
GDF	Growth and differentiation factor
GS	Glycine and serine rich
HEY	Hes-related family bHLH transcription factor with YRPW motif
HHT	Hereditary hemorrhagic telangiectasia
ID	Inhibitor of differentiation
JAG1	Jagged 1
KD	Kinase domain
KLF2	Krüppel-like factor 2
LDL	Low-density lipoprotein
MH1	Mad homology 1
miRNA	MicroRNA
MIS	Müllerian-inhibiting substance
mTORC1	Mechanistic target of rapamycin complex 1
NICD	Notch intracellular domain
NR2F2	Nuclear receptor subfamily 2 group F member 2

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PAH	Pulmonary arterial hypertension
PDGF	Platelet-derived growth factor
PI3K	Phosphatidylinositol-3-kinase
PVH	Pulmonary venous hypertension
RBPJ	Recombination signal binding protein for immunoglobulin kappa J region
RTK	Receptor tyrosine kinase
TGFβ	Transforming growth factor beta
TβRII	Transforming growth factor beta receptor 2
TMEM100	Transmembrane protein 100
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
YAP1	Yes-associated protein-1
ZP	Zona pellucida

Introduction

Ligands within the transforming growth factor-β (TGFβ) superfamily signal via transmembrane receptor complexes and control myriad processes important in development and disease, including embryonic axis patterning, heart and vascular development, epithelial–mesenchymal transition, and fibrosis. Among the signaling receptors, activin A receptor like type 1 (*ACVRL1*, which encodes ALK1) has remarkable cell-type specificity, with predominant expression in arterial endothelial cells (ECs) [1]. Heterozygous mutations in *ACVRL1* cause hereditary hemorrhagic telangiectasia type 2 (HHT2), whereas heterozygous mutations in endoglin (*ENG*), a co-receptor that facilitates ligand binding, cause HHT1 [2, 3]. Together, mutations in these two genes are responsible for 85–96% of cases of HHT [4]. HHT is an autosomal dominant vascular disease that affects approximately 1 in 5000 people worldwide and presents with variable age of onset and expressivity [5–7]. Although HHT is generally attributed to *ACVRL1* or *ENG* haploinsufficiency, the possibility of somatic second hits as vascular lesion drivers [8, 9] has not been thoroughly explored.

Reduced ALK1 signaling predisposes HHT patients to development of arteriovenous malformations (AVMs), which are direct connections between arteries and veins. Because capillaries normally serve to slow blood flow between arteries and veins and to allow for efficient gas exchange with surrounding tissues, high-flow AVMs can lead to hemorrhage and hypoxia. In HHT patients, these vascular lesions appear most often in skin (face and hands), oral cavity, nasal and gastrointestinal tract mucosa, lung, liver, and brain [5–7]. Small AVMs in the skin and mucus membranes are also known as telangiectasias; rupture of these lesions can lead to epistaxis, gastrointestinal bleeding, and anemia. Pulmonary AVMs may cause hypoxemia, embolic stroke, or brain abscess; the latter sequelae result from impaired filtration of small particles and bacteria by lung capillaries [10, 11]. Liver AVMs, if large or numerous, can significantly lower vascular resistance and lead to high-output heart failure [12], whereas brain AVMs may lead to hemorrhagic stroke [13]. Brain and lung AVMs are thought to be congenital lesions, although clinical significance may not manifest until later in life. In contrast, telangiectasias may develop throughout life. The age of onset and expressivity of HHT are variable. For example, nearly all HHT patients exhibit epistaxis by 20 years of age, whereas high-output heart failure is a relatively rare outcome that manifests later in life [5–7].

Although the requirement for ALK1 and ENG in vascular development and AVM prevention has been recognized for more than 20 years [2, 3], we are only beginning to understand how ALK1 signals in arterial ECs, how ECs change their behavior in response to this signal, and how this behavior functions to maintain a hierarchical vascular network and prevent AVMs. In this review, we briefly discuss vascular development and summarize phenotypes of animal models of HHT; highlight the latest developments in our understanding of molecular aspects of ALK1 signaling, including pathway components, crosstalk with other signaling pathways, and interaction with mechanical force; and present evidence gleaned from a variety of model systems that points to a role for ALK1 in EC migration in response to blood flow. We also discuss emerging evidence for roles for ALK1 signaling in other vascular-associated diseases such as pulmonary arterial hypertension, cancer, and atherosclerosis, and close with a discussion of significant knowledge gaps and research needs.

A brief primer on vascular development

Development of the blood vasculature occurs via two basic mechanisms, vasculogenesis and angiogenesis. In vasculogenesis, primitive ECs differentiate from yolk sac or lateral mesoderm and form solid cords that then hollow into interconnected tubes. EC differentiation requires vascular endothelial growth factor A (VEGFA)/VEGF receptor 2 (VEGFR2)-dependent expression of lineage restricted ETS-family transcription factors [14, 15], and ECs are further specified as arterial or venous prior to the onset of blood flow [16]. Arterial specification requires high VEGFA/VEGFR2 and Notch activity [17–21]. In arterial ECs, Notch activation results in cleavage of the Notch intracellular domain (NICD), which translocates to the nucleus and binds to the transcriptional regulator, RBPJ. RBPJ/NICD, along with Sox transcription factors, activate expression of arterial markers such as delta-like 4 (*Dll4*, which encodes a transmembrane Notch ligand) and ephrinb2 (*Efnb2*, which encodes a transmembrane EphB4 ligand) [22]. Venous specification is less well understood. RBPJ (in the absence of NICD) and the orphan nuclear

receptor Nr2f2 repress arterial gene expression in venous ECs [23], but Nr2f2 is not required to specify venous cell fate [24–27]. These data suggest that the arterial program is actively repressed in venous ECs to maintain venous identity.

Depending on the embryonic location, the template assembled by vasculogenesis may take the form of a honeycombed plexus of similar-sized vessels or an elongated tube. This primitive vascular scaffold is subsequently modified by angiogenesis, which includes sprouting of new vessels from the existing network. During arterial angiogenic sprouting, high vascular VEGFA/VEGFR2 activity induces expression of a cohort of genes (including *DLL4*, *ESM1*, *CXCR4, APELIN*) in select arterial ECs, marking these cells as tip cells [28–30]. Tip cells are highly migratory ECs that lead the lagging stalk cells within an angiogenic sprout, charting a course via filopodial or lamellipodial protrusions [31, 32]. Previous reports suggested that tip cell-expressed *DLL4* was required to maintain tip cell position by activating Notch and dampening *VEGFR2* expression in adjacent stalk cells, and that dynamic Notch signaling allowed switching between tip (low Notch activity) and stalk (high Notch activity) cell fates [33–36]. However, recent studies fail to support this assertion [37–39]. These new studies favor an alternative model in which tip cells derive from a clonal population, with asymmetric division transferring high *vegfr2* mRNA and VEGFA responsiveness to just one of two daughter cells, rendering the second daughter a stalk cell to maintain the leader–follower hierarchy [37, 39]. In this model, cell-autonomous *DLL4* is dispensable for tip cell specification or maintenance, and Notch signaling in tip cells induces *CXCR4* expression to set arterial fate and/or allow tip cell-mediated sprout fusion with pre-existing arterial vessels [38, 39]. Venous angiogenic sprouting is less well characterized than arterial sprouting but may involve VEGFC/VEGFR3 and/or bone morphogenetic protein (BMP) signaling, depending on the vascular bed [40–42].

Angiogenic sprouts are elongated primarily by stalk cell division, and navigation is guided by both positive and negative guidance cues that affect tip cell cytoskeletal organization and migration. VEGFA and CXCL12 are promigratory ligands that act on EC receptors VEGFR2 and CXCR4, respectively [43–45]. Other ligands are bifunctional, with effects dictated by the specific receptors expressed on the EC surface [46]. For example, locally produced semaphorins can induce filopodial extension via endothelial plexinB1 or plexinB3 or retraction via endothelial plexinD1 or neuropilin. Netrin ligands are also bifunctional, whereas slits and ephrins are generally repulsive. An integrated response to these guidance cues is critical for generation of normal vascular pattern.

Both vasculogenic and angiogenic vessels become invested at the abluminal surface by support cells, or mural cells, either subsequent to or concomitant with tube formation [47, 48]. These cells contribute to vascular basement membrane and are required to maintain vascular integrity, prevent vessel leakage, and control vessel diameter. Mural cells include pericytes, which sparsely surround small vessels, and vascular smooth muscle cells, which envelop large vessels. The ontological relationship between vascular smooth muscle cells and pericytes is unclear, and their developmental origin is diverse, with progenitors derived from neural crest, mesothelium, somites, second heart field, epicardium, and endocardium [49, 50]. Development of this perivascular sheath requires paracrine signaling between mural cells and ECs via ligands including platelet-derived growth factor-B (PDGF-B), angiopoietins, and TGFβ [47].

As vascular development proceeds, sprouting, pruning, intratubular EC migration, and fusion reshape the vasculature into a hierarchical structure: large arteries taper to arterioles that ramify into highly branched, thin capillary networks, which coalesce to venules that lead to larger veins. This remodeling process, perhaps best characterized in the dramatic transformation of the mouse yolk sac vasculature from a primitive vascular plexus to an exquisite hierarchical tree, is triggered by and dependent on shear stress [51–53]. Shear stress is the frictional force of blood flow that acts directly on ECs, parallel to the direction of blood flow, with a magnitude proportional to flow velocity and viscosity and inversely proportional to the vessel radius. It is this shear stress-induced remodeling process, as opposed to vasculogenesis or sprouting angiogenesis, that seems to require ALK1 signaling.

Animal models of HHT

Although the consequences of disrupted ALK1 signaling are well appreciated, the role of ALK1 in vascular development and homeostasis and the mechanism by which impaired ALK1 function leads to AVMs remain unclear. In embryonic mice, *Acvrl1* is expressed predominantly in arterial ECs [1]. In adult mice, expression is constitutively high only in lung arterial ECs and is induced during wound healing and tumor angiogenesis [1]. Global embryonic deletion of *Acvrl1* results in mid-gestational lethality [embryonic day (E) 11.5] due to vascular defects: although vasculogenesis is unaffected, mice exhibit dilated vessels/AVMs, failed plexus remodeling, disrupted arterial identity, and decreased vascular smooth muscle cell coverage [54, 55]. Tissue-specific deletion demonstrates a requirement for *Acvrl1* in ECs [56, 57]. Conditional global or endothelial-specific *Acvrl1* deletion is also lethal in neonates and adults, with animals exhibiting AVMs and hemorrhage in uterus, lung, gastrointestinal tract, and retina, but not in brain or skin [57–62]. Interestingly, brain and skin AVMs develop in *Acvrl1*-deleted mice in response to VEGF stimulation or wounding, suggesting

that a "second hit" that triggers angiogenesis is required for AVM development in these tissues [56–59, 63–66]. Global deletion in adulthood is also associated with cardiomegaly and high-output heart failure [57, 62] that may be caused, as in HHT patients, by low peripheral vascular resistance. Together, these studies point to a critical requirement for *Acvrl1* in vascular development and maintenance throughout life.

Zebrafish *acvrl1* is expressed exclusively in arterial ECs during embryonic development, and mutants develop embryonic lethal AVMs. However, in this model, arterial expression predominates in those arteries closest to the heart, and in mutants, arterial/venous (AV) identity is unaffected and cranial vessel shunts develop prior to smooth muscle or pericyte recruitment [67, 68]. These observations suggest that neither disrupted AV identity nor failed vessel investment causes HHT-associated AVMs.

Eng is expressed predominantly in ECs in embryonic mice, with high expression in endocardium and capillaries, moderate expression in arteries, and weaker expression in veins [69]. In adult lung, *Eng* is expressed in distal arterioles, capillaries, venules, and pulmonary veins, but absent in pulmonary arteries [70]. Endoglin, also known as CD105, is also expressed on hematopoietic stem cells, mesenchymal stem cells, macrophages, and vascular smooth muscle cells, suggesting additional roles outside of the endothelium [71–75]. *Eng* deletion in mice results in a less severe vascular phenotype compared to *Acvrl1* deletion. Global deletion is embryonic lethal (E10.5–E11.5) with failed plexus remodeling, vessel dilation, decreased vascular smooth muscle cell and pericyte coverage, but no loss or arterial identity and no, or very small, AVMs [76–79]. In these mice, embryonic lethality is likely caused by cardiac defects, including failed cardiac cushion and valve development and ventricular and outflow tract dilation [76, 77, 80], and impaired blood flow may in turn be responsible for vascular abnormalities [51]. Embryonic deletion of *Eng* via SM22α:Cre (which deletes in smooth muscle cells and some ECs) results in brain AVMs by 5 weeks of age, which were attributed to mosaic loss in ECs, whereas deletion in macrophages causes immune function deficits but not vascular lesions [74, 81]. EC-specific *Eng* deletion in neonates causes retinal AVMs, whereas global or EC-specific but not smooth muscle cell-specific deletion in adults is associated with wound-induced or VEGF-stimulated skin and brain AVMs, but no other vascular defects [58, 81, 82]. Notably, global *Eng* deletion in adults causes lethality within 4–10 days, whereas endothelial-specific deletion is not lethal. Together, these data suggest not only a cell-autonomous role for *Eng* in ECs with respect to vascular development and homeostasis, but also additional critical roles in other cell types.

Zebrafish *eng* was recently identified and is broadly expressed in embryonic arterial and venous ECs during embryogenesis [83]. However, in contrast to mice, *eng* homozygous mutants survive to adulthood and exhibit only minor blood vessel defects including increased caliber of and shunt-like flow through embryonic axial vessels; dilated, tortuous vessels in aged brain (21 months); and AVMs in adult tail fin vessels in response to amputation [83]. Similar to zebrafish *acvrl1* mutants, the *eng* embryonic vascular phenotype, which manifests as a temporary high-flow shunt, occurs prior to vessel investment by pericytes or smooth muscle cells [83]. Although these zebrafish studies focused on development of congenital AVMs, it seems likely that ALK1/ENG function would be conserved postnatally and that altered mural cell coverage may be a secondary consequence and not a primary cause of AVM development at any stage.

Although HHT is an autosomal dominant condition and most mutations do not function as dominant negatives (reviewed in [84]), it remains unclear whether HHT-associated AVMs result from haploinsufficiency or a second somatic hit and resulting mosaic loss-of-function. In support of the second somatic hit theory, heterozygous *Acvrl1* mice develop vascular phenotypes with incomplete penetrance and low expressivity, [85], and heterozygous *Eng* mice develop a similarly incomplete penetrant phenotype, but only on the 129/Ola background [77, 86], which is predisposed to vascular defects [87]. In addition, mosaic homozygous but not heterozygous loss-of-function of either gene can generate AVMs in mice [81, 88, 89]. Taken together with the fact that the age of onset and expressivity of HHT vary within families [90], these data support the second somatic hit/mosaic loss of function hypothesis. However, this hypothesis requires formal testing.

ALK1 signaling in ECs

Overview of TGFβ family signaling

In humans, the TGFβ family includes 33 secreted ligands that are categorized into multiple subfamilies based on structure and phylogeny. The BMPs and growth and differentiation factors (GDFs) together comprise the largest subfamily (approximately 20 proteins) and are the ancestral ligands of the family. These ligands preceded evolution of activins, inhibin, Müllerian-inhibiting substance (MIS), and TGFβs, the latter of which are only found in vertebrates [91]. All TGFβ family ligands are generated as proproteins that are processed intracellularly by Golgi-resident proprotein convertases (for example, furin) to generate an N-terminal prodomain and a C-terminal growth factor domain [92, 93]. The proteins are often secreted in a complexed form, hereafter referred to as a procomplex, containing a growth factor domain homodimer non-covalently bound to two molecules

of its cognate prodomain. Growth factor domain homodimers, which in most cases are covalently linked by a single inter-chain disulfide bond, are the active signaling moiety. The function of non-covalently bound prodomains may vary among the TGFβ family ligands, with suggested functions including extracellular matrix targeting and conferral of latency [94, 95].

TGFβ family ligands bind to the extracellular domains of type I and type II transmembrane receptors, which are serine–threonine kinases. There are seven type I receptors (ALK1–ALK7) and five type II receptors (ActRIIA, ActRIIB, BMPRII, TβRII, and AMHRII). Generally, ALK4, ALK5, and ALK7 bind to TGFβ, nodal, and activin ligands, whereas ALK1, ALK2, ALK3, and ALK6 bind BMPs, GDFs, and MIS [96]. The type III transmembrane receptors, betaglycan and endoglin, may also participate in membrane complex formation, with betaglycan capturing TGFβ ligands [97, 98] and endoglin binding BMPs [99]. These non-signaling receptors contain a large N-terminal extracellular domain that directly binds ligand and a short C-terminal cytoplasmic domain that may interact with intracellular proteins [100, 101]. Ligand-mediated complex formation juxtaposes two type II and two type I receptors, allowing type II receptors, which are constitutively active kinases, to phosphorylate multiple serine residues within the cytoplasmic GS (glycine and serine rich)-negative regulatory domain of type I receptors [102]. Phosphorylation within the GS domain derepresses the kinase activity of type I receptors.

Activated type I receptors transduce the extracellular ligand-induced signal by phosphorylating cytoplasmic SMAD proteins on C-terminal serines. Phosphorylated, dimerized SMADs bind to the common partner SMAD4, and this heterotrimeric complex translocates to the nucleus, binds DNA, and recruits coactivators or corepressors to upregulate or downregulate gene expression. The phosphorylated SMAD proteins are generally type I receptor specific, with SMAD2 or SMAD3 transducing signals from ALK4, ALK5, and ALK7, and SMAD1, SMAD5, or SMAD8 transducing signals from ALK1, ALK2, ALK3, and ALK6 [103, 104]. SMAD proteins, with the exception of SMAD2, bind directly to DNA via an 11-residue β-hairpin within their N-terminal Mad homology 1 (MH1) domain. SMAD3–SMAD4 and SMAD1/5/8–SMAD4 complexes bind to distinct *cis* regulatory elements and, therefore, influence expression of different sets of genes. However, SMAD DNA binding affinity is weak and specificity and affinity are influenced by interaction with non-SMAD transcription factors, thereby allowing transcriptional output to reflect the integration of multiple signaling pathways and/or, through interaction with lineage-specific transcription factors, cell-type specificity [105]. How different type I receptors that phosphorylate the same SMAD proteins regulate different gene cohorts and, therefore, different cellular behaviors are not understood.

TGFβ ligands may also activate non-SMAD pathways. For example, ligand-bound receptors may recruit: src homology-domain-containing proteins, to activate a RAS/RAF/MEK/ERK1/2 cascade; TRAF6/TAK1, to activate mitogen-activated protein kinases JNK and p38; or the p85 subunit of phosphatidylinositol-3-kinase (PI3K), to activate AKT/mTORC1 (for review, see [106]). TGFβ ligand-mediated activation of these alternative pathways is SMAD independent but these pathways may feed back, either positively or negatively, on SMAD activity. The relative importance of these alternative pathways in vivo remains poorly understood.

ALK1 ligands BMP9 and BMP10

ALK1 was initially characterized as a TGFβ receptor based on evidence that TGFβ1 and TGFβ3 activate ALK1 extracellular domain (ECD)/ALK5 intracellular domain (ICD) chimeric receptors [107], and that TGFβ1 can enhance ALK1/ALK5 complex formation and activate ALK1-dependent SMAD1/5/8 phosphorylation in cultured ECs [108, 109]. However, the in vivo relevance of TGFβ as an ALK1 ligand is unclear [56]. More recently, BMP9 and BMP10 have emerged as physiologically relevant ALK1 ligands (Fig. 1). BMP9 and BMP10 growth factor domain homodimers are the only TGFβ family ligands that bind ALK1 with high affinity [100, 110–112]. BMP9 is synthesized primarily by the liver, with transcripts reported in hepatocytes, biliary cells, ECs, Kupffer cells, and stellate cells [113, 114]. BMP10 is synthesized throughout trabeculated embryonic myocardium [115–117] and is later restricted to right atrial myocardium [118]. Both ligands are detected in circulation at concentrations above published EC_{50} values [113, 119–123] and would, therefore, be available to activate ALK1 on the apical surface of arterial ECs.

BMP9 and BMP10 are generated as proproteins, cleaved intracellularly by furin, and secreted as procomplexes [113, 124]. The BMP9 prodomain does not inhibit BMP9 activity: BMP9 growth factor homodimer and BMP9 procomplex added to cell culture medium activate ALK1 signaling with similar efficacy [125, 126]. This lack of inhibition may be attributed to the "open-armed" conformation of the BMP9 procomplex [126], which allows the growth factor domain to bind ALK1, BMPRII, ActRIIA, ActRIIB, or ENG, with rapid displacement of the prodomain [121]. However, it remains possible that, similar to BMP7, the BMP9 prodomain might bind fibrillin or some other glycoprotein to effect a "closed-arm" conformation and confer latency [127]. The BMP10 procomplex was initially classified as latent based on activity tests using cultured C2C12 (myoblast) or NIH 3T3 (fibroblast) cells [123, 128]. However, the activity of the

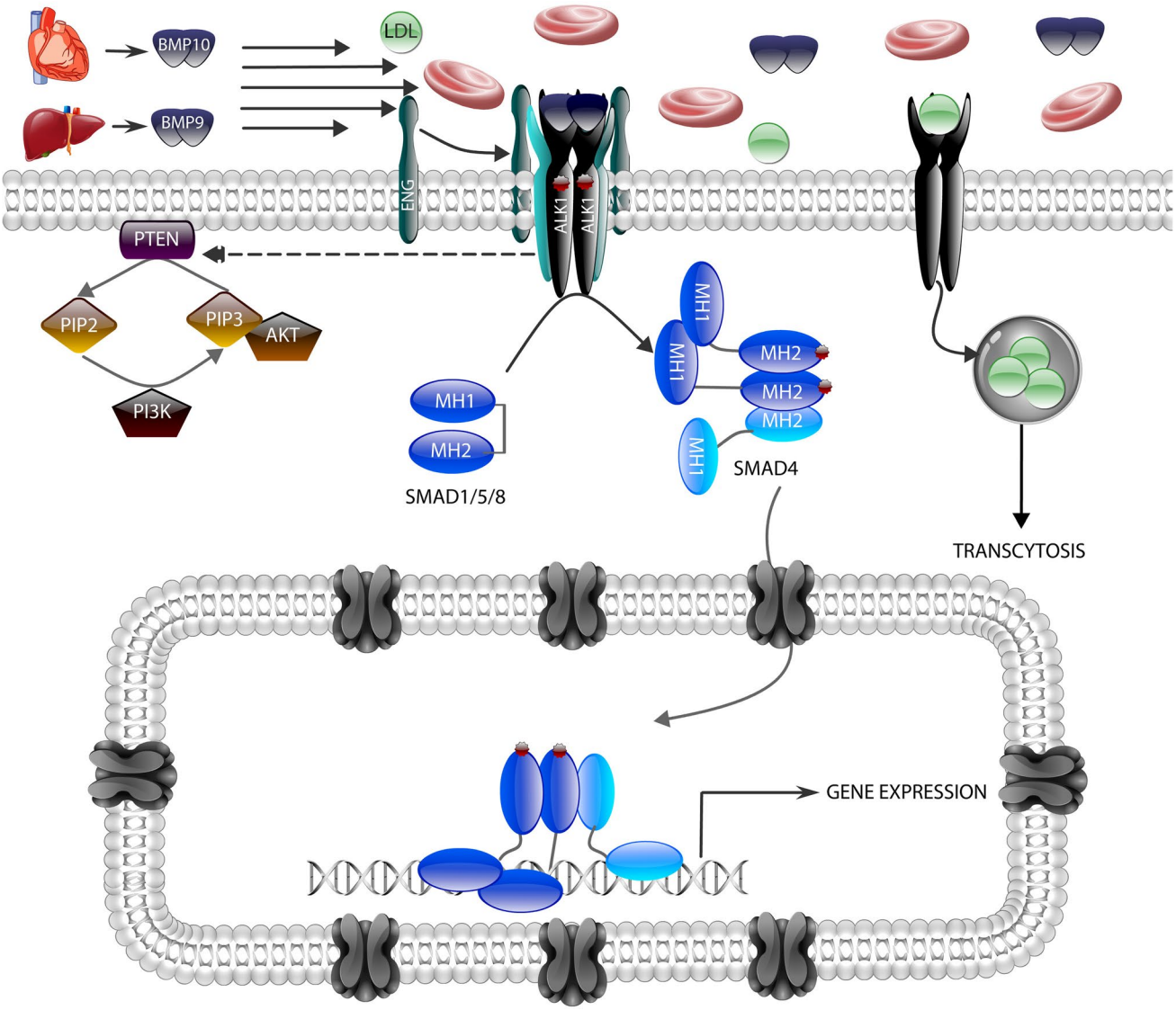

Fig. 1 ALK1/ENG signaling in endothelial cells. Circulating ligands BMP9 (from liver) or BMP10 (from heart) bind to heterotetramers of two ALK1 receptors and two type II receptors. Ligand binding is facilitated by endoglin (ENG). The type II receptors phosphorylate (*red star*) ALK1, which phosphorylates SMAD1, SMAD5, or SMAD8. pSMADs bind SMAD4, enter the nucleus, and regulate gene expression. Through an unknown mechanism, ligand-mediated ALK1 activation may also help to maintain PTEN in an active state, thereby opposing PI3K and favoring AKT inactivation. ALK1 may also play a role in transcytosis of LDL from blood to the subendothelial space; this activity does not require ALK1 kinase activity, type II receptor, or endoglin. Blood flow distributes circulating ligands to ALK1 and imparts shear stress on the endothelium, which enhances the association between ALK1 and ENG

BMP10 procomplex on a variety of ECs was recently shown to approach that of the BMP10 growth factor homodimer [129]. These data suggest that surface proteins on ECs in addition to ALK1 might be important for either stabilizing the binding of the BMP10 prodomain or displacing it from the growth factor homodimer. In support of this interpretation, the BMP10 prodomain can bind to basolaterally located fibrillin [130], although whether binding favors latency or activation is unknown, and whether apically located EC proteoglycans or glycoproteins bind the BMP10 prodomain is unknown.

Although BMP9 and BMP10 similarly activate ALK1 signaling, sorting out the unique and redundant functions of these ligands in vivo is a work in progress. In humans, heterozygous *BMP9* mutations have been identified in three patients with an HHT-like syndrome; these patients presented with epistaxis and telangiectasia-like lesions somewhat atypical of HHT (in terms of size and location), but classical HHT-like AVMs were not reported [131]. Moreover, mouse *Bmp9* knockouts are viable and fertile. These mice exhibit only mild phenotypes, including impaired closure of the ductus arteriosus and enlarged lymphatic vessels

[132–134]. Similarly, zebrafish *bmp9* morphants (wild-type embryos injected with an antisense morpholino-modified oligonucleotide to transiently inhibit *bmp9* translation) fail to phenocopy *acvrl1* mutants, exhibiting only mildly impaired venous remodeling and no AVMs [131, 135].

In contrast to *BMP9*, no human vascular anomalies have yet been attributed to mutations in *BMP10*. However, mouse *Bmp10* knockouts die in utero and exhibit failed cardiac trabeculation and AVMs similar to those observed in *Acvrl1* knockouts [118, 119]. Furthermore, in zebrafish, concomitant knockdown of the duplicate paralogs, *bmp10* and *bmp10-like*, phenocopies AVM development in *acvrl1* mutants [135]. These data support a role for Bmp10 but not Bmp9 as the critical Alk1 ligand during vertebrate embryogenesis. However, the embryonic requirement for *Bmp10* in the mouse vasculature has been attributed to the slightly earlier expression of *Bmp10* (E8.5) compared to *Bmp9* (E9.75–E10), which masks functional redundancy. In support of this hypothesis, insertion of *Bmp9* into the *Bmp10* locus allows *Bmp9* to be expressed in the endogenous spatiotemporal pattern of *Bmp10* and prevents embryonic AVMs [119], and combined postnatal immunodepletion of Bmp9 and Bmp10 is required for development of retinal vascular dysplasia and AVMs [61, 119, 120, 136]. However, replacement of *Bmp10* with *Bmp9* not only fails to rescue trabeculation defects, presumably because of a unique requirement for Bmp10 in inducing cardiomyocyte proliferation independent of Alk1 [137], but also results in dilated, leaky vasculature [119]. As such, whether Bmp9 and Bmp10 have unique or overlapping roles in particular vascular beds or at later times is unknown.

The BMP9/BMP10 transmembrane receptor complex

Type II and type III receptors interact with BMP9/BMP10 and ALK1 at the EC surface to effect signaling (Fig. 1). BMP9 and BMP10 growth factor homodimers bind to F_c-fused extracellular domains of the type II receptors, BMPRII, ActRIIA, and ActRIIB, with BMP9 showing preference for ActRIIB [111]. BMP9 co-immunoprecipitates with ALK1 and BMPRII in bovine aortic ECs and ALK1, BMPRII, and ActRIIB in human umbilical vein ECs [138], and siRNA depletion suggests that BMP9/ALK1 activity requires ActRIIA and BMPRII in human pulmonary artery ECs and umbilical artery ECs [139, 140]. These data suggest that ALK1 can interact with all three BMP type II receptors and that the complement of receptors may vary according to vascular bed. There is some evidence that the type II receptor may compete with the prodomain for growth factor domain binding: BMP9 procomplexes bind to type II receptors with lower affinity than BMP9 growth factor domain dimers [126], and BMPRII can displace the prodomain from the BMP10 procomplex [129]. These observations are consistent with the reported structures [111, 126], which show that the binding sites for the prodomain overlap with that of the type II receptor.

Although the type III receptor, endoglin, is not required for BMP9/BMP10 activation of ALK1, it enhances signaling output as measured by pSMAD1/5/8 [88, 141]. Endoglin was initially described as a co-receptor for many TGFβ family ligands, including TGFβ1, TGFβ3, activin A, BMP2, and BMP7. However, endoglin binding to these ligands requires co-expression of a type I or type II receptor [142, 143]. In fact, among all TGFβ family ligands, endoglin can directly and independently bind only BMP9 and BMP10 growth factor homodimers [99, 100]. Biochemical studies show that endoglin binds BMP9 and BMP10 through its N-terminal orphan domain, whereas the membrane-proximal zona pellucida domain is dispensable for binding [99, 100]. Structural analysis of the endoglin orphan domain bound to the BMP9 growth factor shows that two molecules of the orphan domain symmetrically bind to the BMP9 homodimer [144] (Fig. 2b). The orphan domain binds by contacting the distal region of the ligand knuckle, which is important for several reasons. First, it allows endoglin, which is covalently dimerized through its zona pellucida domain, to bind the growth factor bivalently and thus with very high affinity [99]. Second, it is likely responsible for endoglin's ability to displace the BMP9 and BMP10 growth factor homodimers away from their prodomains [121], as the prodomain and endoglin binding sites overlap (Fig. 2a, b). Third, although endoglin binds BMP9 and BMP10 homodimers with high affinity, the binding interface is restricted to the distal part of the knuckles, which overlaps with the ActRIIB binding site but not the ALK1 binding site, which is located in the wrist [111] (Fig. 2b–d). This observation agrees with data showing that, with respect to BMP9 binding, endoglin neither competes nor cooperates with ALK1, but it does compete with ActRIIA, ActRIIB, and BMPRII [99, 100].

Altogether, the structures and binding studies suggest a mechanism for complex assembly whereby, in the absence of endoglin, the type II receptor displaces the BMP9 or BMP10 growth factor from its prodomain and in turn the type I receptor, ALK1, binds to form the signaling complex. When endoglin is present, it facilitates binding and enhances signaling (Fig. 2). In this model, endoglin binds circulating BMP9 or BMP10 procomplexes and displaces growth factor dimers from their prodomains (Fig. 2a, b). This endoglin-mediated localization of BMP9 or BMP10 homodimers on the membrane facilitates binding of ALK1 (Fig. 2c). Finally, the type II receptor binds and displaces endoglin to form the type I–type II receptor signaling complex (Fig. 2d). Although this mechanism is consistent with many observations, how the type II receptors displace endoglin and how

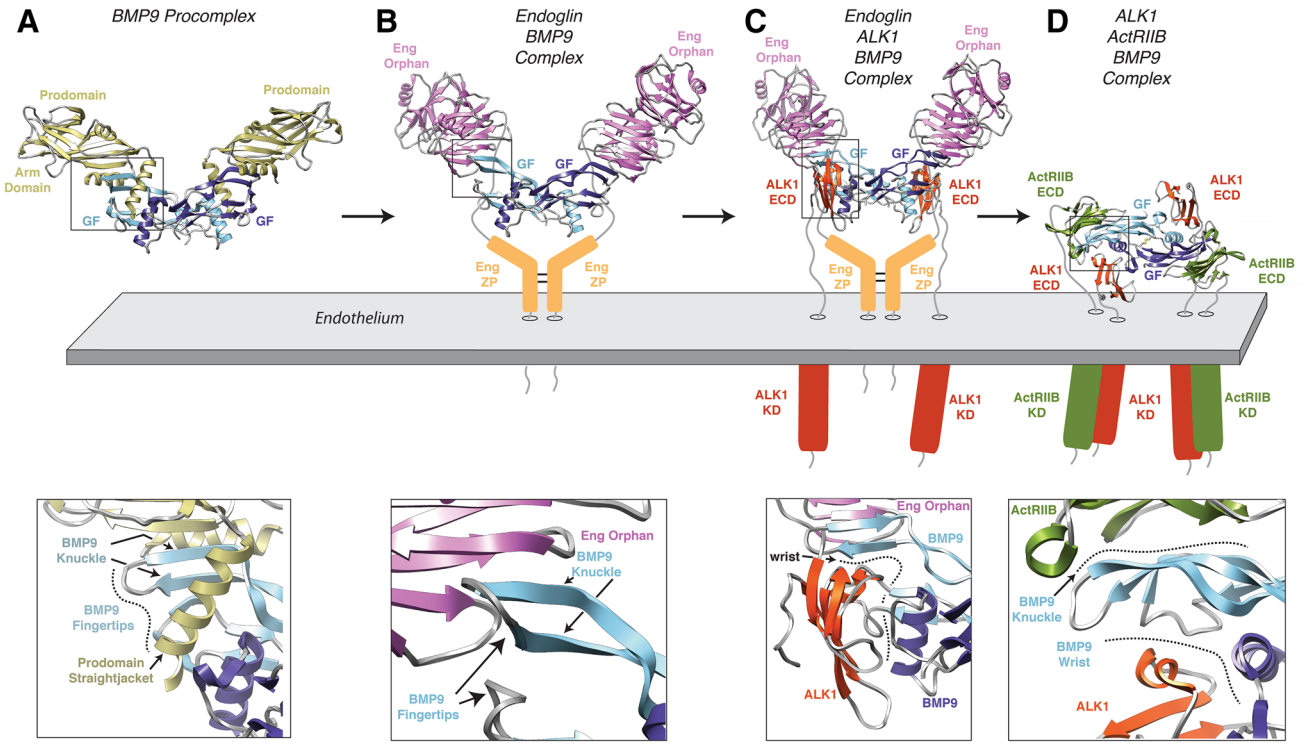

Fig. 2 Structural basis for ALK1 signaling. **a** BMP9 bound in the "open-arm" conformation to its prodomain [126]. The two monomers of BMP9 growth factor (GF) are *cyan* and *dark blue*; the two prodomain monomers are *light green*. **b** BMP9 bound to the endoglin orphan domain [144]. BMP9 is depicted as in **a**; the two bound endoglin orphan domains (Eng Orphan) are *magenta*; the endoglin zona pellucida domain (Eng ZP), which forms a disulfide-linked homodimer, is shown schematically in *orange*. **c** BMP9 bound to the endoglin orphan domain and ALK1. BMP9 and endoglin are depicted as in **a** and **b**, respectively; the two molecules of the ALK1 extracellular domain (ALK1 ECD) are *red*; the ALK1 kinase domains (ALK1 KD) are shown schematically in *red*. **d** BMP9 signaling complex with ALK1 and ActRIIB [111]. BMP9 and ALK1 are depicted as in **a** and **c**, respectively; the two molecules of the ActRIIB extracellular domain (ActRIIB ECD) are *dark green*; the ActRIIB kinase domains (ActRIIB KD) are shown schematically in *dark green*. In each panel, interactions shown in *boxed* region are *highlighted* in further detail below

membrane attachment might influence this process remain unknown.

ALK1 signaling beyond the membrane

Constitutively active ALK1 or BMP9/BMP10 activation of ALK1 strongly induces phosphorylation of SMADs 1, 5, and 8 (hereafter, SMAD1/5/8) in cultured ECs [122, 138, 145, 146] (Fig. 1), and *Alk1* loss of function in mice and zebrafish abrogates arterial endothelial pSMAD1/5/8 [60, 135]. Furthermore, mutations in the common partner *SMAD4* cause a combined juvenile polyposis–HHT syndrome that accounts for <5% of HHT cases [147–149]. However, endothelial-specific deletion of *Smad4* does not cause AVMs [150, 151]. In fact, whether SMAD1/5/8 phosphorylation and complexation with SMAD4 is required downstream of ALK1 in vivo is not certain, and alternative pathways have been suggested. For example, phosphorylated SMADs regulate microRNA (miRNA) processing independent of SMAD4 via recruitment of the RNase, Drosha, to primary miRNAs [152, 153]. This activity has not been investigated with respect to ALK1 signaling and AVM prevention.

In addition to SMADs, several kinases have been implicated downstream of ALK1. ALK1-mediated inhibition of migration of cultured ECs reportedly requires activation of JNK and ERK but not phosphorylation of SMAD1/5/8 [154], whereas ALK1-mediated suppression of PI3K activity (Fig. 1) has been implicated in AVM prevention [61]. In this latter study, *ALK1*-deficient ECs showed enhanced activation/phosphorylation of the PI3K target, AKT (protein kinase B), and its downstream target, FOXO1, and increased prevalence of the phosphorylated, inactive form of PTEN, which normally opposes PI3K. Furthermore, PI3K inhibitors rescued retinal AVMs in *Acvrl1*-deleted or BMP9/BMP10-immunodepleted neonatal mice [61], and similar results were recently obtained in *Eng*-deleted mice [89]. Whether SMAD1/5/8 phosphorylation is required for ALK1/ENG regulation of PI3K activity is unknown.

Genes regulated by ALK1

Downstream of ALK1 activation, few bona fide transcriptional targets have been identified. Microarray experiments comparing the ALK1-inhibited or ALK1-activated condition to baseline identified several established pSMAD1/5/8 targets, including *ID1*, *ID2*, *ID3*, which encode transcriptional repressors, and *SMAD6* and *SMAD7*, which encode inhibitory Smads [155–158]. These studies also identified *ENG* as an ALK1 target [155, 156], a finding later confirmed in numerous studies in cultured cells and in *Acvrl1* mouse mutants [60, 122, 138, 139, 159, 160].

In cultured cells, Notch and ALK1 stimulation demonstrates additive or synergistic effects on mRNA expression of canonical Notch targets (induced by RBPJ/NICD) such as *HEY1* and *HEY2* [161–164], and BMP9 stimulation induces pSMAD1/5/8 binding to these promoters [165]. However, how pSMAD1/5/8, SMAD4, RBPJ, and NICD interact on these gene promoters is not clear. SMAD4 seems to be required for BMP9/ALK1 induction of Notch targets [163–165] and NICD physically interacts with SMAD5 and enhances binding to Smad DNA binding elements [166]. However, there is evidence for [164] and against [162, 167] requirements for RBPJ or NICD in induction of Notch targets downstream of BMP9/ALK1.

Additional genes regulated by ALK1 include *CXCR4* and *DLL4*, which encode arterial markers and promigratory factors that are expressed by endothelial tip cells [168, 169], and endothelin 1 (*EDN1*), which encodes a vasoconstrictive peptide. In zebrafish, disruption of *acvrl1* or *bmp10/bmp10-like* upregulates *cxcr4a* and *dll4* expression and downregulates *edn1* expression in arterial ECs [68, 135, 170], and in cultured human ECs, BMP9/ALK1 signaling downregulates *CXCR4* and induces *EDN1* [156, 160, 171]. However, neither *edn1* loss nor Notch activation is sufficient to phenocopy *acvrl1* mutants, and neither *cxcr4a* loss nor Notch inhibition is sufficient to rescue *acvrl1* mutants. [68, 170]. BMP9/ALK1 signaling also induces connexin 40 (*CX40,* also known as *GJA5*), which encodes a component of gap junctions, in cultured human ECs, and *Acvrl1* and *Cx40* genetically interact in mice in the development of wound-induced skin AVMs [172], supporting the physiological relevance of this target gene. *TMEM100*, which encodes a small transmembrane protein of unknown function, is also induced by BMP9/ALK1 in mice and cultured ECs [173, 174]. *Tmem100* is expressed in embryonic and postnatal ECs, and deletion largely phenocopies *Acvrl1* deletion at embryonic, neonatal, and adult stages [174, 175]. *TMEM100* is induced by endoplasmic reticulum stress [176] and is localized to the plasma membrane [177], suggesting that it may be involved in chaperoning transmembrane receptors through the endomembrane system. Finally, angiopoietin 2 (*Angpt2*), an autocrine-acting pro-angiogenic factor, is increased in mice and cultured ECs with disrupted Alk1 signaling [54, 136]. Whether any of these genes is directly regulated by pSMAD1/5/8 remains to be determined.

Cellular mechanisms of AVM development

Although several genes have been identified as either direct or indirect targets of ALK1 signaling, how these transcript changes alter EC behavior and lead to AVMs is not well understood. One hypothesis is that HHT-associated AVMs arise in response to loss of distinct arterial and venous identities, eliminating a repulsive force required to maintain separation of arteries and veins. This theory is borrowed from Notch gain-of-function (which forces arterial differentiation) and loss-of-function (which forces venous differentiation) studies demonstrating that both conditions result in AVMs [21, 178–180] and is supported by evidence demonstrating that arterial markers *EFNB2* and *JAG1* are induced by BMP9/ALK1 and downregulated in *Acvrl1* knockouts [55, 60, 79, 120, 140, 162]. Evidence that ALK1 cooperates with Notch to induce expression of certain arterial-specific genes further supports this hypothesis [162–164]. However, mouse *Eng* mutants [82] and zebrafish *acvrl1* mutants develop AVMs while maintaining normal AV identities [170], and neither Notch loss-of-function nor Notch gain-of-function can explain AVMs in *acvrl1* mutant zebrafish [170]. These data weaken support for the compromised arterial identity model of HHT-associated AVM development.

HHT-associated AVMs have also been attributed to overactive angiogenic sprouting. In two-dimensional culture, ALK1 activation inhibits EC migration and proliferation, whereas *ACVRL1* or *ENG* knockdown or deletion enhances migration and proliferation [122, 138, 139, 146, 154, 181]. In three-dimensional angiogenic sprouting assays, ALK1 activation inhibits sprouting and favors a stalk cell fate, whereas *ACVRL1* knockdown enhances sprouting and favors a tip cell fate [140, 162]. And in mice, neonatal deletion of *Acvrl1* or *Eng* or sequestration of ALK1 ligands causes retinal hypervascularization associated with increased EC proliferation and increased expression of pro-angiogenic factors such as *Angpt2* [82, 136, 162]. Together, these data support a role for ALK1 signaling in stalk cell maintenance and angiogenic resolution. However, the simple, dilated morphology of embryonic and wound-induced skin AVMs in *Acvrl1* knockout mice and of cranial AVMs in zebrafish *acvrl1* mutants [54, 55, 57, 67], coupled with the recent observation that retinal vascular hypersprouting in *Eng* knockouts is secondary to hypoxia caused by upstream AVMs [89], do not support the idea that exuberant angiogenic sprouting causes HHT-associated AVMs.

Live imaging of AVM development in transparent zebrafish *acvrl1* mutant embryos reveals a third possible

cellular mechanism that may account for HHT-associated AVMs. In these mutants, AVMs invariably develop between 32 and 48 h post-fertilization in cranial vessels that underlie the hindbrain; therefore, AVM development can be imaged in embryos expressing EC-localized fluorescent transgenes using time-lapse confocal microscopy [67, 68, 182]. These imaging studies have revealed that AVM development is a two-step process (Fig. 3). In step 1, loss of *acvrl1* reduces arterial EC migration against the direction of blood flow and enhances migration in the direction of blood flow, resulting in a skewed distribution of ECs toward distal arterial segments. The consequent increase in distal arterial EC number increases distal arterial caliber [68, 182]. In step 2, directly downstream of this enlarged arterial segment, a normally transient arteriovenous shunt is aberrantly retained and enlarged in *acvrl1* mutants, generating an embryonic lethal AVM. Remarkably, the AVM and downstream vein are not *acvrl1* positive, and aberrant migration can be uncoupled from AVM development by stopping blood flow [68]. Taken together, these data suggest that both steps of HHT-associated AVM development involve responses to blood flow. In step 1, an Alk1-dependent response to flow is *abrogated*, allowing arterial EC migration in the direction of blood flow. In step 2, an Alk1-independent response to flow is *activated*, likely due to increased flow rate and shear stress, that prevents pruning of primitive artery–vein connections or selects capillary segments for enlargement. These flow responses are discussed in more detail below.

Interaction between ALK1 signaling and blood flow

Multiple lines of evidence now point to a role for ALK1 in transducing a signal initiated by blood flow. In zebrafish, *acvrl1* is expressed in arterial ECs only after the onset of blood flow, and in mice, *Acvrl1* expression is enhanced by and dependent on blood flow [1, 67, 68, 183]. Furthermore, loss of blood flow phenocopies molecular and cellular defects observed in high-flow regions of the zebrafish *acvrl1* mutant vasculature—including enhanced arterial EC expression of promigratory tip cell markers *cxcr4a* and *dll4*, decreased arterial EC expression of the vasoconstrictive peptide *edn1*, and aberrant arterial EC migration in the direction of flow—and molecular defects caused by flow loss can be rescued by restoration of arterial EC Bmp10/Alk1 signaling [68, 135, 182]. Because ALK1 ligands BMP9 and BMP10 are secreted into the bloodstream, the role of blood flow in ALK1 signaling certainly includes distribution of these endocrine ligands to arterial ECs [123, 135]. However, many ALK1-regulated genes, including *CX40, CXCR4, DLL4, EDN1, SMAD6,* and *SMAD7,* have previously been implicated as mechanoresponsive [184–188], and shear stress imparted by blood flow activates ALK1 signaling [88]. In cultured ECs, shear stress potentiates BMP9-induced ALK1 signaling independently of krüppel-like factor 2 (KLF2) [88], a shear stress-induced transcription factor that coordinately controls expression of numerous shear stress responsive genes [189–192]. Instead, shear stress,

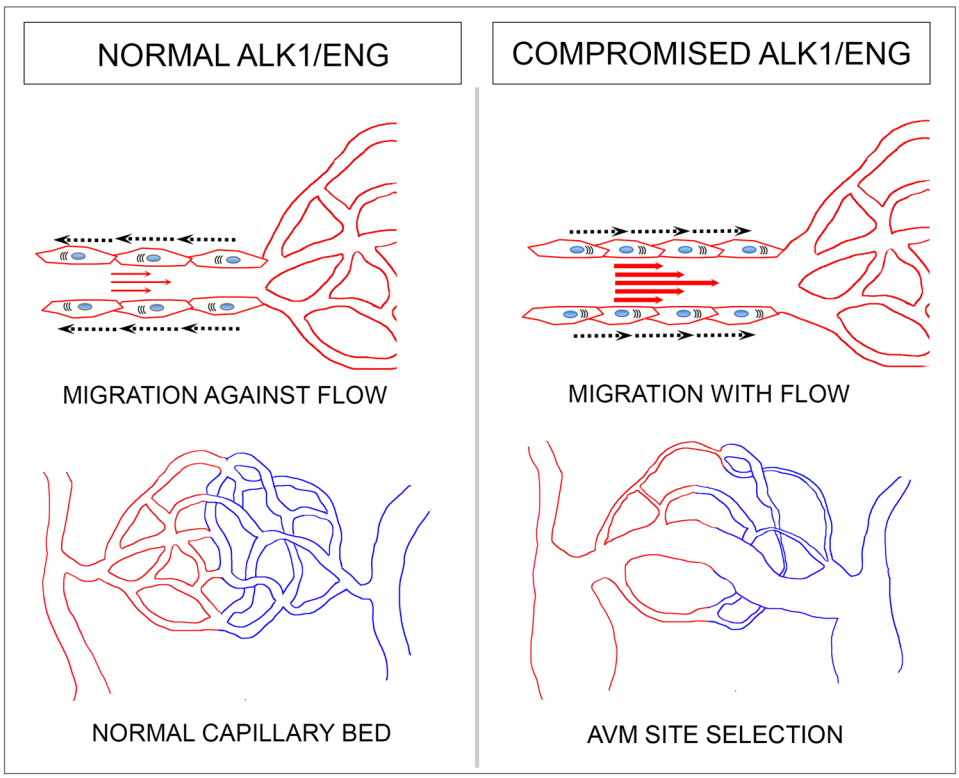

Fig. 3 Two-step hypothesis for HHT-associated AVM development. When ALK1/endoglin signaling is active, arterial endothelial cells in distal segments polarize and migrate against the direction of blood flow. In the absence of ALK1/endoglin, these cells migrate in the direction of flow and increase distal vessel caliber adjacent to temporary arteriovenous connections or capillary beds (Step 1). Increased flow rate in downstream vessels then triggers an adaptive response to normalize shear stress, which may be achieved by maintenance and enlargement of a normally transient AV connection or selective enlargement of a capillary segment (Step 2)

working via an unknown mechanism, increases the physical interaction between ALK1 and endoglin and thereby lowers the effective concentration of BMP9 required for ALK1 activation [88].

As described above, arterial EC migration against the direction of blood flow is impaired in zebrafish *acvrl1* mutants [182], and similar abnormal EC migratory behavior was recently described in *Eng*-deleted ECs in mice. In the latter model, *Eng*-deleted cells migrate aberrantly in the direction of flow and accumulate in arterioles, and enlarged arterioles ultimately become the afferent segments of AVMs [89]. These data strongly support the hypothesis that ALK1 mediates a flow-based signal that controls directional cell migration within the intimal layer of a lumenized artery. How and why blood flow and ALK1 signaling direct EC migration against the direction of flow is an area of active investigation. In vivo, arterial ECs in non-regressing vessels generally polarize against blood flow, with the golgi and microtubule organizing center positioned upstream (with respect to the direction of blood flow) of the nucleus, and migration against flow has been reported in the adult rat aorta and mouse yolk sac arteries [52, 193–197]. In the mouse retina, wall shear stress magnitude positively correlates with the degree of EC polarization against flow and EC density [198]. Together, these data support the notion that arterial ECs within non-regressing arteries migrate against blood flow toward sites of high shear stress. This behavior may be necessary to generate the characteristic hierarchical arterial tree, in which increased distance from the heart correlates with decreased flow rate and decreased arterial caliber, to normalize shear stress across the arterial network.

Whether ALK1/endoglin signaling or shear stress instructs the retrograde migration of arterial ECs is unknown. In zebrafish embryos, *acvrl1*-positive cranial arterial ECs migrate toward the heart, which is the source of the ALK1 ligand BMP10 [135, 182], suggesting that retrograde migration may be a chemotactic response to a BMP10 gradient. However, it is also possible that the direction of flow exclusively dictates the direction of migration. In support of this hypothesis, in cultured ECs, hydrodynamic force can mechanically displace the nucleus to a position downstream of the Golgi, poising the cell for retrograde migration, against the direction of flow [199]. We speculate that ALK1/endoglin signaling may be permissive for this planar polarization or may regulate cell–matrix interactions to allow migration against the direction of flow. In support of these ideas, *ENG* expression alters actin cytoskeletal organization [200], and BMP9 treatment increases the abundance of the actin-polymerizing protein, zyxin [201], in EC focal adhesions [202, 203]. Zyxin and zyxin-related protein-1 both bind to endoglin via its C-terminal intracellular domain [200, 202], and mechanical force or loss of endoglin results in accumulation of zyxin in focal adhesions [201, 203]. These data suggest that zyxin may be sequestered by binding to endoglin, and that either BMP9/ALK1 activation or mechanical force disrupts this interaction to allow relocalization to and maturation of focal adhesions, which are integral to cell migration. BMP9 may also regulate zyxin at the level of transcription: both BMP9 and mechanical force induce yes-associated protein-1 (YAP1) translocation to the nucleus [203, 204], where it can act as a transcriptional coactivator to induce zyxin mRNA expression [205].

AVMs: an adaptive response to altered hemodynamic force?

The ability to uncouple *acvrl1* mutation from AVM development in zebrafish by stopping blood flow suggests that HHT-associated AVMs might represent a physiological, adaptive response to increased shear stress. It is well established that blood vessels remodel when faced with either high or low shear stress, increasing or decreasing caliber to restore shear stress to a desired set point [206–211]. In wild-type zebrafish embryos, transient cranial arteriovenous connections give rise to and temporarily drain the nascent medial arterial system [212]. However, as vascular complexity increases and flow is diverted to downstream arteries, flow rate and shear stress drop in these segments, triggering EC migration toward vessels with higher flow and disconnection of these temporary shunts [53, 212]. In *acvrl1* mutants, at least one of these connections is retained and enlarged [67, 68]. The retained arteriovenous shunt is most often located immediately downstream of the enlarged, *acvrl1*-positive arteries, and is, therefore, subjected to high flow rate and shear stress [68, 135, 170]. These observations, coupled with the requirement for blood flow in shunt retention and the lack of *acvrl1* expression in the shunt and downstream vein [67, 68], suggest that AVMs represent an attempt to decrease shear stress in vessels downstream of the *acvrl1*-dependent enlarged arteries (Fig. 3). As flow increases through this shunt, both upstream arteries and downstream veins continue to enlarge over time. Although shear stress-induced changes in vessel caliber are typically initiated by smooth muscle relaxation or contraction, the zebrafish vasculature is devoid of mural cells at this time [213, 214]. As such, shunt enlargement likely involves shear stress-induced EC hypertrophy, EC proliferation, and/or matrix remodeling.

The location of retinal AVMs in neonatal *Acvrl1*-deleted mice also suggests a role for hemodynamic force in shunt site selection [88]. In neonatal mice, the retinal superficial vasculature originates from the central optic nerve region and extends via angiogenic sprouting to the retinal periphery, forming a radially symmetric network of arteries, veins, and bridging capillaries. In this network, the proximal arterial segments and proximal first-order branches experience the highest shear stress magnitude [215], and in *Acvrl1*-deleted

mice, it is these proximal first-order branches that develop into AVMs [88].

Emerging roles for ALK1 signaling

Although ALK1 signaling has been closely associated with HHT for more than 20 years, emerging evidence suggests roles for this pathway in additional diseases, including pulmonary arterial hypertension (PAH), cancer, and atherosclerosis.

ALK1 and PAH

Hereditary hemorrhagic telangiectasia is associated with dilated vessels, AVMs, low vascular resistance, and possible left heart failure. In contrast, PAH is characterized by enhanced proliferation and vasoconstriction in small pulmonary arteries leading to a high resistance state, low cardiac output, and right heart failure [216]. Yet, paradoxically, rare patients present with a combined HHT/PAH syndrome [217]. From a genetic standpoint, the relationship makes some sense: heterozygous mutations in *BMPR2*, which encodes a type II receptor that can partner with ALK1, account for more than 70% of heritable PAH and up to 20% of the more common idiopathic PAH [218, 219], and causal mutations in *SMAD9* (which encodes SMAD8), which signals downstream of ALK1, have also been reported [220, 221]. Most commonly, patients who present with HHT/PAH harbor heterozygous mutations in *ACVRL1*, or less often, *BMPR2* [217, 222–225]. However, how dysregulation of the same signaling pathway leads to both dilated lung AVMs and obliterative lung arterial vasculopathy is not understood.

It is now recognized that HHT patients are equally likely to present with two different classifications of pulmonary hypertension: PAH (group I pulmonary hypertension), caused by pre-capillary defects, or pulmonary venous hypertension (PVH; group 2 pulmonary hypertension), caused by post-capillary defects secondary to high cardiac output [226–228]. Both PAH and PVH are defined by a mean pulmonary artery pressure, as measured by right heart catheterization, of at least 25 mmHg and can only be distinguished by hemodynamic measurement of pulmonary wedge pressure [229]. Given that vasodilators are a first-line drug in the treatment of PAH but are contraindicated in PVH, it is critical to accurately classify PH in HHT patients [226].

ALK1 and tumor angiogenesis

The concept of inhibiting tumor angiogenesis as a cancer therapy was first postulated in 1971 by Dr. Judah Folkman [230]. Later discoveries of endogenous angiogenesis inhibitors (e.g., angiostatin, endostatin) along with development of antibodies against VEGF helped to support Folkman's hypothesis: these reagents were effective inhibitors of tumor growth in animal models [231–233]. However, clinical trials have generally not been as impressive, in part because tumors circumvent anti-angiogenic insults by turning to alternative pathways to support angiogenesis. Given the important role of ALK1 signaling in blood vessel development, this pathway has more recently emerged as a possible target for anti-tumor angiogenesis. In mouse models of pancreatic cancer, breast cancer, melanoma, and head and neck cancer, immunological sequestration of ALK1 ligands (using ALK1-F_c) or antibody targeting of ALK1 inhibits tumor angiogenesis and tumor growth [110, 234–236]. These promising results led to the development of dalantercept, a human ALK1-F_c fusion. Phase I clinical trials demonstrated reasonable drug tolerance and some anti-tumor efficacy [237], but a monotherapy phase II trial for endometrial cancer was unsuccessful [238]. Combination therapies may hold more promise: dalantercept plus sunitinib, a receptor tyrosine kinase (RTK) inhibitor, was more effective in a mouse model of renal cell carcinoma than either drug alone [239], and dalantercept plus axitinib, another RTK inhibitor, showed promise in a renal cell carcinoma phase I clinical trial [240]. Interestingly, although most studies have reported decreased tumor angiogenesis in response to dalantercept, others have reported dilated tumor vessels and increased blood flow [239]. These observations are in line with effects of *ALK1* loss in humans and animal models and suggest that ALK1 inhibition may actually prove most useful in increasing tumor blood flow and thus enhancing delivery of other chemotherapeutic drugs. However, ALK1 inhibitors can cause undesirable side effects, such as edema, fluid retention, epistaxis, telangiectasias, and congestive heart failure [237, 240], which may limit utility in cancer treatment.

Endoglin targeting is also being explored as an anti-cancer therapy. The endoglin antibody, TRC105, has demonstrated efficacy in dampening vascularity, tumor growth, and/or metastasis in mouse models of various cancers [241–243] and has shown variable efficacy, either alone or in combination with bevacizumab or sorafenib (RTK inhibitor), against a variety of cancers in phase I and phase II clinical trials [244–248].

ALK1 and atherosclerosis

Until recently, there was little evidence for a connection between ALK1 and atherosclerosis: one report in mouse demonstrated strong Alk1 expression at arterial curvatures and bifurcations, which are sites of disturbed flow that are prone to atherosclerosis [249], and one report demonstrated increased ALK1 expression in the endothelium, neointima, and media of human coronary artery atherosclerotic lesions

[250]. Intriguingly, ALK1 is now directly implicated in atherosclerosis via a mechanism seemingly unrelated to its role in HHT [251]. Briefly, the apically localized extracellular domain of ALK1 can bind to circulating ApoB100-containing low-density lipoproteins (LDL) with relatively low affinity (therefore, likely relevant only in a dyslipidemic state) and mediate transcytosis of LDL to the subendothelial space (Fig. 1), initiating an atherosclerotic lesion. Surprisingly, this activity does not require BMP9/BMP10, endoglin, BMPRII, or ALK1 kinase activity, and BMP9/BMP10 cannot compete out LDL binding, suggesting that these ligands bind to different ALK1 residues. As such, targeting LDL/ALK1 interaction may be a viable approach to development of new drugs to prevent atherosclerosis.

ALK1 signaling: future perspectives

Since the initial discoveries of *ACVRL1* and *ENG* as causal genes in HHT [2, 3], our understanding of how ALK1 transmits signal and why this signal is important in maintaining a hierarchical vasculature has improved dramatically. ALK1 is no longer an orphan receptor, with ligands now defined as BMP9 and BMP10 [119, 122, 125, 135]. However, we do not fully understand the unique and overlapping functions of these two ligands in cardiovascular development and disease. Inside the cell, we know that phosphorylation of SMAD1/5/8 can relay ALK1 signal to the nucleus [54], and that PI3K activity is enhanced in the absence of ALK1/endoglin function [61, 89]. However, our understanding remains incomplete regarding the cohort of genes regulated by ALK1-induced pSMAD1/5/8, the mechanism by which ALK1 represses PI3K, and the in vivo relevance of additional intracellular pathways that have been implicated in ALK1/endoglin signaling in various model systems. Regarding cell behavior, we now know that loss of ALK1/endoglin function results in aberrant arterial EC migration in the direction of blood flow, enhancing vessel caliber upstream of capillaries or transient arteriovenous shunts and leading to hemodynamic changes that likely precipitate AVMs [89, 182]. However, we do not know how ALK1 signaling interacts with mechanical force to guide EC migration against the direction of blood flow.

To date, medical therapy for HHT has been limited to approaches that indiscriminately block angiogenesis or enhance clotting. For example, the VEGF inhibitor bevacizumab, when administered by submucosal injection, may curtail nosebleeds in HHT patients and, when administered by intravenous injection, may improve heart function in patients with liver AVMs [252–255]. However, not all HHT patients respond to bevacizumab, and two prospective clinical trials spanning multiple institutions reported no efficacy of topical intranasal bevacizumab with respect to epistaxis severity [256, 257]. As another example, tranexamic acid is an antifibrinolytic agent prescribed to some HHT patients. Although this drug may reduce epistaxis severity [258, 259], it is not consistently effective [257] and it may carry risk of thromboembolism [260]. As such, there is a pressing need to develop targeted therapeutics, based on pathway knowledge, for HHT patients. One approach may be to enhance expression of or signaling through wild-type receptors. For example, the selective estrogen receptor modulators raloxifene and bazedoxifene increase *ACVRL1* and *ENG* expression [261, 262]. These drugs decreased HHT-associated epistaxis in small, uncontrolled studies [261, 262] and are now recognized as orphan drugs for HHT. As a second example, tacrolimus (FK506) both derepresses type I receptor activity and increases *ACVRL1* and *ENG* expression, and promising results with this drug in PAH animal models and patients suggest utility in HHT [263–266]. Generally, these approaches will be most effective if HHT is indeed caused by gene haploinsufficiency and not somatic loss of function. If somatic loss of function is required for HHT-associated lesions, then correcting the pathway downstream of receptors may be a more fruitful approach, particularly with respect to HHT2.

Finally, it is important to consider that ALK1 and endoglin are not simply two cogs in the same machine. Although ALK1 and endoglin clearly interact in a common signaling pathway, HHT1/*ENG* patients typically present with more severe disease than HHT2/*ACVRL1* patients, and HHT1 patients are more susceptible to lung AVMs whereas HHT2 patients are more susceptible to liver AVMs [5, 6]. These surprising genotype–phenotype correlations suggest that there are unique aspects of ALK1 and endoglin function that we still do not understand. Furthermore, both ALK1 and endoglin seem to have independent functions outside of their interaction in vascular development and AVM prevention. The recent implication of ALK1 but not endoglin in atherosclerosis [251] opens up a new field of study for this enigmatic receptor, and endoglin but not ALK1 has been implicated in mesenchymal stem cell maintenance [267] and preeclampsia [268]. As such, new approaches to HHT therapeutics should be stratified based on underlying genetics and must take into account functions of ALK1 and endoglin beyond HHT.

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