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The pathobiology of vascular malformations: insights from human and model organism genetics

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Abstract

Vascular malformations may arise in any of the vascular beds present in the human body. These lesions vary in location, type, and clinical severity of the phenotype. In recent years, the genetic basis of several vascular malformations has been elucidated. This review will consider how the identification of the genetic factors contributing to different vascular malformations, with subsequent functional studies in animal models, has provided a better understanding of these factors that maintain vascular integrity in vascular beds, as well as their role in the pathogenesis of vascular malformations.

Keywords

Sturge-Weber syndrome; Klippel-Trenaunay syndrome; Parkes-Weber syndrome; capillary malformation-arteriovenous malformation; cerebral cavernous malformation; moyamoya disease; hereditary haemorrhagic telangiectasia; CADASIL/CARASIL; arterial tortuosity syndrome; Loeys-Dietz syndrome; glomuvenous malformations; venous malformations; blue rubber bleb nevus syndrome

Loss of integrity in small-calibre vessels

Capillaries are small-calibre vessels distributed throughout the body for nutrient, gas, and waste exchange. These vessels consist of a single layer of endothelial cells, supported by overlying pericytes, and thus lack the structural support found in the arterial and venous vascular beds. Four syndromes, Sturge-Weber syndrome (SWS), Klippel-Trenaunay syndrome (KTS), Parkes-Weber syndrome (PWS), and capillary malformation-arteriovenous malformation (CM-AVM) are characterized by capillary malformations [1-5], but the locations of these lesions as well as other distinguishing features differentiate each of these syndromes. In SWS, the presence of leptomeningeal malformations in the brain, often

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resulting in seizures, calcification, and stroke-like episodes, due to poor blood flow through the affected vessels, is a defining feature; however, capillary malformations on the face, usually in the region of the trigeminal nerve, and lesions in the eye, resulting in early-onset glaucoma, are common [4,5]. KTS is characterized by capillary and venous malformations anywhere on the body [1]. Additionally, local overgrowth, which may include the underlying bones and soft tissues, is a defining feature of KTS [1]. Similarly to KTS, patients with PWS have capillary malformations and limb overgrowth; however, arteriovenous fistulas are also present [1,2]. Finally, like PWS, CM-AVM is defined by capillary malformations and arteriovenous fistulas, which may occur anywhere on the body, but in contrast, limb overgrowth is not observed in CM-AVM [6]. Given the similarities between them, it seems likely that similar underlying molecular defects would cause these four syndromes, with the phenotypic differences arising from the distinct cell populations involved.

Uncovering the genetic basis for SWS, KTS, PWS, and CM-AVM has posed a challenge, as these syndromes usually occur sporadically, although some cases of autosomal dominant inheritance of PWS and CM-AVM have been described [3,6,7].

From the familial cases of PWS and CM-AVM, loss-of-function (LOF) mutations in *RASA1* have been identified [3,6-8]. Recently, a second somatic mutation from a CM lesion has been reported, supporting the theory that complete loss of *RASA1* expression through a second somatic hit is required for lesion formation [9]. *RASA1* LOF mutations have also been reported in a few cases of familial isolated capillary malformations, indicating that this pathway is crucial for regulating vascular development and maintaining integrity [10]. *RASA1* facilitates the inactivation of Ras p21, by enhancing the GTPase activity of the Ras proteins [11]. Thus, loss of *RASA1* function amplifies downstream signalling, with numerous consequences, including aberrant proliferation and angiogenesis [12]. Recently, Norden et al have demonstrated that loss of *RASA1* *in vitro* results in enhanced tubular network development of endothelial cells, coupled with increased ERK activation [13]. Through the use of a conditional *Rasa1* knockout mouse, Lapinski et al demonstrated that *RASA1* is expressed in a variety of adult tissues, including but not limited to the spleen, brain, lymph nodes, kidney, and lung [14]. Lubeck and colleagues generated a knock-in mouse line expressing the p.R780Q *Rasa1* mutation, to study the effect of this mutation on vascular development [15]. This study confirmed that *RASA1* is essential for life and that loss of *RASA1* function disrupts vascular development [15]. Despite the generation of a conditional *Rasa1* knockout animal and the *Rasa1* p.R780Q knock-in mouse, an animal model that recapitulates PWS or CM-AVM has yet to be developed.

In SWS and isolated capillary malformations, a somatic mutation has been identified in affected tissue. These lesions are caused by activating mutations in the gene *GNAQ*, encoding the $G\alpha_q$ subunit, due to a single-nucleotide change resulting in the substitution of glutamine for arginine at position 183 (p.R183Q) [16]. G-alpha subunits, of which $G\alpha_q$ is a member, are responsible for initiating signalling cascades downstream of G-protein coupled receptors (GPCR) when activated by the binding of GTP (reviewed in [17]). Early studies by Kleuss et al on GTPase activity of $G\alpha$ subunits revealed that mutations of R178 in $G\alpha_i$ (analogous to R183 in $G\alpha_q$) significantly attenuate GTPase activity of the alpha subunit [18], resulting in constitutive subunit activation. During *Xenopus* development, Fuentealba

et al have found that *GNAQ* is robustly expressed in the developing nervous system and neural crest lineages [19]; however, it is currently unknown whether this expression pattern is conserved in higher organisms. Recent work has determined that the p.R183Q *GNAQ* mutation is primarily present in the endothelial cells within SWS and capillary malformation lesions, suggesting a cell-autonomous effect, although the effects of the R183Q mutation on endothelial cell biology are not currently understood [20].

In a subset of KTS cases, a somatic mutation of *PIK3CA* involving amino acids E542, E545, or H1047 has been identified, leading to kinase activation [21]. As discussed later in this review, similar mutations in *PIK3CA* have been identified in venous malformations [22], suggesting that conserved pathways are involved in regulating vascular integrity in both of these conditions [21]. *PIK3CA* is a catalytic subunit of PI3 kinases, resulting in the phosphorylation, and thus activation, of downstream phosphoinositides. This action results in the activation of many cellular responses, including: proliferation, angiogenesis and survival [23]. Through the study of global and endothelial-specific *Pik3ca* knockout mice, Yoshioka et al clearly demonstrated the essential role for this protein in the regulation of angiogenesis [24]. Since this study demonstrated attenuated formation of the vascular network *in vivo* [24], it is not surprising that over-formation of the vascular network (such as in KTS and venous malformation) is associated with activating mutations in *PIK3CA*. Given that KTS occurs sporadically, it seems that somatic mutations in early progenitor cells for the blood vessels and underlying bone may result in KTS, whereas somatic mutations solely in venous endothelial cells may give rise to VM.

Thus far, studies of SWS, KTS, PWS, and CM-AVM have been largely limited to the direct study of tissue collected from lesions, highlighting the need for cell culture and animal models to study these syndromes. Currently, other than the *Rasa1* p.R780Q knock-in mouse model described above [15], there are no published animal models for the remaining syndromes, leaving a deficit in our understanding of the roles of these genes and their corresponding mutations in lesion development and progression. Together, the examination of these distinct syndromes indicates that signalling downstream of PI3K in endothelial cells, due to a convergence of $G\alpha_q$ and Ras signalling pathways, may be crucial for regulating vascular development and integrity. Further studies are required to determine if mutations related to the PI3K signalling pathway are present in sporadic cases of KTS, PWS, and CM-AVM, and to identify the cell type(s) harbouring the mutation in these lesions.

Cerebral cavernous malformations (CCMs)

Occurring predominately within the central nervous system, CCMs are clusters of dilated blood vessels with a loss of surrounding parenchymal cells. These ectatic vessels have a loss of endothelial integrity and are prone to haemorrhage. Patients with CCMs present with headaches, focal neurologic deficits, seizures, stroke, and death. CCMs occur sporadically as well as in an autosomal dominant heritable pattern [25]. The sporadic disease generally presents with solitary lesions, while the familial form has an earlier onset and results in multiple lesions. Knudson's two-hit mutation hypothesis (first invoked to explain the

formation of tumours from loss of a tumour suppressor gene) has been shown to apply to CCM lesion pathogenesis, as described below [26].

Three causative genes for CCM have been identified: *CCM1* or *KRIT1* (*KREVI/RAP1A interaction trapped-1*) [27,28], *CCM2* or *OSM* (*osmosensing scaffold for MEKK3*) [29], and *CCM3* or *PDCD10* (*programmed cell death 10*) [30]. The loss-of-function mutations in these genes include missense mutations, nonsense mutations and large, multiple exon deletions, such as those observed in *CCM2* [31]. Each of these genes encodes a cytosolic protein; these together can form a heterotrimeric complex of unknown function as well as independently interact in a variety of other protein complexes to regulate endothelial cell-cell junction stability, polarity, migration, angiogenesis, actin remodelling, vesicle trafficking, and apoptosis [32,33]. The study of the three CCM genes in a variety of model organisms has enhanced our understanding of the physiological and pathological roles of these proteins, as well as identifying novel therapeutic targets.

Studying the CCM protein orthologues in zebrafish and *C. elegans* has provided valuable information about the function of each protein and their possible roles in the mammalian system. Loss of the *CCM1* and *CCM2* zebrafish orthologues, *santa* and *valentine*, respectively, results in a shared phenotype of dilated heart with a single layer of myocardial cells [34] and dilation of major vessels [35]. *CCM3* has two zebrafish orthologues, *ccm3a* and *ccm3b*, which have been knocked down in the zebrafish model; however, different cardiovascular phenotypes have been reported. Knockdown studies by Voss et al [36] demonstrated an identical phenotype to previous studies of zebrafish *ccm1* and *ccm2*. Contrastingly, Yoruk et al [37] observed an absence of zebrafish heart dilation and the presence of abnormal cerebral vasculature following the knockdown of both *CCM3* orthologues. As the latter group notes, these phenotypic differences suggest that *CCM3* may contribute to vascular lesions via a mechanism distinct from that of *CCM1* and *CCM2*. Similar conclusions, of a *CCM3* pathway independent of *CCM1* and *CCM2*, were reached with *ccm-3* deletions in the invertebrate *C. elegans* model organism [38]. In the nematode, *ccm-3* is involved in regulating lumen extension of the excretory canals, actin dynamics, Golgi stability, and endocytic recycling [38]. These abnormalities of the excretory canals in *ccm-3* mutant *C. elegans*, given the absence of *CCM2* in the nematode, suggest that *CCM3* contributes to lumen morphology through interactions with the STRIPAK (striatin-interacting phosphatase and kinase) complex, distinct from signalling through interactions with the other CCM proteins [38]. Studies in the zebrafish and nematode suggest that loss of *CCM3* results in a different pathogenic mechanism when compared to the loss of the other two CCM genes. This is consistent with developmental and phenotypic observations in mice [39] as well as patients [40] with loss of *CCM3*, which results in a more severe form of disease when compared to loss of *CCM1* and *CCM2*.

Mice deficient in *Ccm1*^{-/-} [41] or *Ccm2*^{-/-} [42,43] suffer mid-gestation lethality, as these genes contribute significantly to the development of systemic vessels. Mice heterozygous for *Ccm1* and *Ccm2* are viable, and develop vascular lesions that resemble the human CCMs; however, these occur at a low frequency [42,44]. The lesion burden was bolstered by increasing the likelihood of a second mutation within the *Ccm* heterozygous mice by creating homozygous deletions of tumour suppressor *p53* [45] or DNA mismatch repair gene

Msh2 [46]. More recently, the cre/lox technology has been utilized to create inducible *Ccm* deletion mouse models with tissue specificity and temporal control, to further investigate the pathogenesis and development of lesions. These inducible knockout (iKO) models have enabled the targeted deletion of genes implicated in CCM development to further understand the early signalling events in pathogenesis.

Several *in vitro* and *in vivo* studies have identified a variety of endothelial signalling pathways that are dysregulated in CCMs. One of the first pathways implicated in CCM was increased RhoA GTPase activity from loss of the *Ccm1/Ccm2* interaction [42,47,48]. The RhoA downstream targets, Rho-associated kinases ROCK1 and ROCK2, increase endothelial actin stress fibre formation and lead to a decrease in endothelial junction integrity [48]. The importance of the RhoA/ROCK pathway was further supported by the use of fasudil, a ROCK inhibitor, to decrease the vascular permeability and lesion burden in CCM1 and CCM2 models [48,49]. Multiple groups have observed increased expression of *Klf2*, *Klf4*, and *Adamts4* in CCM lesions [50,51]. One explanation for this observation is that the early lesion genesis is driven by an increase in MEKK3/KLF2,4 signalling upstream of the RhoA pathway [50]. The lesion burden in a *Ccm1* iKO model was significantly reduced by individually deleting *Map3k3*, *Klf2*, and *Klf4* within endothelial cells [50]. An alternative explanation is that *Klf4* is a transcription factor of *Bmp6* [52]. An increase of *Bmp6*, which activates the TGF- β /BMP/SMAD pathway, resulted in an endothelial-to-mesenchymal transition of the endothelial cells lining the cavernomas in a *Ccm1* iKO mouse model [52]. Treating the *Ccm1* iKO mice with TGF- β inhibitors decreased CCM formation [52]. Furthermore, the knockout of *Klf4*, the upstream transcription factor, in the same model also decreased the lesion burden [51]. The TGF- β /BMP/SMAD pathway was further studied in a *Ccm3* iKO model, in which lesion development was decreased in mice treated with sulindac, an anti-inflammatory drug that decreases β -catenin transcriptional activity [53]. In an effort to identify and repurpose drugs originally developed for other indications that may have efficacy in treating CCM, a high-throughput, machine-learning algorithm with subsequent *in vitro* and *in vivo* assays was recently developed [54]. Analysis of over 2,100 developed drugs identified cholecalciferol (vitamin D₃) and tempol, a membrane-permeable radical scavenger, as potential therapeutic options. Both of these drugs decreased lesion burden in a *Ccm2* iKO mouse model [54]. Consistent with the efficacy of tempol, previous *in vitro* studies found that loss of *Ccm1* increases intracellular reactive oxygen species production [55]. Recent work with a *Ccm3* iKO mouse model defined a mechanism by which *Ccm3* regulates exocytosis. In both the mouse and human loss of CCM3 resulted in increased angiopoietin 2 in the lesion. Lesions in these mice were decreased by knocking out *Unc13b*, a gene involved in vesicle trafficking, as well as treating the mice with anti-angiopoietin antibodies [56]. As a whole, these studies illustrate the diverse signalling pathways in which the CCM proteins interact, and potential therapeutic targets for developing future interventions.

These animal models have led to the identification of several signalling pathways that are dysregulated in CCMs, and have demonstrated the efficacy of various therapies. Together, these studies show promise for translating findings in model organisms into clinical trials for CCM patients. The concerted effort of the field to develop, analyse, and further improve

CCM models will continue to enhance our understanding of this disease and drive development of disease-modifying therapies.

Compromised vascular integrity in arterial vascular bed

Within the arterial vascular beds, a loss of vascular integrity as a result of genetic insults manifests in several distinctive malformative diseases. One such disease that appears to indicate the importance of endothelial cells in maintaining vascular integrity within the arterial system is moyamoya disease (MMD). Moyamoya vessels, from which the name MMD is derived, are abnormal collateral vessels visible on cerebral angiogram [57-59], which presumably arise to compensate for decreased blood flow due to narrowing of the internal carotid arteries [58]. Histologically, the affected cerebral arteries exhibit intimal hyperplasia, contributing to occlusion of the vessel, along with atrophy of the overlying media layer and aberrations in the elastic lamina [60].

Through the analysis of familial cases, two independent groups identified the p.R4810K mutation of *RNF213* as a susceptibility gene for MMD in East Asian populations arising from a founder mutation [57,60]. Although *RNF213* mutations were found in a small percentage of Caucasian individuals with MMD, the mutations identified differed from the p.R4810K mutation found in East Asians and occurred at a much lower frequency, suggesting genetic heterogeneity of this disease across populations [60]. Interestingly, mutations in *RNF213* are present in approximately 1% of East Asians [60] although the prevalence of MMD is much lower, highlighting its incomplete penetrance and lending support to the theories that unidentified factors, such as inflammation [61,62], may be required for the manifestation of MMD.

Currently, it remains unclear how mutations in *RNF213*, which has ATPase and ubiquitin ligase domains [60,63], affect protein function in MMD. Several approaches have been used to address the effect of *RNF213* in vascular development and maintenance. Using zebrafish lacking both copies of the duplicated fish orthologues of *Rnf213*, Liu et al found that while vascular development in the trunk of the fish appeared largely normal, loss of *Rnf213* resulted in abnormal vascular sprouting in the head, especially in the eye, along with potential differences in vessel thickness [60]. In contrast to the findings in zebrafish, *Rnf213* knockout mice have no obvious differences in vascular development under baseline conditions [64,65]. Interestingly, with permanent ligation of the femoral artery, Ito and colleagues found that blood flow to the hind limb was restored more quickly with *Rnf213* deletion, presumably due to the increase in angiogenesis observed following ischemia initiation [66], suggesting that loss of function of *RNF213* may promote the cascade of events resulting in MMD. However, this study was unable to demonstrate an increase in angiogenesis with temporary ligation of the middle cerebral artery; the authors speculate that this is attributable to the short duration of the ischemic event [66]. More recently, Kobayashi and colleagues have explored the role of p.R4757K *RNF213* over-expression in smooth muscle cells or endothelial cells using transgenic mice [67]. Exposure of these animals to hypoxia suggests that endothelial-specific expression of this mutation may inhibit neovascularization in the brain following hypoxic exposure [67], supporting *in vitro* findings of reduced angiogenic potential with the p.R4810K *RNF213* mutation [67-69]. Although the

authors did not notice any vascular changes consistent with MMD in this study, and are inconsistent with the findings of Ito et al [66], it is possible that the duration of the ischemia was insufficient to recapitulate MMD progression, as discussed by Ito et al [66]. Finally, due to the incomplete penetrance of the *RNF213* sequence variants, some have suggested that additional factors, such as inflammation [61,62], may be required for promoting MMD. Support for this hypothesis comes from *in vitro* studies showing regulation of *RNF213* by inflammatory cytokines, such as interferon- β and interferon- γ [67,69], although this connection has not been validated *in vivo*. Thus, current data suggest that pathological signals originating from the endothelium may be promoting the intimal dysfunction, and thus disease pathology is observed, but this has yet to be directly tested and verified.

A more heavily studied arterial vascular disease, that results from the disruption of endothelial integrity, is hereditary haemorrhagic telangiectasia (HHT), also known as Rendu-Osler-Weber syndrome. This autosomal dominant disorder leads to mucocutaneous telangiectasias and solid organ arteriovenous malformations (AVMs). The dilated and fragile vessels of mucocutaneous telangiectasias lead to recurrent epistaxis and gastrointestinal haemorrhage. The cutaneous telangiectasias are found predominantly on the face, mouth, and hands [70]. The formation of AVMs results from the abnormal loss of capillary beds and direct connection of the arterial and venous vessels. HHT patients develop AVMs most commonly in the lungs, liver, and brain. The dysplastic AVMs can lead to life-threatening haemorrhage. There is a significant range in clinical presentation among not only the general HHT population, but also within families harbouring the same genetic mutation [71].

To date, three genes involved in the TGF- β /BMP/SMAD signalling pathway have been identified as causing HHT. A large variety of mutations in endoglin (*ENG*) [72], activin receptor like-kinase 1 (*ACVRL1* or *ALK1*) [73], and mothers against decapentaplegic homolog 4 (*SMAD4*) [74] have been identified in HHT1, HHT2, and juvenile polyposis HHT (JP-HHT), respectively. The most common mutations of *ENG* and *ALK1*, which together account for approximately 85% of patients genetically tested for HHT, are missense and deletion mutations [75]. These loss-of-function mutations result in decreased amount of their respective proteins and suggest a haploinsufficiency model that is supported by studies of protein levels from peripheral and umbilical cells collected from individuals harbouring HHT1 and HHT2 mutations [76]. Endoglin is a transmembrane accessory receptor that interacts with the type I and type II serine/threonine kinase cell surface receptors of endothelial cells. ALK1 is a type I receptor for the TGF- β superfamily of ligands. It has now been shown to be a receptor for BMP9 and BMP10 ligands, and importantly, missense mutations in ALK1 result in aberrant signalling via these ligands, furthering support that these ligands are involved in HHT pathogenesis [77-79]. Unlike the endothelial transmembrane proteins, SMAD4 is a cytoplasmic protein involved in the downstream transcriptional regulation of vessel formation and endothelial homeostasis. Our understanding of the role of these genes in the pathogenesis of HHT has been enhanced through the creation and investigation of various *in vivo* models.

Early *in vivo* experiments aimed at understanding the role of these proteins in vascular development and disease examined the development of *Eng*^{-/-} [80,81] and *Alk1*^{-/-} [82] embryos. These genotypes were lethal at mid-gestation, due to abnormal yolk sac

vasculature, angiogenesis, and cardiac development. The heterozygous *Eng*^{+/-} [80,83] and *Alk1*^{+/-} [84] mice share the phenotype of epistaxis, mucocutaneous telangiectasias, and AVMs with HHT patients. An important observation in the *Eng*^{+/-} mice was the decrease in vascular smooth muscle cells (VSMCs) and abnormal extracellular collagen and elastin matrix [83]. This is consistent with previous studies of the *Alk1*^{-/-} embryos, in which abnormal recruitment and differentiation of VSMCs were observed [82]. These studies suggest an important role of endothelial and VSMC signalling in the pathogenesis of these dilated and haemorrhagic vessels. While these heterozygous mice were the first viable models to reproduce the vascular lesions of HHT, and they exhibited low penetrance and an age-dependent development of disease similar to HHT patients, there was a need for a more tractable HHT mouse model with which to perform basic research.

To create mouse models with more robust phenotypes, as well as temporal and cellular control of *Eng* and *Alk1* expression, inducible knockout (iKO) models were generated utilizing cre/lox technology [85,86]. The systematic use of cre recombinase expression in VSMCs, macrophages, pericytes, and endothelial cells has suggested that the loss of function of *Eng* and *Alk1* in endothelial cells is necessary for the development of vascular abnormalities [87-94]. Interestingly, loss of either *Eng* or *Alk1* is not sufficient for the development of AVMs; an inflammatory, injury, or pro-angiogenic stimulus is also necessary to induce cutaneous [90,91,95] and brain [88] malformations in adult mouse models. This has led to the proposal of a “three event hypothesis” for the development of AVMs, consisting of 1) heterozygous germline mutation of *Eng* or *Alk1*, 2) second somatic mutation or local loss of protein through shedding, and 3) an angiogenic trigger [96]. One exception to this proposed sequence of AVM pathogenesis is the development of gastrointestinal AVMs in the *Alk1* iKO adult model without an apparent additional angiogenic stimulus [95].

The zebrafish has also provided important insights into the role of endothelial cells and blood flow in the development of AVMs. Studies utilizing *alk1* mutation-carrying transgenic zebrafish embryos, which develop cranial AVMs, have shown that *alk1* expression and the formation of AVMs both depend upon blood flow [97-99]. Interestingly, this model suggests that the loss of *alk1* does not alter endothelial cell proliferation or apoptosis, but rather, changes the pattern of endothelial cell migration, from the wild-type migration (against the direction of flow) to *alk1*-mutant endothelial cells migrating in the direction of flow to more distal parts of the arterial vasculature [100]. With the ability to perform live imaging of AVM formation with endothelial cell resolution, the zebrafish has complemented the mouse well as a model for HHT.

Animal models of HHT have thus provided valuable information regarding the roles of endothelial cell mutations of *Eng* and *Alk1*, loss of VSMCs in dilated and haemorrhagic vessels, pro-angiogenic stimuli, such as inflammation and wound healing, and blood flow in the pathogenesis of the vascular malformations in this disease.

Cerebral arteriopathy with subcortical infarcts and leukoencephalopathy is a heritable small vessel vasculopathy with autosomal dominant (CADASIL) and recessive (CARASIL) modes of inheritance. CADASIL leads to transient ischaemic attacks or strokes, cognitive

impairment, progressive dementia, pseudobulbar palsy, migraine with aura, and psychiatric disturbances [101,102]. Patients with CARASIL experience strokes and cognitive impairment, as well as alopecia and spondylosis deformans [103]. Most of the pathology in these diseases results from lacunar infarcts in the deep branching arterioles in the white matter of the brain. CADASIL is characterized by pathognomonic accumulation of granular osmiophilic material (GOM) on the surface of degenerating vascular smooth muscles [104] and fibrotic thickening and stenosis of the arterioles [105]. CARASIL does not exhibit GOM, but does have atherosclerosis and loss of vascular smooth muscle cells [103]. While they share similar phenotypes, CADASIL and CARASIL have distinct genetic causes and disease pathogenesis.

The gene causing CADASIL was identified as *NOTCH3*, which encodes the Notch3 receptor protein [106]. In adult human tissues, Notch3 is exclusively expressed in vascular smooth muscle [107]. Some data show that the GOM deposited on the VSMC surface of CADASIL patients contains the extracellular domain of the Notch3 receptor [108]. In the wild-type form, Notch3 has an even number of cysteine residues in its extracellular domain; however, all reported *NOTCH3* mutations result in an odd number of cysteine residues [109]. The two competing hypotheses are (i) that the mutations in Notch3 result in loss of function and decreased canonical signalling or (ii) that they lead to a novel gain of function for the Notch3 extracellular domain. Two groups have studied the ability of *NOTCH3* transgenes harbouring different mutations to rescue either the ischaemic stroke susceptibility [110] or the arterial defects [111] observed in *Notch3*-deficient mice. These studies reached different conclusions regarding the canonical function of Notch3 mutants; the ischemic susceptibility study found a decreased ability of the mutated transgene to rescue the animal from an induced stroke, while the arterial defect study found that the mutated and wild-type *NOTCH3* transgenes could both rescue the arterial differentiation and maturation. The alternative hypothesis, that the Notch3 mutants result in a gain of function for the receptor, is supported by a recent study analysing CADASIL brain vasculature from patients and mouse models [112]. This indicated that the aggregation of the Notch3 extracellular domains caused by disulphide bonds between the odd number of cysteine residues may act as a nidus for tissue inhibitor metalloproteinases 3 (TIMP3) and vitronectin (VTN) aggregation [112]. An increased amount of TIMP3 in the vessel lysates of CADASIL patients was also observed [112]. TIMP3 inhibits the activity of metalloproteinase degradation of the extracellular matrix. The arteriole wall thickening and fibrosis of CADASIL patients is consistent with the observed increase of TIMP3. These are interesting results that suggest a gain-of-function mutation in *NOTCH3* is responsible for the pathogenesis of CADASIL [112]. The current standard of care for CADASIL patients is symptom management. Greater understanding of how the Notch3 mutation contributes to disease may identify novel targets for therapeutic interventions.

The gene underlying CARASIL is *HTRA1* (high-temperature requirement serine peptidase A1) [113]. HTRA1 is a serine protease responsible for repressing TGF- β signalling [113]. Its normal function is to cleave pro-TGF- β 1 in the endoplasmic reticulum, resulting in the degradation of the TGF- β 1 cytokine [114]. Mutations of *HTRA1* decrease the serine protease activity, with a failure of ER-associated TGF- β 1 degradation, and an increased level of mature TGF- β 1 [113,114]. TGF- β 1 is essential for vascular development and

extracellular matrix homeostasis; its increased activity is proposed to result in the arteriolar fibrosis observed in CARASIL [115]. TGF- β 1 is also involved in VSMC recruitment and development, which may explain the loss of VSMCs seen in CARASIL [116]. As with CADASIL, there is no therapy available for CARASIL but anti-TGF- β therapy has been proposed [115].

In addition to the importance of endothelial and vascular smooth muscle cells in maintaining the vasculature, proper maintenance of the extracellular matrix (ECM) composition is crucial for ensuring vascular integrity. One disease that highlights the importance of the interaction between vascular cells, including VSMCs and the ECM, is the autosomal recessive arterial tortuosity syndrome (ATS) [117]. In patients with ATS, the major arteries are abnormally twisted, narrowed, and prone to aneurysm formation [117]. Notably, the connective tissue overlaying the vessels and throughout the body is disorganized, leading to increased flexibility, and probably reduced strength, of the vessel wall, contributing to a loss of vascular integrity [118]. Several collagens and ECM components were excluded as causative genes in ATS [119]. Homozygosity mapping and sequencing revealed that recessive LOF mutations in *SLC2A10*, encoding glucose transporter type 10 (GLUT10), cause ATS [118,120,121]. The cloning of *SLC2A10* revealed structural similarities to other facilitative glucose transporters [122], indicating that glucose transport may be a main function of this protein. Expression of *SLC2A10* has been identified in many tissues, with high expression noted in the pancreas, stomach, aorta, and adipose tissue [122-124]. Furthermore, within the aorta, VSMCs have been shown to express high levels of *SLC2A10* [124,125]. Interestingly, GLUT-10 is the most highly expressed glucose transporter in differentiated VSMCs [125], hinting at the importance of this transporter in maintaining vascular integrity after vessel maturation.

Based on phenotypic overlap with Loeys-Dietz Syndrome (LDS), Coucke and colleagues investigated whether transforming growth factor-beta (TGF- β) signalling was altered in the VSMCs and fibroblasts from individuals with ATS [118]. Immunohistochemical analysis of a limited number of patient samples showed increased levels of pSmad2 and connective tissue growth factor (CTGF), indicating elevated TGF- β signalling [118]. As described below, inactivation of *SLC2A10* appears to increase cellular oxidative stress, ultimately resulting in TGF- β signalling via α v β 3-dependent mechanisms [126]. Thus, it seems probable that pathologic elevation of TGF- β signalling in the VSMCs and connective tissue may contribute to the improper assembly of the ECM, ultimately leading to compromised vascular integrity.

To understand better the role of *SLC2A10* in ATS and the maintenance of vascular integrity, mice harbouring the p.G128E mutation of *SLC2A10* were generated [127,128]. Although initial studies by one group did not reveal changes in arterial morphology [128], this may have been due to insufficient aging of the mice to allow for disruption of vascular integrity, since Cheng et al identified pathological alterations in the elastic lamina overlaying several major vessels at ten months of age [127]. More recently, several groups have shown that *SLC2A10* deficiency is correlated with an increase in reactive oxygen species (ROS) in VSMCs and fibroblasts from patients [124,126]. These studies have demonstrated that *SLC2A10* can transport oxidized vitamin C (dehydroascorbic acid) into mitochondria, to

mitigate damage caused by ROS accumulation [124,126,129]. Zoppi and colleagues go on to suggest that increased ROS generation and resulting stress activates the non-canonical TGF- β signalling pathway, ultimately resulting in ECM dysregulation [126]. Together, these studies begin to reveal the role of transporter proteins in maintaining vascular integrity during steady-state homeostasis.

Loeys-Dietz syndrome (LDS) was first described in a small cohort of families sharing similar cardiovascular, craniofacial, and skeletal phenotypes, inherited in an autosomal dominant pattern. Patients presented with arterial tortuosity, aortic aneurysms and dissections, as well as hypertelorism, bifid or broad uvula, and various skeletal abnormalities [130]. Mutations of several genes involved in TGF- β signalling, including ligands, receptors and transcription factors, have been identified in LDS. The initial LDS probands were found to have heterozygous mutations in *TGFBR1* and *TGFBR2*, which encode type I and type II receptors for TGF- β , respectively [130]. Heterozygous mutations of *SMAD3* (mothers against decapentaplegic homolog 3) [131][132] and *TGFB2* (transforming growth factor β 2) [133] have also been identified as causing aortic aneurysms and multisystem abnormalities. Patients with *SMAD3* mutations also experience osteoarthritis [131] and aneurysms of cerebral vessels, leading to haemorrhage and stroke [132]. *SMAD3* is an intracellular protein that is phosphorylated by activated TGF- β receptors and enters the nucleus as part of a protein complex to regulate transcription of TGF- β signalling genes [131,132]. Histological analyses of aortic samples from patients with *TGFBR1*, *TGFBR2*, *SMAD3*, and *TGFB2* mutations have demonstrated reduced and disorganized elastin as well as increased collagen, consistent with increased TGF- β signalling [130,131,133].

Given the various missense mutations involving several members of the TGF- β signalling cascade in LDS, there has been interest in studying mice with knockouts of the involved genes, to better understand how they contribute to vascular development and function. These studies have revealed the importance of the receptors *TGFR1* and *TGFBR2*, as well as the ligand *TGFB2*, during cardiovascular development. Homozygous deletion of any of these three genes results in embryonic lethality during mid-gestation, due to defects in the development of the vasculature and heart [134-136]. Specifically, abnormal vascular development in the yolk sac was noted in the *TGFBR1* [134] and *TGFBR2* [135] knockout lines, while many defects in the heart, including ventricular septal defects, improper numbers of vessels leaving and entering the heart, and abnormalities in the valves, were described in the *TGFB2* knockout line [136].

Interestingly, studies of mice heterozygous for deletion of either TGF- β receptor did not reveal any differences in diameter of the aorta, or increased tortuosity [134,137]. Additionally, the elastic fibres and VSMCs in the aortic walls were comparable to wild-type animals [134,137], indicating that the pathology of LDS is not due to reduced TGF- β signalling. However, in mice harbouring either a p.M318R mutation in *TGFBR1* or a p.G357W mutation in *TGFBR2*, several hallmark features of LDS, including breakdown of the elastic fibres within the aortic wall, increased vascular tortuosity, excess collagen deposition, and aortic dissection, were recapitulated [137]. This study also recapitulated the paradoxical increase of TGF- β signalling, which has been reported in aortic samples from patients with LDS [130,137,138]. Notably, by inhibiting signalling through the angiotensin

II type 1 receptor by the drug losartan, these authors observed normalization of TGF- β signalling to wild-type levels and protection of vascular integrity in both the p.M318R TGFBR1 and p.G357W TGFBR2 mice [137]. Thus, pathways external to the TGF- β pathway appear to modulate the degree of signalling through this pathway in the presence of LDS mutations. Another study demonstrated mild changes in the integrity of the aortic wall with haploinsufficiency of TGFB2, mirroring some of the clinical findings of LDS [133]. This study, similarly to that by Gallo et al [137], noted a trend towards increased TGFB1 production as a potential factor contributing to the observed increase in TGF- β signalling. Important advances have thus been made in understanding the role of the TGF- β in maintaining vascular integrity; however, further studies are required to fully comprehend the complex interaction of pathways involved in maintaining the integrity of major arteries throughout life.

Maintenance of vascular integrity in the venous beds

With the exception of the capillary beds, vascular smooth muscle cells (VSMC) overlie the endothelial layer of the vasculature, to provide strength and regulate blood flow through the vessels. From the histology of different vascular malformations, it is clear that alterations to VSMC morphology or interference with VSMC recruitment affects vascular integrity. Although VSMCs are present in both arterial and venous beds, the proper function of VSMCs appears to be especially critical in the venous system, as evidenced by their perturbation in several venous malformations. Below, we examine several vascular malformations in greater detail, with the goal of uncovering similarities between malformations that disrupt VSMC function, to gain a better understanding of the roles played by VSMCs in vascular integrity and the genes involved in coordinating proper interactions between these cells and the vasculature.

Glomuvenous malformations (GVM) represent one clear example demonstrating the link between VSMC dysfunction and vascular malformation development. The lesions of GVM are often painful to the touch, and generally do not empty on external compression [139]. Histological examination reveals dilated veins, lined with endothelial cells that are normal in outward appearance, and the presence of immature VSMCs, termed glomus cells [139]. GVMs are inherited in an autosomal dominant fashion with high penetrance [139-142], most lesions developing by the third decade of life [139,143]. Germline LOF mutations in the glomulin gene have been implicated in GVM pathology [139-142]. More recently, Amyere et al confirmed the presence of a second somatic *GLMN* mutation exclusively within GVM lesions [144], resulting in a complete loss of glomulin within the affected tissue, thereby explaining the existence of unaffected carriers and the variation in lesion localization. It has also been demonstrated that glomulin is highly expressed in VSMCs [145]. Together, these findings have prompted additional studies of mouse and human tissues to better understand how loss of glomulin promotes GVMs.

Studies of glomulin knockout mice have revealed that glomulin is essential for viability, and plays an important role in regulating development of the embryonic vasculature [146]. Specifically global loss of glomulin resulted in immature vascular beds, due to failure to undergo appropriate remodelling [146]. This may explain why homozygous loss of

glomulin, due to a second-hit somatic mutation, has, to date, only been found within the GVM lesion, and germline biallelic mutations have not been observed [144]. Through *in vitro* studies, Tron and colleagues have demonstrated that glomulin normally interacts with Rbx1 (Ring box protein 1, an E3 ubiquitin-protein ligase) to decrease target protein ubiquitination [146]. Interestingly, this study also found that loss of glomulin results in decreased levels of Fbw7 (an F Box protein), promoting an increase cyclin E and c-myc due to a loss of ubiquitination and subsequent protein degradation [146]. Since glomulin harbouring various GVM-causing mutations did not rescue Fbw7 levels, and these mutated glomulins failed to bind Rbx1, the authors conclude that regulation of the Rbx1-Cul1 complex by glomulin is crucial for regulating ubiquitination [146]. Thus, it appears that increased levels of proliferative proteins in VSMCs contribute to the formation of vascular malformations in venous beds. How this change specifically causes GVMs though, remains unclear. Perhaps the glomus cells present in the lesion provide insufficient stability for the vessels as blood accumulates, resulting in localized dilation.

Like GVMs, the lesions present in mucocutaneous venous malformations (referred to as venous malformations (VMs) here), and those arising in blue rubber bleb nevus syndrome (BRBNS), show abnormalities in the VSMCs overlaying the venous endothelium [147,148]. However, in contrast to GVMs, inadequate recruitment of VSMCs to the veins (rather than defects in VSMC maturation) contributes to the pathology of the lesions in VMs and BRBNS [148]. The lesions in BRBNS and VMs are easily emptied of blood with external pressure, unlike GVM lesions [148]. Patients generally report pain in VMs after prolonged periods of inactivity, whereas GVMs are usually only painful following compression of the lesion [148]. Thus, the physical presentation of these lesions seems to correlate with VSMC coverage.

Although VMs and BRBNS mostly occur sporadically, some cases have been reported of autosomal dominant inheritance of VMs [149]. Genetic analysis reveals that germline activating mutations in *TEK* (*TIE2*) are present in these families [149]. These mutations result in ligand-independent phosphorylation of the TIE2 receptor, and thus, aberrant downstream signalling [149]. A venous malformation is seeded by a “second-hit” somatic mutation in the remaining wild-type copy of the gene. Activating *TEK/TIE2* mutations have also been identified within the affected tissue of patients with spontaneous VMs [150].

Interestingly, lesions from sporadic VMs tend to have more strongly activating *TIE2* mutations, indicating that these particular mutations may be incompatible with embryonic development, and thus, cannot be inherited [150]. The TIE2 receptor is expressed primarily on endothelial cells [151], with some expression detected in haemopoietic stem cells [152]. The two ligands for TIE2, angiopoietin-1 and angiopoietin-2, have mutually antagonistic roles [153,154]. While Tie2 activation by angiopoietin-1 promotes the recruitment of vascular support cells, and thus maturation of the existing vessels [155,156], angiopoietin-2 is largely responsible for facilitating neovascularization in the presence of additional angiogenic factors, such as VEGF [155]. Thus, constitutive activation of this receptor in VMs would be expected to contribute to aberrant vascular development in the affected vessels. Activating somatic mutations of *PIK3CA* have recently been identified from lesions of VMs that lack mutations in *TEK/TIE2* [22]. As described in an earlier section, *PIK3CA*

encodes the catalytic subunit of PI3-kinase and initiates signalling to several pro-angiogenic pathways [23]. Notably, Yoshioka et al determined that endothelial-specific loss of *Pik3ca* in mice resulted in reduced smooth muscle coverage of the blood vessels [24], supporting a role for this protein in endothelial cells for recruiting VSMCs. Genetic studies to uncover the mutations involved in BRBNS development are limited, although some studies suggest that, similar to VMs, activating *TEK/TIE2* mutations may be present [157].

Characterization of the effects of activating *TEK/TIE2* and *PIK3CA* mutations in endothelial cells have revealed that these mutations cause similar changes in downstream signalling pathways [22,158]. To date, *in vitro* studies have reported an up-regulation of Akt signalling and STAT1 phosphorylation with the familial *TEK/TIE2* mutation, p.R849W, and the sporadic mutation, p.L914F [158,159]. Generally, these studies found that the p.L914F mutation had a stronger effect on downstream signalling, and promoted dramatic changes in the expression of many genes relevant to vascular development, suggesting that this mutation may disrupt vascular development to an extent that is incompatible with survival of embryos harbouring germline mutations [158]. Interestingly, secretion of PDGFB, a mesenchymal mitogen, was severely reduced in endothelial cells containing either activating *TEK/TIE2* mutation [158] or three different *PIK3CA* mutations [22]. Together, these findings suggest that reduced PDGFB levels from the endothelial cells in these lesions contribute to the improper recruitment of VSMCs, although this observation has not yet been validated in histological sections of BRBNS lesions. Together, BRBNS and VMs demonstrate the importance for vascular development of proper communication between the endothelium and overlying VSMCs.

Conclusions

The elucidation of the genetic causes of vascular malformations found in inherited syndromes, as well as the identification of specific somatic mutations in both inherited and sporadic forms, has played an invaluable role in understanding normal and pathological blood vessel structure and function. *A priori*, based on the abnormal structure and microanatomy seen in vascular malformations, one might have hypothesized that the genes underlying these phenotypes would have encoded structural proteins of the endothelial or smooth muscle cells, or possibly extracellular matrix proteins. Instead, these vascular malformation genes invariably encode components of the myriad signalling pathways that modulate normal endothelial or smooth muscle cell function. Some of these, such as the TGF- β and phosphatidylinositol 3-kinase signalling pathways, were already well known from years of study in cancer biology, and yet their role in vascular biology and pathobiology was either unknown or underappreciated. Other proteins encoded by some of these genes, such as RNF213 and glomulin, await further functional characterization, but especially in these cases, the genetic discoveries should be credited with the identification of a novel gene/protein/pathway with a critical role in vascular biology and pathology. Further study of these proteins will open up new vistas in vascular pathobiology and suggest novel approaches to therapy.

The CCM gene products were likewise essentially completely unknown prior to their identification as the cause of cerebral cavernous malformations. These scaffold proteins

appear to modulate multiple signalling pathways, and by such biochemical multi-tasking, illustrate the inter-connectivity of these different signalling pathways. The biochemical connections between these pathways might also suggest that at least in some cases, therapies developed for one type of vascular malformation might show some efficacy for others.

The biochemical studies of these proteins have been invaluable in our understanding of the role of the mutant proteins in vascular malformation pathogenesis. Nonetheless, the importance of *in vivo* studies using genetically-engineered animal models cannot be overstated. The biochemical function of the wild-type and mutant proteins can be studied *in vitro* in appropriate cell types, but only in the *in vivo* setting can we fully understand the development of normal vessels, and especially the pathobiology of the vascular malformation. The mouse has led the way in new advances, but the zebrafish and even the nematode, that has no circulatory system, have also provided important information. A particular advantage of the mouse is the ability either to knock out the relevant gene, thereby providing knowledge of the role of the encoded protein in normal (vascular) development, or to “knock-in” specific mutations, affording the ability to distinguish loss-of-function and gain-of-function effects of disease-causing missense mutations, as illustrated in the murine studies of Loeys-Dietz Syndrome. These *in vivo* models also have shown the critical roles that factors other than the original gene mutation play in the pathogenesis of the vascular malformation; thus, only the animal models were capable of elucidating the importance of inflammation (CCM and HHT) and hemodynamic blood flow (HHT) in the development of the respective vascular lesion. We anticipate that these models will continue to uncover new biology and a better understanding of vascular malformations, and provide the best route forward towards scientifically based therapies.

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