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Genetics of vascular anomalies

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A B S T R A C T

Vascular anomalies are developmental defects of the vasculature and encompass a variety of disorders. The identification of genes mutated in the different malformations provides insight into the etiopathogenic mechanisms and the specific roles the associated proteins play in vascular development and maintenance. A few familial forms of vascular anomalies exist, but most cases occur sporadically. It is becoming evident that somatic mosaicism plays a major role in the formation of vascular lesions. The use of Next Generating Sequencing for high throughput and "deep" screening of both blood and lesional DNA and RNA has been instrumental in detecting such low frequency somatic changes. The number of novel causative mutations identified for many vascular anomalies has soared within a 10-year period. The discovery of such genes aided in unraveling a holistic overview of the pathogenic mechanisms, by which *in vitro* and *in vivo* models could be generated, and opening the doors to development of more effective treatments that do not address just symptoms. Moreover, as many mutations and the implicated signaling pathways are shared with cancers, current oncological therapies could potentially be repurposed for the treatment of vascular anomalies.

Introduction

Vascular anomalies refer to a wide variety of disorders that result from disruptions in the development and maintenance of the vasculature. The majority of vascular anomalies occur sporadically; however, several can be inherited, predisposing patients to developing multifocal vascular lesions. Identification of causative genes in the rare familial cases set the initial fundamental understanding of potential molecules and signaling pathways involved in vascular development and maintenance. In these rare cases, great variability in phenotypic penetrance, age of onset, and severity of lesions was seen in several distinct disorders despite the loss-of-function (LoF) of relevant genes.[1] It was shown that this is due to Knutson's two-hit mechanism (like in certain hereditary cancers), in which the combination of a germline and post-zygotic mutation within a lesion results in complete localized loss of gene function. Evidence has been reported for several disorders, for example, glomuvenous malformation and cerebral cavernous malformations.[2–4]

It was initially demonstrated that 50% of resected sporadic venous malformations were associated a gain-of-function (GoF) TIE2 mutation, which was absent in blood.[5] The discovery of low frequency mosaic 2nd hit mutations within tissues emphasized the importance of somatic changes in lesion development and drove focus on such mutations as an explanation for the sporadic cases. Because detection of somatic alterations is difficult, research has benefitted from the high-throughput Next Generation Sequencing (NGS) technology, which is sensitive enough to detect tissular alterations as low as 1%. Since the princeps demonstration in 2009 that 50% of sporadic venous malformations were associated with a somatic GoF TIE2 mutation,[5] many novel associated genes for sporadically occurring vascular anomalies have been found (Table 1).

Revelation of causative genes and corresponding mechanistic pathways has unveiled potential molecular targets for pharmacological treatment of vascular anomalies. For the most part, it appears that fast-flow vascular lesions are affected by inappropriate mitogen-activated protein kinase (MAPK) and the transforming growth factor-β (TGFβ) signaling pathways, while slow-flow lesions are caused by hyperactivation of the phosphatidylinositol 3-kinase (PIK3)/AKT signaling pathway. Some of the same genes are responsible for seemingly distinct disorders, thus common pharmacological treatments could be possible and early proof-of-principle studies have shown interesting promise.

Hemangiomas

Infantile hemangiomas (IH) are common benign tumors found in 5–10% of children. Definitive diagnosis of IH is determined by positive staining for Glucose transporter-1 (GLUT-1) on hemangioma specimens. However, the etiology of IH is not known. Amino acid substitutions in VEGF receptor-2 (VEGFR2) and tumor endothelial marker-8 (TEM8) were found in a few cases. *In vitro* studies indicate that these changes result in increased signaling via VEGFR2, which seems to be characteristic of hemangioma-derived endothelial cells (ECs).[6]

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Table 1
Genes mutated in vascular anomalies.

Vascular anomaly	Locus	Gene	Inheritance	Mutation affect, type	Normal protein function or involvement
Vascular tumors					
Congenital Hemangioma	9q21	GNAQ	Sp	GOF, somatic	α-subunit in Gq class of proteins,
Vascular malformations	19p13.3	GNA11	Sp	GOF, somatic	regulation of GPCR
Capillary Malformations	9q21	GNAQ	Sp	GOF, somatic	α-subunit in Gq class of proteins,
Capillary malformation (CM) (port-wine	19p13.3	GNA11	Sp	mosaicism	regulation of GPCR
stain)	9q21	GNAQ	Sp	GOF, somatic	same as above
Sturge-Weber Syndrome (SWS)				GOF, somatic	α-subunit in Gq class of proteins,
Arteriovenous Malformations				mosaicism	regulation of GPCR
Arteriovenous malformations (AVM)	15q22.31	MAP2K1	Sp	GOF, somatic	Intracellular MAP kinase
	7q34	BRAF	Sp	GOF, somatic	Intracellular MAP kinase
(cerebrovascular)	11p15.5	HRAS	Sp	GOF, somatic	GTPase for MAPK signaling
	12p12.1	KRAS	Sp	GOF, somatic	GTPase for MAPK signaling
Hereditary Hemorrhagic Telangiectasia (HHT)					
HHT1	9q34.11	ENG	AD	LOF, germ (+ som)	TGF-β/BMP co-receptor
HHT2	12q13.13	ALK1	AD	LOF, germ (+ som)	TGF-β/BMP type I receptor
HHT3	5q31.3-q32	–	AD	–	–
HHT4	7p14	–	AD	–	–
Juvenile polyposis-HHT (JP-HHT)	18q21.2	MADH4/SMAD4	AD	LOF, germline	TGF-β signaling co-mediator
HHT-like	10q11.22	GDF2/BMP9	–	LOF, germline	BMP signaling ligand
Capillary malformation-arteriovenous malformation	5q14.3	RASA1	AD/sp	LOF	RasGTPase-RAS signaling
CM-AVM1	7q22.1	EPHB4	AD/sp	LOF	Receptor tyrosine kinase for ephrins
CM-AVM2					
Parkes Weber syndrome	5q14.3	RASA1	AD	LOF, germline	RasGTPase-RAS signaling
Cerebral cavernous malformations (CCM)					
CCM1	7q21.2	KRIT1	AD/sp	LOF, germline + somatic 2nd hit/somatic	Suppress RhoA-GTPase signaling
CCM2	7p13	Malcavernin	AD/sp	Same	Suppress RhoA-GTPase signaling
CCM3	3q26.1	PDCD10	AD/sp	Same	Apoptosis
Venous anomalies					
Sporadic venous malformation (VM)	3q26.32	PIK3CA	Sp	GOF	110-kD catalytic α-subunit of PI3K
	9p21.2	TEK/TIE2	Sp	GOF, som	EC-specific tyrosine kinase receptor
Familial cutaneomucosal VM (VMCM)	9p21.2	TEK/TIE2	AD	GOF, germ + somatic 2nd hit	for angiopoietins
Blue Rubber Bleb Nevus Syndrome (BRBN)	9p21.2	TEK/TIE2	Sp	GOF, som	Same as above
Glomuvenous malformation (GVM)	1p22.1	Glomulin	AD	LOF,	Intracellular signaling, cell cycle
Verrucous VM	17q23.3	MAP3K3	Sp	germ + somatic 2nd hit GOF?	regulation Intracellular kinase
Lymphatic Malformations					
Lymphatic malformation (LM)	3q26.32	PIK3CA	Sp	GOF	110-kD catalytic α-subunit of PI3K
Primary Lymphedema (LE)					
Primary congenital LE (Nonne-Milroy disease)	5q35.3	FLT4/VEGFR3	AD/AR/Sp	LOF, germ/som	EC-specific tyrosine kinase receptor
Nonne-Milroy-like disease	4q34	VEGFC	AD	LOF, germline	Ligand for VEGFR3
Choanal atresia-LE	1q41	PTPN14	AR	LOF, germline	Protein tyrosine phosphatase
LE-distichiasis-yellow nail syndrome	16q24.1	FOXC2	AD	GOF & LOF, germ	Transcription factor
Hypotrichosis-LE-telangiectasia (HLT) syndrome	20q13.33	SOX18	AD/AR/Sp	LOF?/DN, germline	Transcription factor
Hennekam syndrome 1	18q21.32	CCBE1	AR	LOF	Regulates ADAMTS3-mediated activation
	4q28.1	FAT4	AR	LOF	of VEGFC
Hennekam syndrome 2	4q13.3	ADAMTS3	AR	LOF	Cadherin-related protein
Hennekam syndrome 3					Zinc-dependent protease
Microcephaly with or without chorioretinopathy, LE, or mental retardation (MCLMR)	10q23.33	KIF11	AD/Sp	LOF, germline/somatic	Spindle motor protein
X-linked syndrome anhydrotic ectodermal dysplasia with immunodeficiency, osteopetrosis, and LE (OLEDAID)	Xq28	IKBKG/NEMO	X-linked	Hypomorphic	NF$\kappa\beta$ transcription regulation
Hereditary LE II (Meige disease)	1q41–42	GJC2/CX47	AD	Missense, germline	Gap junction protein
Oculodentodigital dysplasia-LE	6q22.31	GJA1/CX43	AD	Missense, germline	Gap junction protein
Primary LE-myelodyplasia (Emberger syndrome)	3q21.3	GATA2	AD	LOF, germline	Transcription factor
Combined/Complex Syndromes					

(*continued on next page*)

Table 1 (*continued*)

Vascular anomaly	Locus	Gene	Inheritance	Mutation affect, type	Normal protein function or involvement
Congenital, lipomatous overgrowth, vascular malformations, epidermal nevi and scoliosis/skeletal/spinal anomalies (CLOVES)	3q26.32	PIK3CA	Sp	GOF, somatic mosaicism	110-kD catalytic α-subunit of PI3K
Klippel-Trenaunay syndrome	5q13.3/ 11p15.1 3q26.32	AGGF1/VG5Q PIK3CA	Sp/AD? -	Somatic/GOF -	Pro-angiogenic factor Same as above
Proteus syndrome	14q32.33	AKT1		Som	PI3K/AKT signaling
PTEN hamartoma tumor syndrome (PHTS)	10q23.31	PTEN	AD	Som	Dual-specificity protein phosphatase for PI3K/AKT signaling

AD, autosomal dominant; AR, autosomal recessive; DN, dominant negative; Sp, sporadic; LOF, loss-of-function; GOF, gain-of-function; EC, endothelial cell; ECM, extracellular matrix; Underlined = discovered via NGS; Som = somatic mosaicism, Germ = germline mutation.

Congenital hemangiomas (CH) occur less frequency than IH and are present at birth. The two main forms of CH are based on whether the lesion rapidly disappears (rapidly involuting CH, or RICH) or persists (non-involuting CH, NICH). Somatic missense mutations at position 209 in the genes encoding guanine nucleotide binding proteins, Q polypeptide (GNAQ) and $\alpha-11$ (GNA11) have been found in RICH and NICH tissues.[7] These act as α-subunits of heterotrimeric G proteins, which couple seven-transmembrane domain receptors and activates downstream signaling pathways. The relevant cell type or pathways in CH has not been determined yet.

Capillary malformations

Capillary malformations (CM163000 OMIM) are commonly known as “port-wine” stains. They are usually sporadic, unifocal red, flat, localized or spread lesions most often located on the head and neck. Somatic missense mutations in GNAQ and GNA11 have also been found in CM.[8,9] However, the most common changes affect the arginine residue at position 183, and mutations are enriched within the blood endothelial cells (BECs). The pathological mechanism underlying GNAQ and GNA11 mutations regarding the vasculature has not been elucidated. However, similar GNAQ and GNA11 alterations have been studied in uveal melanoma. The likely consequence is hyperactivation of the MAPK signaling pathway. This is supported by a study into a complex syndrome in which CM is often seen, Sturge-Weber syndrome (SWS, OMIM 185,300). SWS is a sporadic disorder characterized by leptomeningeal angiomatosis associated with facial CMs and/or glaucoma. The same arginine183-to-glucine (R183Q) *GNAQ* variant is recurrent in resected tissues from SWS patients. The substitution was found in cutaneous and cerebral lesions but not in unaffected skin. When HEK 293T cells were transfected with the R183Q mutant, a mild increase in ERK activation, compared to control cells, was seen.[10] Each mutation would need to be explored in specific cells types to understand why the mutations in the same gene could give rise to so many different phenotypes.

Arteriovenous malformations

Most AVMs arise sporadically and are the most problematic vascular lesions to manage. When the compromised vessels rupture, the consequences can be devastating, especially when found within the brain. Somatic mutations in several members of the MAPK signaling pathway have been implicated in sporadic AVMs. These include changes that result in hyperactivation of the ubiquitously expressed GTPase HRAS or two intracellular kinases, BRAF and MAP2K1.[11–14] Mutations in another GTPase, KRAS, and BRAF have been attributed to about 60% of intracranial AVMs.[15–17]

Interestingly, KRAS (and BRAF) are well-established proto-oncogenes; the most common KRAS alterations in AVM are glycine to aspartate or valine substitutions (G12D or G12V), which are the same hotspot mutations in a variety of cancers. However, the endothelium appears to be the only cell type affected, as mutations were detected in EC isolated from AVMs.[12] Additionally, isolated ECs and human umbilical vein endothelial cells (HUVEC) transfected with a KRAS-G12V or KRAS-G12V had elevated ERK1/2 phosphorylation, while non-endothelial cells or HUVEC-KRAS-WT cells did not.[14,15,17]

The findings introduce a hypothesis that MAPK signaling has an essential endothelial-specific role that has would need to be explored more. To support this, an endothelial-specific inducible (iEC) Hras-V12 mouse model developed dilated, proliferative blood vessels in the brain and intracerebral hemorrhaging; mutant mice died within 3 weeks of induction.[18] Furthermore, Braf(V600E) and Map2k1(Q58del) expression in zebrafish lead to vascular defects and hindered blood flow.[13] It appears that inhibitors against MAPK signaling would be good candidates for treatment of associated AVM. When the Braf and Mapk2k1 mutant zebrafish were treated with the BRAF(V600E) inhibitor vemurafenib for two days, blood flow was restored.[13] Additionally, child with an AVM possessing a MAP2K1 mutation was treated with the MEK inhibitor trametinib and this resulted in reduced AVM size.[19] The affected pathway appears to be more geared towards the MAPK signaling, compared to PI3K signaling, as vascular defects worsen in iEC–Hras(V12) mice treated with the PI3Kα inhibitor BYL719 (alpelisib).[18]

AVMs are hallmarks in two familial disorders in which predisposing causative genes have been identified. CM-AVM (OMIM 608,354) is a disorder that is transmitted in an autosomal dominant manner. CMs in CM-AVM are characteristically small, multifocal, and commonly surrounded by a pale halo. There are two types of CM-AVM, based on which gene is affected. CM-AVM1 (OMIM 608,354) is caused by mutations in *RASA1*,[20] and CM-AVM2 in the tyrosine kinase receptor EPHB4 (OMIM 618,196). The various mutations in CM-AVM1 cause LoF of the encoded the RAS negative regulator protein p120-RasGAP. This loss is thought to lead to overstimulation of Ras/MAPK signaling, causing aberrant cell growth, differentiation and proliferation.[21] CM-AVM1 is also characteristic of Parkes Weber Sydrome (PWS, OMIM 608,355).[22] Affected PWS individuals experience hypervascularity in limbs early in life with multiple small AV fistulas. The EPHB4 receptor plays an essential role in A-V specification and directly interacts with RASA1. Loss of EPHB4 also results in enhanced MAPK signaling. Vein of Galen malformation (VOGM), in which a large vein deep within the brain is enlarged, is a rare entity that is closely associated with CM-AVM. Mutations in both the CM-AVM1 and −2 genes have been found.[23] Moreover, SNPs in to gene encoding the EPHB4 lig- and Ephrin-B2 (EFNB2) show some association.[24] Thus, it appears

that EphrinB2-EphB4-RASA1-MAPK signaling pathway is essential in CM-AVM pathogenesis, at least within the cerebral vasculature.

Hereditary hemorrhagic telangiectasia (HHT, OMIM 187,300) is associated with disruption in TGFβ signaling. LoF mutations in the co-receptor Endoglin (ENG), activin kinase-like receptor-1 (ALK1/ACVRL1), and the common intracellular mediator SMAD4 account for over 85% of HHT patients.[25–28] Moreover, exome sequencing was used to find mutations in bone morphogenetic protein (BMP)−9 in three patients with HHT-like symptoms. BMP9 is a likely ligand for ACVRL1.[29] This strongly supports the notion that BMP signaling pathway is involved in the development of HHT. Despite knowledge that the TGFβ signaling pathway is canonical, the underlying pathogenetic mechanism remains controversial. Patients' phenotypic severity varies widely, even within the same family, and lesions tend to be focal. Likewise, heterozygous *Eng* and *Alk1* mice develop HHT-like phenotypes but with inconsistent penetrance.[30,31] As in other inherited multifocal vascular malformations, the HHT lesions need a somatic genetic second-hit to be formed, leading to localized homozygous genetic loss.[32] In addition, it appears that other pro-angiogenic environmental cues contribute to lesional progression. Conditional EC-specific *Alk1, Eng,* and *Smad4* knockout mice, in which both alleles have been deleted postnatally, develop robust AVMs after stimuli, such as wounding or VEGF exposure.[33–36]

Cerebral cavernous malformations (CCM, OMIM 116,860) are composed of dilated vessels or "caverns" that are filled with blood that mainly affect the central nervous system, but can also be found in the retina and skin.[37] Approximately 20% of CCM cases are inherited in an autosomal dominant manner. The LoF of three genes have been linked to CCMs: Krev Interaction Trapped-1 (*KRIT1*), malcalvernin (*CCM2)* and Programmed cell death 10 (*PDCD10).*[37] A second-hit causing complete localized deletion of one of the CCM genes is needed for the formation of CCMs.[3,4] The three proteins interact with each other and yet have differential functions.[38,39] KRIT1 is involved in the vasculature in regulating endothelial cell-cell junctions via the Delta-Notch pathway.[40] CCM2 is a scaffolding protein for mitogen-activated protein kinase (MEKK3).[41] In CCM3, which possess PDCD10 mutations, apoptosis and VEGF signaling may be altered.[42] Additionally, delta-like ligand 4 (DLL4) may be a downstream target for CCM3.[43] Unlike many of the other vascular malformations, there is no predominant signaling pathway associated with CCM. The three proteins interact to form CCM signaling complexes (CSC) that help regulate angiogenesis. The CSC can then interact with molecules from many different signaling pathways and process, notably WNT/β-catenin, TGFβ/BMP, and Notch signaling.[44]

Venous malformations

The slow-flow venous malformations can be divided into four groups. Sporadic venous malformations (VM) are the most common, accounting for 94% of venous anomalies. Cutaneomucosal venous malformations (VMCM, OMIM 600,195) and glomuvenous malformations (GVM, OMIM 138,000) are inherited in an autosomal dominant manner. They are estimated to account for 1% and 5% of patients, respectively. The fourth entity is sporadic Blue Rubber Bleb Nevus Syndrome (BRBN, OMIM 112,200). For VMCM and GVM a somatic second-hit is needed for lesions to form.[2,5] The most common second-hit in GVMs is acquired uniparental isodisomy, which renders the inherited mutation homozygous in the affected tissues.[45]

The causative gene for most VMs is the EC-specific tyrosine kinase receptor TIE2/TEK.[46] The most frequent mutation is a somatic change in which a leucine is changed to a phenylalanine at position 914 (L914F), while a weaker Arg849 to Tryptophan (R849W) substitution is detected in more than half of inherited VMs.[5,46–49] BRBN Syndrome is characterized by appearance of several cutaneous, visceral, and gastrointestinal VMs, which may hemorrhage. Somatic *TIE2* mutations have also been identified in most patients. Yet, they differ from VM and VMCM causative mutations, by occurring in double, on the same allele.[50]

TIE2 predominantly functions via the PIK3/AKT signaling pathway. Mutated TIE2 receptors aberrantly activate AKT in a ligand-independent manner, resulting in reduced platelet-derived growth factor-B (PDGFB) production and secretion.[51,1] To underscore the importance of the PI3K/AKT signaling in VM pathogenesis, mutations in phosphatidylinositol-4,5-biphosphate 3-kinase catalytic subunit-α (PIK3CA) were identified in another 20% of sporadic VM.[52] PIK3CA encode for the p110α, which is the catalytic subunit of PI3K. The most frequent change found is an oncogenic hotspot histidine-to-arginine change at position 1047 (H1047R). Several preclinical VM mouse models have been generated using different approaches that phenocopy patient VM. Transplantation models were made by subcutaneously injecting either HUVECS transfected with the TIE2-L914F mutation or (TIE2 and PI3KCA-mutant) patient derived ECs into athymic mice. Additionally, *Pik3ca* was conditionally activating in transgenic mice.[53–57] Rapamycin (sirolimus), a mTOR inhibitor, was able to control VM progression in the xenograft mouse model with HUVECs transfected with the TIE2-L914F, leading to its use in Phase II/III clinical trials.[53,58] Alternative molecular treatments are being explored, such as PI3K inhibitors; in particular BYL719, which has shown promise both *in vitro* and *in vivo*.[52,56] TIE2 can signal via the MAPK pathway as well, thus inhibitors of that pathway may also be beneficial. Treatment of HUVECs overexpressing TIE2 with the MAPK inhibitor PD98059 helped normalize cell morphology.[55]

GVMs resemble VMs yet are distinct. The severity of GVMs varies among patients, from punctate harmless blue spots to large, painful debilitating lesions. New lesions potentially form over time.[59] Nearly all GVMs are caused by mutations leading to LoF of glomulin (*GLMN*).[2] There is a paucity of information about GLMN function; however, the specificity of the lesions indicate that this molecule plays an important role in the vasculature, particularly in vascular smooth muscle cells (vSMC). Glomulin plays a role in Fbw7-mediated protein ubiquitination and degradation. Glomulin inhibits the E3 ubiquitin ligase activity of the Cul1-RING-ligase (CRL1) complex by binding to the RING domain of Rbx1.[1,60] *In vitro* studies suggest that GLMN is regulated by its binding with different FK506-binding proteins (FKBP), particularly to FKBP12.6 and FKBP51, however, the relationship to lesion progression and potential pharmacological targets remains unknown.[61]

Recently, a vascular anomaly known as verrucous venous malformation (VVM) has been recategorized from a hemangioma to a venous malformation (ISSVA). A missense isoleucine-to-methionine change at position 441 (I441M) of the MAP kinase-3 (MAP3K) was seen in VVMs.[62] Based on the *Mekk3* conditional knockout mouse models, the gain of function changes of *Mekk3* within endothelial cells is pathogenic. Compound deletion of *Mekk3* and CCM2 in mice helped prevent development of CCM lesions.[63] *In vitro* studies indicate that MAP3K3 is downstream of angiopoietin-1/TIE2 signaling, however, the relationship still needs to be evaluated.[64]

Lymphatic malformations

Lymphatic malformations (LM) consist of enlarged lymphatic vessels with fibrotic walls of variable thickness. They are commonly classified into macro- and micro-cystic lesions. They are found in the skin and deeper in soft tissue, typically on the head and neck. LMs are commonly localized but can be more widespread on the thoracic and abdominal areas. There is no evidence of familial forms. Activating mutations in PIK3CA, enriched within lymphatic ECs (LEC), have been reported in LM pa-

tients.[57–66] As in some sporadic VM, the most frequent reported change is PIK3CA(H1047L), resulting in hyperphosphorylation of AKT in LECs.

Lymphedema is another lymphatic anomaly, in which the initial and/or collecting lymphatics are affected. Lymphedema defects are not localized but rather affects entire limbs. In general, more females than males are affected. Lymphedema is typically seen in older patients, and the affected regions of the body gradually become enlarged as lymph fluid is not efficiently cleared. Yet, some forms are already present at birth, such as the Nonne-Milroy disease. In lymphoscintigraphy, lymphedema is characterized by hypoplasia, aplasia, or hyperplasia of lymphatic channels.[67]

There are two main categories of lymphedema. "Secondary" refers to lymphedema that develops in response to a trauma, such as surgery. When the cause is not known, lymphedema is considered "primary". Most cases of primary lymphedema are sporadic; however, 35% of patients have a positive family history.[68] At least 30 genes have been linked to lymphedema, though a clear pathogenetic mechanism connecting these is unknown. Just a few will be highlighted in this review. The VEGFC/VEGFR3 and PI3K-AKT signaling pathways related to the development and maintenance of lymphatic valves seem to be major players.[67]

Familial congenital lymphedema, or Nonne-Milroy disease, is linked to mutations in the vascular endothelial growth factor receptor 3 (VEGFR3, OMIM 153,100).[69] Patients with *VEGFR3* mutations tend to have bilateral lower limb lymphedema. VEGFR3 is a tyrosine kinase receptor expressed in ECs, essential for angiogenesis and later for lymphangiogenesis in embryonic development. Mutations lead to lack of receptor phosphorylation and decreased downstream signaling. *Vegfr3* KO mice are embryonic lethal at midgestation due to cardiovascular defects.[70]

Rare families with inherited primary lymphedema have a LoF mutation in the ligand that binds VEGFR3 specifically in lymphatic vessels, VEGFC.[71,72] The *Chy3* mouse has *Vegfc* ablated and characteristically develops chylous ascites, in addition to lymphedema in the hindlimbs.[73] *Vegfc* heterozygous mice are born with hypoplastic lymphatic vessels and develop lymphedema in adulthood.[73]

A homozygous deletion of exon 7 of the protein tyrosine phosphatase-14 (PTPN14) was discovered in one consanguineous family. The mutation leads to a premature stop codon that ultimately results in nonsense-mediated mRNA decay. *Ptpn14* knockout mice develop hyperplasia of vessels and lymphedema, recapitulating the human syndrome. As PTPN14 is recruited to VEGFR3 via VEGFC stimulation in order to dephosphorylate the active VEGFR3, the lymphatic defects may arise from hyperactivity of VEGFR3 in the absence of PTPN14.[74]

Hereditary Lymphedema II often develops in combination with distichiasis (and to a lesser degree, ptosis, and yellow nails). It is linked to truncating and missense mutations in the forkhead box protein C2 (FOXC2) transcription factor; both loss and gain of FOXC2 function have been found to be deleterious.[75,76] FOXC2 has many functions, notably in angiogenesis by regulating the expression of important endothelial target genes, such as *Ang-2, integrinβ3, D114* and *Hey2*.[77] *Foxc2* heterozygous mice develop lymphatic vessel and lymph node hyperplasia, in which increased pericyte recruitment interferes with proper function of collecting lymphatics.[78] FOXC2 is also crucial for lymphatic valve development. It is a downstream target of VEGFC/VEGFR3 signaling.[67,79]

A rare form of syndromic lymphedema with variable onset is called Hypotrichosis-Lymphedema-Telangiectasia (HLT, OMIM 607,823). Both recessive and dominant mutations in the transcription factor SRY-containing box 18 (SOX18) have been reported. SOX18 regulates the essential initiator of lymphangiogenesis, prospero homeobox 1 (PROX1), which in turn regulates VEGFR3 expression. The reported dominant nonsense mutation is found within the transactivation domain of SOX18, while recessive substitutions lie within the DNA-binding domain.[80] Mice with disrupted *Sox18* only have a mild coat phenotype, which may be due to compensatory mechanisms by other Sox transcription factors.[81]

Hennekam syndrome is inherited in autosomal recessive manner and three subtypes have been established. LoF alterations within the collagen and calcium-binding EGF domain-containing protein-1 (CCBE1, OMIM 235,510), FAT atypical cadherin 4 (FAT4), and a metalloproteinase with thrombospondin motifs-3 (ADAMTS3) have been reported.[82,83] In the lymphatics, CCBE1 is bound to the extracellular matrix and influences ADAMTS3-mediated proteolytic activation of VEGFC.[84] Hennekam syndrome is characterized by extensive lymphedema with visceral involvement, mental retardation, and distinct facial features (flat facies, hypertelorism and a broad nasal bridge); occasionally hydrops fetalis is seen.[85] Knockdown of the zebrafish homolog *full of fluid* (*fof*) indicates that CCBE1 is involved in lymphangioblast budding and sprouting from the venous endothelium.[86]

Microcephaly associated with or without chorioretinopathy, lymphedema, or mental retardation (MCLMR, OMIM 152,950) is linked to the homotetrameric kinesin-like protein-11 (KIF11)/EG5.[87] KIF11 is a motor protein involved in chromosome positioning and centrosome separation during mitosis, by forming a bipolar spindle. KIF11 inhibition activates PI3K/AKT signaling. *Kif11* null mice are early embryonic lethal, but *Kif11* heterozygotes are phenotypically normal.[88] It is unclear how KIF11 mutations lead to lymphedema.

Inhibitor of Kappa Light Polypeptide gene enhancer in B-cells (IKBKG), also known as NEMO, is altered in the X-linked syndrome osteopetrosis, lymphedema, anhidrotic ectodermal dysplasia, and immunodeficiency (OLEDAID, OMIM 308,300). In this form of lymphedema, mutations are hypomorphic. Thus, IKBKG is greatly reduced, but not completely lost.[89] IKBKG indirectly regulates VEGFR3 expression by inducing nuclear factor-$\kappa\beta$ activation and consequently PROX1.[90]

Defective lymphatic valve development/maintenance can also result from defects in two connexins (CX), CX47 and CX43.[91,92] The reported mutations are amino acid substitutions that alter connexin activity. Yet, *Cx47* knockout mice do not have lymphatic problems, indicating compensation by other proteins may be involved.[93,94] Loss of CX47 is seen in the autosomal recessive disorder hypomyelinating leukodystrophy, which affects the central nervous system, without lymphedema. Only one patient was reported to have the CX43 mutation along with oculodentodigital dysplasia.[95]

The zinc-finger transcription factor *GATA2* is mutated in Emberger syndrome (OMIM 614,038); patients are susceptible to develop acute myeloid leukemia in addition to lymphedema.[96,97] GATA2 is mainly expressed within ECs and hematopoietic stem cells, as well as progenitors and valves of the lymphatic vessels. The lymphatic defects are due to loss of GATA2 function. *Gata2*-null mice are embryonic lethal at midgestation due to severe anemia and lowered myeloid-erythroid progenitor cells. However, no vascular defects were observed. This may be due to redundancy among GATA family members.[98,99]

The use of NGS has been an asset in finding some of these genes. It enables to study even small families, in which classical linkage analysis is not informative. Its increased systematic use for diagnostic testing will likely uncover more novel mutations and help explain the large variability in clinical phenotypes, even in patients carrying the same Mendelian mutation.

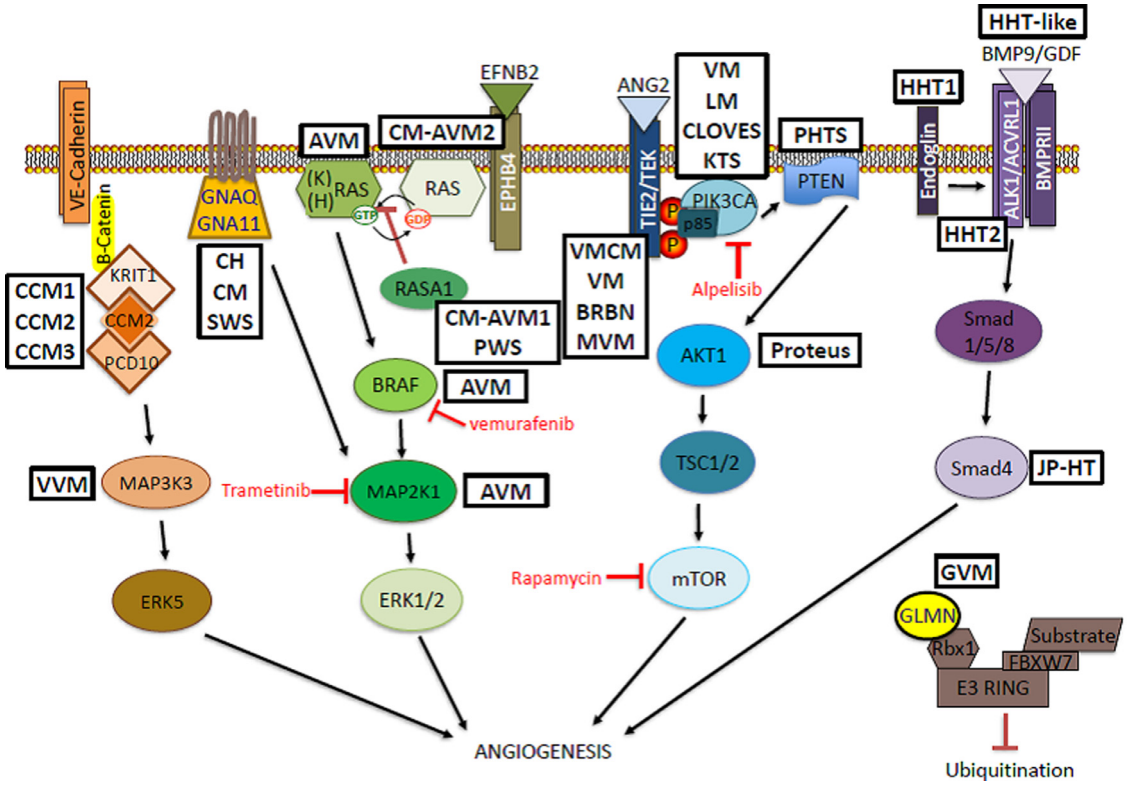

Fig. 1. Summary of major signaling pathways affected in vascular anomalies. Names of anomalies are written next to associated protein. Inhibitors that have been reported to effectively alleviate vascular defects in animal models and some patients, are also shown (in red), in relation to their target. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Combined and complex disorders

Vascular malformations are occasionally found as one of several symptoms in complex syndromes, in which hyperplasia of subcutaneous and soft tissues and bones are also present. As in the simple vascular malformations, somatic or mosaic mutations are likely the major cause of these phenotypes. The associated genetic changes identified thus far are related to the PI3K-AKT signaling pathway. PROS (PIK3CA-related overgrowth syndrome) is a blanket term for a group of syndromes in which PIK3CA mutations are common. Vascular malformations are a prominent feature in several types, such as in congenital lipomatous asymmetric overgrowth disorder with vascular malformation, epidermal nevi and skeletal anomalies (CLOVES, OMIM 612,918).[100] WES has been integral in revealing the genetic mutations and in enabling more accurate diagnosis. Additionally, as some patients with overgrowth syndromes may be predisposed to cancer, the identification of the causative genes should help evaluate prognosis and identify more therapeutic molecules.

The Klippel-Trenaunay syndrome (KTS, OMIM 149,000), is characterized by slow-flow capillo-lymphatico-venous malformations with soft tissue overgrowth. It occurs sporadically, and like CLOVES, it is due to post-zygotic modifications in PIK3CA.[100] Rare chromosomal translocations between 8q22.3 and 14q13, as well as 5q13.3 and 11p15.1 have also been reported. The latter resulted in the identification of a gain of function effect of angiogenic factor with G patch and FHA domains 1 (AGGF1).[101] AGGF1 is likely a venous cell fate determining factor that functions via AKT. Zebrafish overexpressing *aggf1* exhibited enhanced venous differentiation and an increase in AKT signaling.[102]

Proteus Syndrome (PLS, OMIM 176,920) is another non-inherited overgrowth syndrome recognized by skin, connective tissue, and brain hyperplasia, which often appears patchy, with a susceptibility to developing tumors. It is associated with a recurrent somatic *AKT1* mutation. The aberrant genetic variant likely results in GoF of AKT1.[103] A transgenic mouse expressing activated *Akt1* exhibited skin overgrowth.[104] Additionally, cartilage calcification, a major event in Proteus syndrome, is induced when *Akt1* is activated in murine chondrocytes *in vitro*.[105]

PTEN hamartoma tumor syndrome (PHTS) is inherited in an autosomal dominant manner. PHTS encompasses several disorders: Crowden syndrome (OMIM 158,350), Bannayan-Riley-Ruvalcaba Syndrome (OMIM 153,480), PTEN-related Proteus syndrome and Proteus-like syndrome. In addition to the appearance of slow- and fast-flow vascular anomalies, PHTS patients may exhibit tissue overgrowth, gastrointestinal polyps, and macrocephaly. PHTS is diagnosed when a pathogenic phosphatase and tensin homolog (PTEN) mutation is identified. PTEN is a tumor suppressing phosphatase that counteracts PI3K signaling. In the absence of PTEN, PI3K/AKT signaling is dysregulated.[106, 107]

As with VM, other slow-flow lesion-associated disorders and syndromes involve overactive PI3K signaling. Thus, inhibitors towards this pathway are attractive medicinal choices. Sirolimus has shown promise in effectively reducing lesion progression in LM, KTS, and PHTS.[108–112] However, as PIK3CA is commonly mutated in distinct disorders, a more direct inhibitor, like BYL719 is of great interest. Indeed, BYL719 was able to restore defective vessels and organ dysfunction in an inducible *Pik3ca* CLOVES/PROS mouse model. Additionally, symptoms were greatly improved in a small cohort of patients given BYL719.[113]

Concluding remarks

Vasculogenesis, angiogenesis, and lymphangiogenesis are tightly regulated and complex processes that involve several signaling pathways. Discovery of causative genes and understanding the molecular mechanisms underlying them would ideally identify po-

tential targets for treatment (Fig. 1). As most cases occur sporadically, tissue heterogeneity has been an obstacle in the search for alterations. Standard sequencing methods initially used to search for genetic mutations were not sensitive enough to detect such low-level somatic mutations. NGS, and especially targeted NGS of the (likely) pathogenic genes, is a great tool to expose etiopathogenic variants in resected heterogeneous tissues. Identification of causative somatic mutations will lead to a better molecular clarification of vascular anomalies, and address time-point relatedness influencing severity and localization of lesions.

The power of NGS has been demonstrated by the identification of novel causative genes for several vascular anomalies at a rapid pace. Interestingly, many of the same genes are mutated in seemingly distinct malformations, underscoring the complicated processes that are involved. A noticeable pattern has formed, though: fast-flow anomalies involve the RAS/MAP signaling pathway, while slow-flow lesions express predominantly aberrant PI3K-AKT signaling. However, more research needs to be done to even better understand genotype:phenotype correlations and the processes relevant to vascular development and maintenance. Focus will need to be on cell types affected; for example, PIK3CA mutations in the BECs give rise to VM, while those enriched in LECs lead to LM. Additionally, functional defects of specific mutations should be evaluated further in the context of the vascular ECs. For example, GNAQ/GNA11 mutations at the position Gln209 are associated with CH, while those at the Arg183 residue cause CM. It was reported in uveal melanoma, that a change at Gln209 is more deleterious than at Arg183, indicating functional differences with a specific change could lead to distinct outcomes.[114] Such studies will help better define all vascular malformations, thus aiding precise diagnostics. As our understanding of the processes relevant to vascular development and maintenance become clearer, preclinical animal models can also be developed. The relevant models can be used to screen for beneficial effects of therapeutic molecules in use for other disorders and to develop precise, targeted treatments.

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