

JB Review

The vasohibin family: a novel family for angiogenesis regulation

Received August 28, 2012; accepted September 24, 2012; published online October 25, 2012

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Angiogenesis, a formation of neovessels, is regulated by the local balance between angiogenesis stimulators and inhibitors. A number of such endogenous regulators of angiogenesis have been found in the body. Recently, vasohibin-1 (VASH1) was isolated as a negative feedback regulator of angiogenesis produced by endothelial cells (ECs) and subsequently vasohibin-2 (VASH2) as a homologue of VASH1. It was then explored that VASH1 is expressed in ECs to terminate angiogenesis, whereas VASH2 is expressed in cells other than ECs to promote angiogenesis in the mouse model of angiogenesis. This review will focus on the vasohibin family members, which are novel regulators of angiogenesis.

Keywords: angiogenesis/endothelial cell/SVBP/vasohibin-1/vasohibin-2.

Abbreviations: EC, endothelial cell; FGF-2, fibroblast growth factor 2; KO, knockout; MNC, mononuclear cell; SVBP, small vasohibin-binding protein; VASH1, vasohibin-1; VASH2, vasohibin-2; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor.

The vasculature is primarily composed of luminal endothelial cells (ECs) and surrounding mural cells (smooth muscle cells or pericytes). ECs are multifunctional cells covering the entire luminal surface of all blood vessels. ECs remain G₀ phase of the cell cycle and form an interface between the circulating blood between the lumen and the rest of the vessel wall, and maintain vascular homeostasis. Physiological function of ECs includes the transport of various molecules across the vascular wall, the regulation of the adhesion of leukocytes for extravasation, the manipulation of vascular tonus and the prevention of thrombotic events. However, when stimulated by angiogenic factors, ECs migrate, proliferate and form neovessels for angiogenesis. The initial step of angiogenesis is the extrication of mural cells from endothelial tubes for vascular destabilization. Subsequently, specialized ECs, the so-called tip cells, migrate by extending numerous filopodia, whereas following ECs, the so-called

stalk cells, proliferate causing elongation of the sprouts to form immature tube-like structures. Finally, redifferentiated mural cells affix themselves to the newly formed vessels for vascular restabilization. By this process, ECs stop their proliferation, thus terminating angiogenesis (1).

The body contains a number of endogenous angiogenesis stimulators and inhibitors, and the local balance between them regulates this process of blood vessel formation. Angiogenesis stimulators are mostly growth factors and cytokines including vascular endothelial growth factor (VEGF), whereas angiogenesis inhibitors are variable and include hormones, chemokines, proteins accumulated in the extracellular matrix, proteolytic fragments of various proteins and so forth. In addition, the majority of angiogenesis inhibitors are extrinsic to the vasculature; some are constitutively expressed and act as barriers to prevent the invasion of sprouts, and the others are generated in response to stimuli and counteract this process (2).

Isolation of VASH1 and VASH2

It could be hypothesized that ECs themselves might produce either angiogenesis stimulators or inhibitors as an autoregulatory or a feedback fashion. To test this hypothesis, cDNA microarray analysis was performed to detect VEGF-inducible genes in ECs (3). Among a variety of VEGF-inducible genes, an attention was focused on genes whose functions were undefined. *In vitro* functional assays for angiogenesis were performed, and one gene having anti-angiogenic activity was isolated. This gene was designated as *vasohibin-1* (*VASH1*), and its *in vivo* anti-angiogenic activity was further confirmed (4). The gene for human *VASH1* gene is located on chromosome 14q24.3 and consists of seven exons (Fig. 1). There are two isoforms of human *VASH1*: full-length *VASH1A* and the spliced variant *VASH1B* (Fig. 1). Human *VASH1A* protein is composed of 365 amino acid residues, whereas human *VASH1B* protein is composed of 204 amino acid residues, and this splicing variant maintains anti-angiogenic activity (5, 6).

By searching in the database, one gene homologous to *VASH1* was found and designated as *vasohibin-2* (*VASH2*) (7). The gene for human *VASH2* is located on chromosome 1q32.3. So far, nine exons for the *VASH2* gene have been shown in the database to form multiple transcripts owing to alternative splicing (Fig. 1). The full-length human *VASH2* was found to be expressed in cultured cells, which is composed of 355 amino acid residues (7). The overall homology between full-length human *VASH1* and *VASH2* is 52.5% at the amino acid level.

The phylogenetic tree of vasohibin family proteins reveals that parasite or sea squirt possesses one common

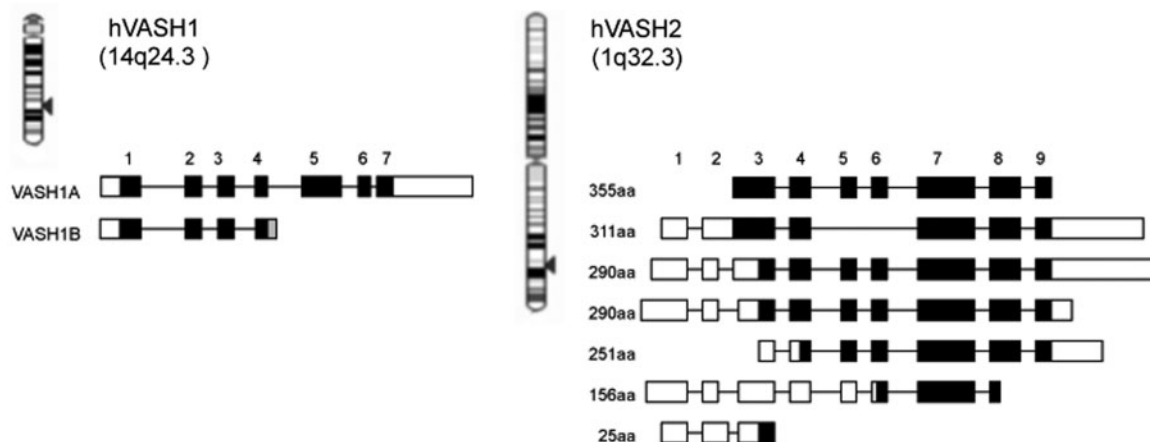


Fig. 1 *VASH1* and *VASH2* genes and their transcripts. Human *VASH1* gene is encoded in 14q24.3, whereas human *VASH2* gene is encoded in 1q32.3. There are multiple transcripts in both human *VASH1* and *VASH2*. Black squares indicate encode proteins.

ancestry vasohibin gene, while vertebrates have *VASH1* and *VASH2* (Fig. 2). The homology between sea squirt vasohibin and human *VASH1* or human *VASH2* is about 40%. Moreover, amino acid sequences of vertebrate *VASH1* and *VASH2* are well conserved. Thus, a common ancestry gene seems to be divided into *VASH1* and *VASH2* during the evolution to vertebrate. No known functional motifs were found in the amino acid sequences in either *VASH1* or *VASH2*. This makes extremely difficult to estimate the functions and compare three-dimensional structures of these two molecules. Instead, the order/disorder orientation of *VASH1* and *VASH2* proteins estimated by Protein Disorder Prediction System (<http://prdos.hgc.jp/cgi-bin/top.cgi>) would provide useful information. The order region defines stable in a three-dimensional composition, whereas the disorder region defines unstable in a three-dimensional composition. In addition, the disorder region is more important for determining the function of proteins. As shown in Fig. 3, *VASH1* and *VASH2* contain disorder regions in both N-terminus and C-terminus ends and order region in the centre. The overall order/disorder probability lines of *VASH1* and *VASH2* are considerably resemble, indicating the correspondence of these two molecules. However, when similarity of order and disorder area was compared, disorder areas are less resemble (Fig. 3). The differences in the disorder regions may indicate the distinctive function of *VASH1* and *VASH2*.

Isolation of Small Vasohibin-Binding Protein

To understand the undefined characteristics of vasohibins, their possible binding partners were searched by using a yeast two-hybrid technique, and one candidate gene was discovered (8). This gene was registered in the database as hypothetical protein LOC374969 or coiled-coil domain containing 23. The binding of this protein to *VASH1* and *VASH2* was confirmed by using the BIAcore system. Because this protein is composed of 66 amino acids, this molecule was renamed as small vasohibin-binding protein (SVBP) (8). The database search revealed that SVBP is highly conserved

between species. The analysis of the function of SVBP revealed that SVBP binds to vasohibins within the cells, makes a heterodimer with vasohibins and facilitates the secretion of vasohibins. The knockdown of SVBP impedes the secretion of vasohibins, and vasohibins remained in the cells are degraded via the proteasome–ubiquitin system (8). Because vasohibins lack classical signal sequence for secretion, it has been obscure whether vasohibins are secreted. The isolation of SVBP verifies vasohibins as secretory proteins, and SVBP acts as a secretory chaperone of vasohibins.

Expression and Function of *VASH1* and *VASH2*

As one can see from its discovery, the expression of *VASH1* in ECs is inducible. The VEGF receptor (VEGFR)-induced expression of *VASH1* in ECs is mediated via VEGFR2 and its downstream PKC δ (5). *VASH1* is induced not only by VEGF but also by fibroblast growth factor 2, another potent angiogenic factor (4, 5), and this induction is also mediated by PKC δ (5). Accordingly, the principal signalling pathways for the induction of *VASH1* by these two representative angiogenic growth factors considerably overlap. Interestingly, this induction of *VASH1* in ECs disappears under a hypoxic condition or in the presence of inflammatory cytokines, tumour necrosis factor- α and interleukin-1 (5).

In contrast, the expression of *VASH2* seems to be not inducible but constitutive as it is not modulated by growth factors and cytokines. In connection with this expression pattern of *VASH2*, recently, *VASH2* is found as the target of mir-200 (9). There is a cluster of miR-200bc/429 binding sites in the 3' untranslated region of *VASH2* mRNA, and mir-200b silences the expression of *VASH2*. Thus, the expression of *VASH2* is augmented when the expression of mir-200b declines (9).

Large collections of microarray data contain information about concerted changes in transcript levels in the datasets beyond the original purpose of each dataset, and the co-expressed gene database (<http://>

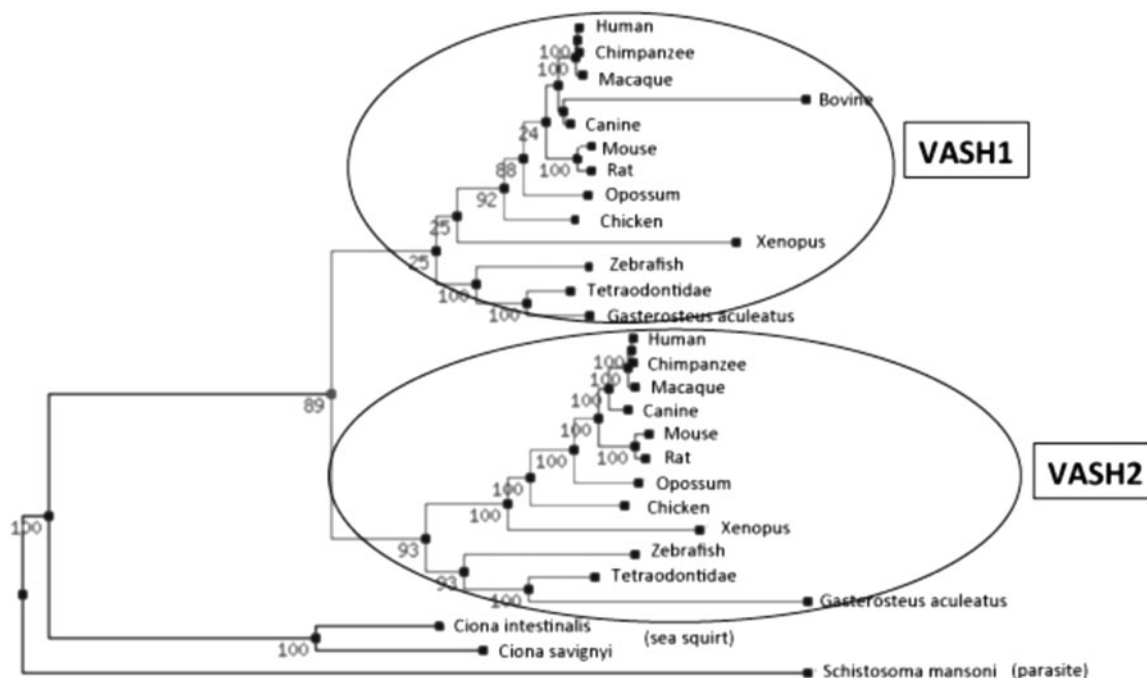


Fig. 2 The phylogenetic tree of vasohibin family proteins. Parasite and sea squirt have one vasohibin ancestry gene, whereas vertebrates have *VASH1* and *VASH2*. A common ancestry gene seems to be divided into *VASH1* and *VASH2* during the evolution to vertebrate.

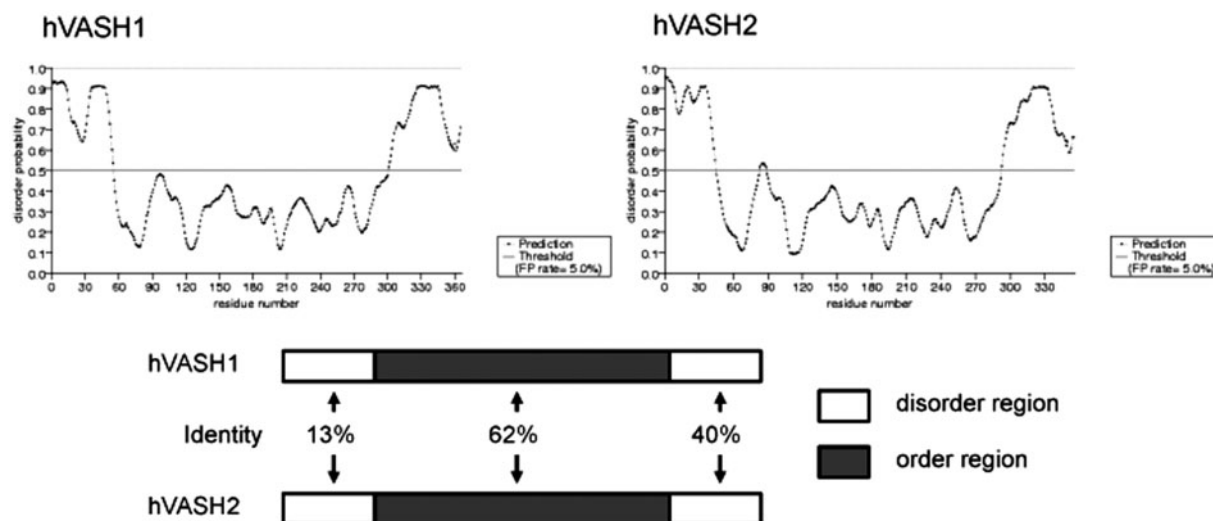


Fig. 3 Order/disorder configuration of human *VASH1* and *VASH2* proteins. Order/disorder probability lines of human *VASH1* and human *VASH2* are shown on the top. Area above the line of disorder probability 0.5 is regarded as disorder region. Similarities of order and disorder regions are shown at the bottom.

coexpressdb.jp/) using those datasets will determine the co-expressed genes with *VASH1* and *VASH2* are compared by the use of this database, and the results revealed that co-expressed genes with *VASH1* and *VASH2* are completely different (Fig. 4). This information may suggest that the cells expressing *VASH1* and *VASH2* are different.

To disclose the precise relationship of *VASH1* and *VASH2*, their spatiotemporal expression and function during angiogenesis were examined (10). The analysis using the mouse subcutaneous angiogenesis model revealed that *VASH1* is expressed not in ECs at the sprouting front but in newly formed blood vessels

behind the sprouting front where angiogenesis terminates. In contrast, *VASH2* is expressed preferentially in mononuclear cells (MNCs) that are mobilized from the bone marrow and infiltrate the sprouting front. *VASH1* and *VASH2* knockout (KO) mice were generated and used in this analysis. *VASH1* KO mice contain numerous immature microvessels in the area where angiogenesis should be terminated. This phenotype was gene dosage sensitive, as more enhanced angiogenesis was observed in *VASH1* ($-/-$) mice (10). Importantly, newly formed immature microvessels in *VASH1* ($-/-$) mice are functional, as indicated by blood flow (10). In contrast, *VASH2* KO mice

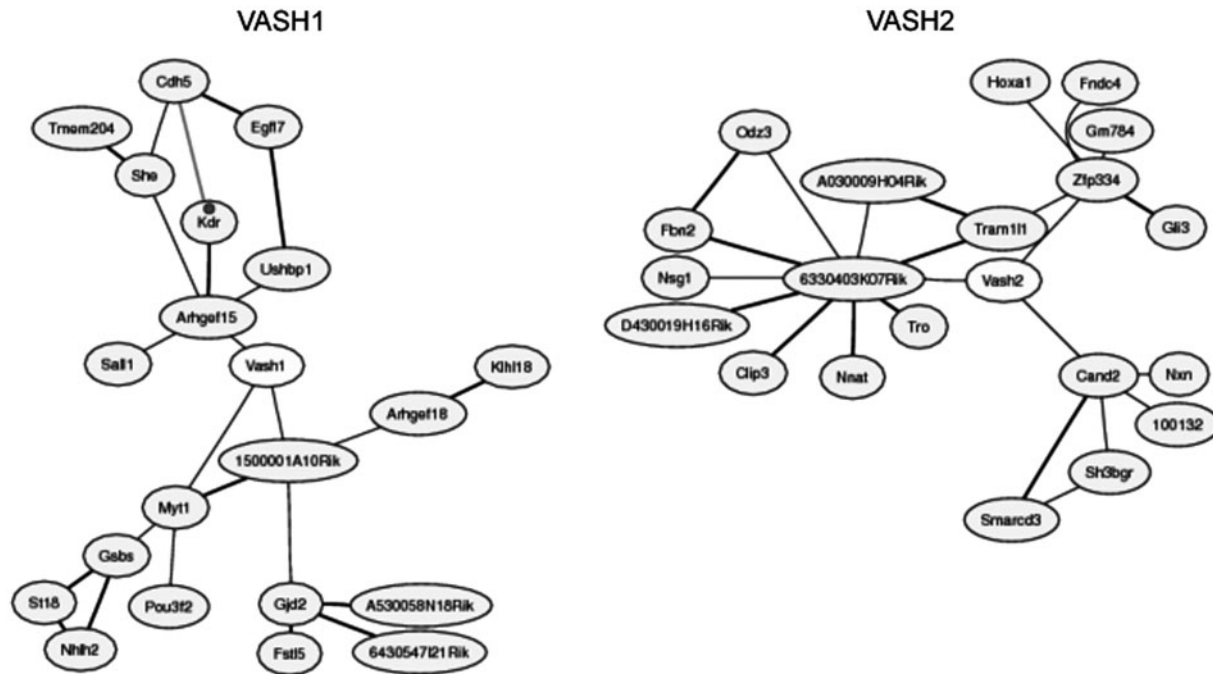


Fig. 4 Co-expressed genes with VASH1 and VASH2. Co-expressed genes with VASH1 are shown on the left and those with VASH2 are on the right.

contain less neovessels in the sprouting front of angiogenesis. This phenotype was also gene dosage sensitive, as more impaired angiogenesis was observed in *VASH2* ($-/-$) mice (10). Importantly, the infiltration of MNCs in *VASH2* ($-/-$) mice is identical to that of wild-type mice (10). As described earlier, the expression of VASH1 is low in proliferating ECs at the sprouting front but high in non-proliferating ECs (10). In addition, angiogenesis in the *VASH2* KO mice was deficient at the sprouting front (10). These results indicate that VASH1 is expressed in ECs in the termination zone of angiogenesis to terminate angiogenesis, whereas VASH2 is mainly expressed in MNCs in the sprouting front and promotes angiogenesis (Fig. 5).

VASH1 and VASH2 in Pathophysiology

Immunohistochemical analysis has revealed that VASH1 protein is present in ECs in the developing human or mouse embryo, but it is reduced in expression in the post-neonate (7). Nimmagadda *et al.* independently showed by *in situ* hybridization that VASH1 mRNA is expressed in a wide range of tissues and organs in the chicken embryo and suggested that the expression of VASH1 might not be limited to ECs (11). Indeed, VASH1 mRNA was detected in bone marrow hematopoietic stem cells (12) and striated muscles (13). Although the significance of these expression is not clear (14), immunohistochemical analysis preferentially detects VASH1 protein in ECs at the site of angiogenesis (4, 7). The expression of VASH1 was further investigated under various conditions accompanied by pathological angiogenesis and a related condition. The presence of VASH1 in ECs is evident in various cancers, atherosclerotic lesions, age-dependent macular

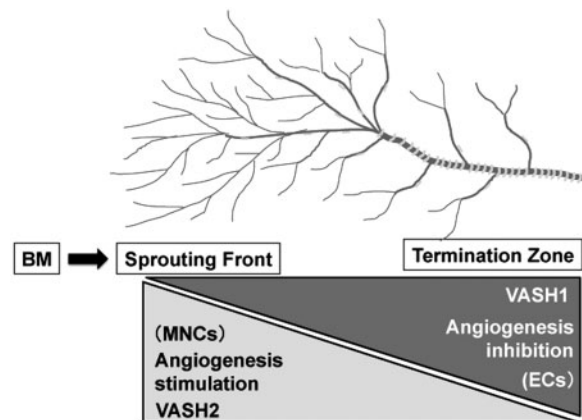


Fig. 5 Spatial expression and function of VASH1 and VASH2 in the regulation of angiogenesis. VASH1 is mainly expressed in ECs at the termination zone and halts angiogenesis. In contrast, VASH2 is mainly expressed in MNCs at the sprouting front mobilized from bone marrow (BM) and promotes angiogenesis.

degeneration, diabetic retinopathy, rheumatoid arthritis and arterial re-endothelialization after denudation (15–27). Even under pathological conditions, the extent of angiogenesis may vary. Tumours inoculated into *VASH1* ($-/-$) mice contain numerous immature vessels, resulting in a growth advantage of the tumours (20). These observations suggest that VASH1 should regulate the course of angiogenesis under pathological conditions as well. Interestingly, Lu *et al.* recently reported that EZH2 silenced the VASH1 expression in ovarian cancers and that made the prognosis of ovarian cancers worse (28).

The initial analysis on VASH2 revealed that VASH2 protein was also present in ECs in the developing human or mouse embryo and faded in the

post-neonate (7). However, when the expression of VASH2 was examined in postnatal angiogenesis, it was shown not in ECs but in infiltrating MNCs (10). The analysis was extended to pathological conditions including cancers thereafter and showed the expression of VASH2 in cancer cells of gastric cancer (29), hepatocellular carcinoma (30) and ovarian serous adenocarcinoma (9). This increased expression of VASH2 in cancer cells can be mediated by modulating the methylation of its promoter region (27) or the decrease of mir-200b (9). Importantly, the knockdown of VASH2 expression in cancer cells prominently inhibited both tumour growth and tumour angiogenesis (9, 27). These results indicate that VASH2 is expressed in cancer cells and promotes tumour growth by stimulating angiogenesis.

Therapeutic Application of Vasohibin

Because of the anti-angiogenic potential, VASH1 can be applied to treat diseases associated with angiogenesis. Its efficacy in anti-angiogenic treatment has been reported in animal models of cancers, ocular (retinal or choroidal) angiogenesis, arterial intimal stenosis, diabetic nephropathy and pulmonary diseases such as pulmonary fibrosis and bronchiolitis obliterans (4, 14, 19, 31–41).

Anti-angiogenic drugs that are now available in the clinics target VEGF-mediated signals. Those drugs are used for the treatment of cancers and age-related macular degeneration, but have some problems. Because VEGF acts as survival factor of ECs, such drugs cause cardiovascular toxicities including hypertension and proteinuria due to the impairment of normal ECs (42). However, VASH1 does not cause hypertension and proteinuria (43). Another problem is drug resistance due to the switch from VEGF to other angiogenic stimulators (44). As VASH1 inhibits not only VEGF-induced angiogenesis but also angiogenesis induced by various angiogenesis stimulators (34), VASH1 can be an alternative option for the treatment of pathological angiogenesis.

Because of the broad-spectrum anti-angiogenic activity of VASH1, the possible effect of VASH1 on lymphangiogenesis was examined (34). Peripheral lymphatic vessels are composed of a single layer of lymphatic ECs without mural cell coverage, and their function is to collect fluid lost from blood vessels and to maintain immune responses, lipid uptake and tissue homeostasis. Recently, attention has focused on lymphangiogenesis, because it has been shown to be related to lymph node (LN) metastasis of cancers. Angiogenesis is counter balanced by various endogenous inhibitors. However, little is known about endogenous inhibitors of lymphangiogenesis. The effect of VASH1 in the corneal micropocket assay was carefully examined, and it revealed broad-spectrum anti-angiogenic and anti-lymphangiogenic activities of VASH1 (34). In addition, VASH1 inhibited tumour lymphangiogenesis and tumour LN metastasis (34). Accordingly, VASH1 is the first molecule intrinsic to the endothelium that exhibits anti-lymphangiogenic activities.

Concluding Remarks

This study has focused on the VASH family members, VASH1 and VASH2. VASH1 and VASH2 are highly conserved between species, and their roles in the regulation of angiogenesis are distinct and perhaps contradictory. VASH1 is mainly expressed in ECs for the termination of angiogenesis, whereas VASH2 is expressed in infiltrating MNCs or cancer cells to stimulate angiogenesis. It has been recently described that VASH1 induces prolyl hydroxylase-mediated degradation of hypoxia-inducible factor-1 α in human umbilical vein ECs (45). However, its significance on the effect of VASH1 is unclear. Apparently, further studies including their receptor and its downstream signalling are required to disclose the entire function of these unique family proteins.

Funding

This author is supported by a grant from the program Grant-in-Aid for Scientific Research on Priority Areas from the Japanese Ministry of Education, Science, Sports and Culture; Health and Labour Sciences research grants (22112006); Third Term Comprehensive Control Research for Cancer (H22-3-008); Japanese Ministry of Health, Labour, and Welfare (22300323).

Conflict of interest

None declared.

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