International).

Generation of VASH1 and VASH2 knockout mice

For the construction of the *VASH1* targeting vector, a 7.6 kb genomic fragment upstream of exon 1 and a 2.4 kb genomic fragment downstream of exon 2 were subcloned. The region was designed such that the short homology arm (SA) extends 2.4 kb to 5' end of loxP/FRT flanked Neo cassette, and the long homology arm (LA) starts at the 3' side of loxP/FRT flanked Neo cassette. For the construction of the *VASH2* targeting vector, a 7.8 kb genomic fragment upstream of exon 3 and a 2.4 kb genomic fragment downstream of exon 3 were subcloned. The region was designed such that the SA extended 2.4 kb to the 5' side of the Neo cassette, and the LA started at the 5' side of LacZ cassette. The loxP/FRT flanked Neo cassette replaced 6.6 kb of the *VASH1* gene including exon 1 (including the ATG start codon), whereas exon 3 of the *VASH2* gene was replaced with the LacZ/Neo cassette.

Targeted alleles were generated by homologous recombination in embryonic stem (ES) cells of C57BL/6 background. The ES cells carrying each of the targeted alleles were injected to C57BL/6 mouse blastocysts to produce chimeric mice. Chimeras were mated with C57BL/6 females to obtain F1 mice carrying each of the targeted alleles.

Immunohistological analysis

For immunohistochemical analysis of the skin flap, specimens were frozen in OCT compound (Sakura, Tokyo, Japan), sliced into 10 μm sections, and fixed in methanol for 20 min at -20°C. Primary antibody reactions were performed at a dilution of 1:200 for CD31 (rat anti-mouse CD31 mAb, Research Diagnosis Inc., Flanders, NJ, USA), alpha smooth muscle actin (αSMA) (mouse anti-mouse αSMA mAb, SIGMA, St. Louis, MO, USA), lymphatic vessel endothelial hyaluronate receptor 1 (LYVE-1) (rabbit anti-mouse LYVE-1 polyclonal Ab, Acris antibodies, Himmelreich, Germany), PCNA (mouse anti-mouse PCNA mAb, Santa Cruz Biotechnology, Santa Cruz, CA, USA), CD11b (goat anti-mouse CD11b polyclonal Ab, Santa Cruz Biotechnology) and macrophage specific antigen F4/80 (rat anti-mouse F4/80 mAb, Acris antibodies) and at 1:400 for mouse VASH1 (rabbit anti-mouse VASH1 polyclonal Ab)[16] and VASH2 (rabbit anti-mouse VASH 2 polyclonal Ab)[16] overnight at 4°C. Specificities of anti-mouse VASH1 and anti-mouse VASH2 antibodies have been shown previously.[16] Secondary antibody reactions were performed at a dilution of 1:1,500 of the appropriate Alexa 488, Alexa 568, or Alexa 594 conjugated donkey secondary Abs (Molecular Probes, Eugene, OR, USA) for 1 hour at room temperature. The vascular luminal area was calculated from 5 different high power fields.

For whole mount immunohistochemical analysis of ear skin, the ear skin was prepared according to the method described by Oike et al.[19] Briefly, ear skin was fixed with 4% paraformaldehyde (PFA) in PBS for 2 hours, permeabilized with methanol, and blocked in 5% sheep serum in 0.3% Triton X-100 (SIGMA) in PBS. Primary antibodies and secondary antibodies were incubated overnight at 4°C.

All the samples were analyzed using a confocal fluorescence microscope (Olympus, Tokyo, Japan). Objective lens: UPLSAP x10 or x40. Temperature: room temperature. Fluorochromes: Alexa488, Alexa 594, FITC or GFP. Software: OLYMPUS FLUOVIEW (FV1000).

Scanning electron microscopy (SEM)

Ear skin was obtained and fixed with 3% glutaraldehyde in 0.1 mol/L phosphate buffer (pH 7.4). The method of SEM observation was as previously reported.[20] In brief, the specimens were treated with 1-2% Sodium Hypochlorite solution for 45-100 seconds, hydrolyzed with 8N HCl for 30 minutes, postfixed with 1% OsO_4, treated with 1% Tanninc acid solution and again with 1% OsO_4. After a brief rinse, the specimens were dehydrated through a graded series of ethanol, immersed in t-Butyl Alcohol, freeze-dried, coated with platinum, and observed with an SEM (Hitachi S-800, Tokyo, Japan).

The detection of vessel perfusion

For the detection of blood vessels with perfusion, mice were infused with FITC labeled concanavalin A (SIGMA) via intracardiac injection prior to the collection of skin flap. Thereafter, samples were analyzed using a confocal fluorescence microscope (Olympus).

Bone marrow transplantation

Wild-type mice were lethally irradiated with 1 dose of 9 Gy. Thereafter, bone marrow cells harvested from GFP-mice (generous gift from Dr. Okabe, Osaka University) that had been purified by density centrifugation (Ficoll-paque PLUS; Amersham Biosciences, Uppsala, Sweden), were transplanted into the wild-type mice (5×10^6 cells/animal). All the recipient mice were given a minimum of 6 weeks rest to allow for complete bone marrow reconstitution. The engraftment efficiency was determined by fluorescent-activated cell sorting (FACS: FACS Vantage, Becton Dickinson, San Jose, CA, USA) for GFP expression in the bone marrow of mice receiving transplants of GFP positive bone marrow cells.

Cells

Human umbilical vein endothelial cells (HUVECs) were obtained from KURABO industries Ltd. (Osaka, Japan) and were cultured on type-1 collagen coated dishes (IWAKI, Chiba, Japan) in 10% FBS/EBM (Clonetics, Walkersville, MD, USA). THP-1, a human monocytic cell leukemia cell line was obtained from the Cell Resource Center for Biomedical Research at our institute. THP-1 cells were cultured in 10% FBS/RPMI 1640 (Nissui Pharmaceutical Co., Tokyo, Japan). GM7373, a chemically immortalized bovine aortic endothelial cell line, was cultured in Dulbecco's modified Eagle medium (DMEM) (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% FCS. MS1, an immortalized cell line with a SV40 large T antigen from mouse pancreatic ECs, was purchased from American Type Culture Collection (Manassas, VA, USA), and were cultured in αMEM (Invitrogen, Carlsbad, CA, USA) supplemented with 10% FCS.

All the cells were cultured at 37°C in a humidified atmosphere with 5% CO_2.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) Analysis

Total RNA was extracted from HUVEC and THP-1 cells by the AGPC method using ISOGEN (Nippon Gene, Toyama, Japan) according to the manufacturer's instructions. Total tissue RNA was extracted from several organs of male BALB/c mice at 4 weeks or from placentas of female BALB/c mice by the AGPC method using ISOGEN-LS

(Nippon Gene). First-strand cDNA was generated using a first-strand cDNA synthesis kit for RT-PCR (Roche Diagnostics Corp., Mannheim, Germany). RT-PCR was performed using a DNA thermal cycler (Takara, Tokyo, Japan). PCR conditions consisted of an initial denaturation step at 95°C for 10 min followed by 40 cycles consisting of 15 s at 95°C, 5 s at an annealing temperature (as described below), and 15 s at 72°C. The primer pairs used were: mouse β-actin forward 5-ACAATGAGCTGCGTGTGGCT and reverse 5-TCTCCTTAATGTCACGCACGA (annealing temperature 58°C); mouse VASH1 forward 5-AGATCCCCATACCGAGTGTG and reverse 5-GGGCCTCTTTGGTCATTTCC (annealing temperature 58°C); mouse VASH2 forward 5'-ATGCCTTGAAGCTGTCATCC and reverse 5' –TGGCATATTTCTCCAGCTCC (annealing temperature 60°C). PCR products were analyzed by 1% or 1.5% agarose gel electrophoresis.

Quantitative real time RT-RCR

Total RNA was extracted from the skin flap using the RNeasy mini kit (QIAGEN) according to the manufacturer's instructions. First-strand cDNA was generated using a first-strand cDNA synthesis kit for RT-PCR (Roche Diagnostics Corp.). Quantitative real time RT-PCR was performed using a Light Cycler System (Roche Diagnostics

Corp.) according to the manufacturer's instructions. The amount of PCR product was measured as a fluorescence signal that was proportional to the amount of the specific target sequence present. PCR conditions consisted of an initial denaturation step at 95°C for 10 min, followed by 40 cycles consisting of 15 s at 95°C, 5 s at an annealing temperature (as described below) and 15 s at 72°C. The primer pairs used were: β-actin forward 5-TCGTGCGTGACATCAAAGAG and reverse 5-TGGACAGTGAGGCCAGGATG; mouse VASH1 forward 5-GATTCCCATACCAAGTGTGCC and reverse 5-ATGTGGCGGAAGTAGTTCCC (annealing temperature 62°C); mouse VASH2 forward 5'-GGCTAAGCCTTCAATTCCCC and reverse 5' CCCATTGGTGAGATAGATGCC (annealing temperature 64°C). Each mRNA level was measured as a fluorescent signal corrected according to the signal for β-actin.

Northern blot analysis

Northern blotting was performed as described.[11] Briefly, cells were starved in 0.1 % FBS/αMEM. In some experiments, cells were stimulated with VEGF (SIGMA) for 12 hours. Total RNA was then extracted by ISOGEN according to the manufacturer's instruction, and was separated on a 1% agarose gel containing 2.2 M formaldehyde and transferred to a Hybond N^+ membrane. The membrane was hybridized with a

^{32}P-labeled VASH1 cDNA probe containing an open reading frame (464-1417). Autoradiography was carried out on an imaging plate and analyzed with an FLA2000 (Fuji Film, Tokyo, Japan).

Intracellular localization of VASH1 and VASH2

To make a hemaglutinin (HA)-tagged construct, human VASH2 cDNA was cloned into the EcoR1-Xho1 site of internal ribosome entry site (IRES)-humanized Renilla green fluorescent protein (hrGFP) 2a vector (Stratagene, La Jolla, CA) (VASH2: HA-IRES-GFP vector). To make GFP-fusion constructs, human VASH2 cDNA was cloned into CT-GFP TOPO vector (VASH2: CT-GFP vector) or NT-GFP TOPO vector (VASH2: NT-GFP vector) (Invitrogen). Transfection was performed using Fugene 6 (Roche) according to the manufacturer's instructions. GEF-fusion protein was detected by the use of confocal microscopy.

Establishment of VASH1or VASH2 expressing MS1 clones

To improve the activity of transcription, the CMV promoter of the pcDNA3.1/Hygro plasmid (Invitrogen) was replaced with the chicken β-actin promoter derived from pCALL2.[21] This vector, pCALL2- pcDNA3.1/Hygro, was used for the transfection in this study. Human VASH1 or VASH2 cDNA was cloned into the pCALL2-pcDNA3.1/Hygro vector at multiple cloning sites (Xho-I and Not-I). MS1 was

transfected with the expression vector by using Effectene transfection reagent (QIAGEN) according to the manufacturer's protocol. After the transfection, the cells were selected by hygromycin (500 μg/mL) (Invitrogen).

Statistical analysis

The statistical significance of differences was evaluated by unpaired analysis of variances (ANOVA), and probability values were calculated with the Student's t test. A value of $p<0.05$ was considered statistically significant.

RESULTS

A mouse model of postnatal subcutaneous angiogenesis

To explore the roles of VASH family in the regulation of angiogenesis, we employed a mouse model of postnatal subcutaneous angiogenesis. In this model, 2 parallel skin incisions were made on the back which penetrated the cutis, dermis, and underlying adipose tissue, a silicon sheet was inserted beneath the skin flap, and the incision closed (Supplemental figure 1A). The skin flap became hypoxic because the inserted silicon sheet blocked the blood supply from the deeper layer, and new vessels were distributed from the areas left undissected. Because of the substantial length of the skin incision, the central region became necrotic over the experimental period (Supplemental figure 1B). After 7 days, the skin flap was harvested and oriented parallel to the original incision for sectioning (Supplemental figure 2A). Pimonidazol staining revealed that hypoxia was evident at the edge adjoining the necrotic tissue (Supplemental figure 2B).

We evaluated the skin flap every 2 mm interval from the necrotic edge and determined the vessel distribution in each area (Fig. 1A). In this way, the entire progress of angiogenesis could be observed from a single section. The area 0-4 mm from the necrotic edge contained mostly small vessels with PCNA positive proliferating ECs (Fig. 1A). This area was thus defined as the sprouting front of angiogenesis. The elevation of

vascular luminal area and vascular numbers was most prevalent in the area 2-4 mm from the necrotic edge (Fig. 1B). The area further than 4 mm from the necrotic edge contained hierarchical vasculature of small and large vessels with PCNA negative non-proliferating ECs surrounded by αSMA positive mural cells (Fig. 1A). This area was thus defined as the termination zone of angiogenesis. Vascular luminal area and vascular numbers were decreased in this area (Fig. 1B).

The spatio-temporal expression profile of VASH1 and VASH2 during angiogenesis

During the postnatal period, the expression of VASH1 and VASH2 proteins is only faintly shown in arterial ECs under the basal condition.[16] Here we determined VASH2 mRNA in various organs in mice (Supplemental figure 3). This restricted expression pattern of VASH2 was comparable to that of VASH1[11], and further confirmed that basal expression of these 2 proteins is quite low. However, we have already reported that VASH1 is selectively present in ECs at the site of angiogenesis.[11,14,15]

Here we determined their expression during angiogenesis more precisely. Immunostaining of VASH1 protein showed that PCNA-positive ECs in small vessels at the sprouting front were faintly stained, whereas PCNA-negative ECs surrounded by mural cells in the termination zone were intensely stained (Fig. 2A). Quantitative real

time RT-PCR further confirmed that VASH1 mRNA was lowest in the area 0-2 mm from the necrotic edge, increased and was most prevalent in the area of 4-6 mm from the necrotic edge, and then declined thereafter (Fig. 2B). As VASH1 was identified as a VEFG-inducible molecule,[11] we initially thought that ECs in the sprouting front expressed VASH1 abundantly. However, it was not the case. We therefore reevaluated the expression pattern of VASH1 by using cultured HUVECs. What we found was that the expression level of VASH1 was dependent on the culture conditions. The basal expression of VASH1 in exponentially proliferating HUVECs was extremely low, increased in subconfluent to confluent cultures, although VEGF-inducibility was maintained (Fig. 2C). Thus, the expression profile of VASH1 in the skin flap correlated with that we found in the culture condition.

We then determined the expression of VASH2 in this system. We noted that VASH2 protein was preferentially localized in infiltrating MNCs in the area 0-2 mm from the necrotic edge (Fig. 3A). These MNCs were CD11b positive but F4/80 negative (Fig. 3A). Although we showed the specificity of our antibodies previously[16], the specificity was further confirmed, as VASH1 was negative in MNCs in the sprouting front (Fig. 2A) whereas VASH2 was negligible in ECs in the termination zone (Supplemental figure 4). Quantitative real time RT-PCR further confirmed that VASH2

mRNA was highest in the area 0-2 mm from the necrotic edge (Fig. 3B). We could also show that monocytic TPH-1 cells expressed VASH2 mRNA more abundantly than HUVECs (Fig. 3C).

It has been well accepted that bone marrow-derived cells contribute to angiogenesis.[22] We therefore hypothesized that these MNCs were derived from bone marrow. To prove this, we lethally irradiated wild-type mice and transplanted them with bone marrow cells from mice ubiquitously expressing green fluorescent protein (GFP-mice). After confirming bone marrow reconstitution, we performed the subcutaneous angiogenesis analysis. We observed that most of the VASH2 positive MNCs were positive for GFP (Fig. 3D, arrow heads). We could hardly detect GFP-positive cells integrated in the wall of neo-vessels.

These results indicate that VASH1 is expressed by ECs in the termination zone, while VASH2 is mainly expressed by bone marrow-derived MNCs infiltrating the sprouting front.

Effects of exogenous VASH1 or VASH2 on angiogenesis

To evaluate the effect of exogenous VASH1 or VASH2, we injected AdVASH1 or AdVASH2 into the tail vein of mice to cause expression of these genes in the liver. We

confirmed the expression of VASH1 or VASH2 in the liver (data not shown). As described previously, this procedure supplied sufficient proteins to regulate angiogenesis in the remote site.[15] Adenovirus-mediated transfer of the VASH1 gene inhibited angiogenesis at the sprouting front where endogenous VASH1 was scarce, but did not influence vascularity in the termination zone where endogenous VASH1 was enriched (Fig. 4A and 4B). Adenoviral-mediated transfer of the VASH2 gene did not cause any changes in the sprouting front, but sustained the increased vascularity in the termination zone (Fig. 4A and 4B). These opposing effects of VASH1 and VASH2 were further confirmed *in vitro* by the stable transfection of the VASH1 or VASH2 gene into cultured ECs (Supplemental figure 5). Both VASH1 and VASH2 lack classical signal sequences.[11,16] We have previously shown that VASH1 is an endoplasmic reticulum (ER)-independent secretory protein. To compare the intracellular localization of VAS1 and VASH2 proteins, we constructed the GFP-fused human VASH2 expression vectors and transfected them into GM7373 cells. We simultaneously transfected human VASH1 gene by the use of AdVASH1. As shown in Supplemental Figure 6, the intracellular localization of VASH1 and VASH2 was comparable.

In consequence, exogenous VASH1 inhibits angiogenesis at the sprouting front, whereas exogenous VASH2 prolongs angiogenesis in the termination zone.

Function of endogenous VASH1 or VASH2 on angiogenesis

To further clarify the function of endogenous VASH1 and VASH2, we generated *VASH1* or *VASH2* knockout mice by conventional homologous recombination (Fig. 5A). We examined the steady-state vascular architecture of ear subcutis of survived mice. We could not find any significant changes of vascular architecture in either *VASH1* knockout or *VASH2* knockout mice (Fig. 5B).

We then subjected the *VASH1* knockout and *VASH2* knockout mice to the model of subcutaneous angiogenesis. The degree of vascular area at the sprouting front was almost identical between wild-type, *VASH1* (+/-) and *VASH1* (-/-) mice (Fig. 6B). The vascular area significantly decreased in the termination zone in the wild-type mice where endogenous VASH1 was enriched, but that was maintained in higher degree in *VASH1* knockout mice (Fig. 6A). Quantitative analysis showed that this change was gene-dosage sensitive (Fig. 6B). Lectin staining indicated that new vessels of *VASH1* (-/-) mice were patent and maintained blood flow (Fig. 6C). Supplementation of the deficient proteins by adenoviral-mediated gene transfer normalized the abnormal angiogenesis patterns in *VASH1* knockout (Fig. 6D).

In contrast, the vascular density was lower in entire areas in the *VASH2* knockout mice

(Fig. 7B). This change was gene-dosage sensitive at the sprouting front where endogenous VASH2 should be enriched (Fig. 7A and 7B). Importantly, the extent of MNC infiltration in the sprouting front was not altered in *VASH2* (-/-) mice (Fig. 7C). Again, supplementation of the deficient proteins by adenoviral-mediated gene transfer normalized the abnormal angiogenesis patterns in *VASH2* knockout mice (Fig. 7D).

DISCUSSION

We characterized the roles of the two members of the vasohibin family in the regulation of angiogenesis using a mouse model of subcutaneous angiogenesis. The spatio-temporal expression pattern and substantial effects of these two molecules indicate that VASH1 and VASH2 control the promotion and termination of angiogenesis in a complementary manner.

The expression of endogenous VASH1 was augmented in non-proliferating ECs in the termination zone of postnatal angiogenesis. This VASH1 in the termination zone should be involved in halting angiogenesis, as angiogenesis persisted in the termination zone in *VASH1* knockout mice in a gene-dosage manner. This result also implies that endogenous VASH1 is not a principle regulator limiting the sprouting. However, when applied exogenously, VASH1 can inhibit angiogenesis at the sprouting front where endogenous VASH1 is scarce. In addition, exogenous VASH1 exhibits little effect, if any, in the termination zone where endogenous VASH1 is present. Accordingly, the present results show the distinctive acting points of endogenous and exogenous VASH1 and further support the legitimacy of employing exogenous VASH1 as an anti-angiogenic treatment.

Endogenous VASH2, in contrast, was expressed mainly by infiltrating bone

marrow-derived MNCs at the sprouting front in our model. The present result contrasts with our previous observation regarding VASH2 expression in ECs of developing embryos without obvious MNC infiltration.[16] With regard to the expression of VASH2 in MNCs, monocytic THP-1 cells were found to express VASH2 more abundantly than ECs in culture. Hence, MNCs can be the main source of VASH2 when they infiltrate.

Bone marrow-derived cells including endothelial progenitor cells (EPCs) contribute to postnatal angiogenesis.[22] However, we could hardly detect the integration of bone marrow-derived cells in the neo-vessels in our model, indicating that EPCs might not play a major role in our model. What we observed was that most of bone marrow-derived cells infiltrated in the sprouting front were MNCs. It is described that bone marrow-derived MNCs stimulate angiogenesis by producing angiogenic factors including VEGF and several matrix metalloproteinases (MMPs).[23,24] Along these lines, we propose that VASH2 produced by bone marrow-derived MNCs takes part in the promotion of postnatal angiogenesis, as angiogenesis at the sprouting front is significantly impaired in *VASH2* knockout mice even in the presence of MNC infiltration. Appropriately, exogenous VASH2 inhibited the termination of angiogenesis in the termination zone where endogenous VASH2 staining was faint.

We previously reported that, when applied exogenously, VASH-2 exhibited the

anti-angiogenic activity in the mouse cornea.[16] However, to our surprise, the present study rather indicated the pro-angiogenic activity of VASH2. Amino acid sequence of VASH2 is 52.5% homologous to that of VASH1 in humans, and 51.9% homologous in mice.[16] We therefore hypothesize the role of VASH2 as follows. On the analogy to angiopoietin-1 and angiopoietin-2, VASH1 and VASH2 may share the same receptor. VASH2 is a weak agonist and antagonizes VASH1 in a certain condition, although the receptor for the VASH family is not yet identified. We are currently testing this hypothesis.

There are a number of endogenous angiogenesis inhibitors in the body, but it is still not clear why the body needs so many angiogenesis inhibitors. Systematic analysis to show how these endogenous angiogenesis inhibitors orchestrate the control of angiogenesis is lacking. Delta like 4 (Dll4) is the ligand of Notch1, which determines the arterial specification of ECs.[25,26] However, recent evidence indicates that ECs at the sprouting tip express Dll4, and that this Dll4 negatively regulates the formation of appropriate numbers of sprouting tips.[27-31] Therefore, Dll4 and VASH1 are 2 inhibitors that are expressed in ECs, but the apparent difference between VASH1 and Dll4 is their temporal expression patterns. Dll4 is selectively expressed in tip cells, whereas VASH1 is expressed in ECs in the termination zone. Hence, VASH1 and Dll4 are expressed in

different phases of angiogenesis and should negatively tune this phenomenon distinctively. It is described that inactivation of Dll4 increases sprouting microvessels without proper blood perfusion.[32,33] Importantly, increased number of microvessels in the termination zone of *VASH1* (-/-) mice were patent and maintained blood perfusion. This difference in blood perfusion further proposes the distinctive roles of VASH1 and Dll4 in the regulation of angiogenesis.

In summary, the vasohibin family members, VASH1 and VASH2 participate in the regulation of angiogenesis in a previously unrecognized manner. VASH1 is expressed in ECs in the termination zone to halt angiogenesis, whereas VASH2 is expressed mainly in infiltrating MNCs at the sprouting front to promote angiogenesis. Discovery of these molecules should provide novel approaches to both anti-angiogenic and pro-angiogenic treatments. Further study is currently underway to clarify the underlying mechanism how VASH1 and VASH2 regulate angiogenesis in an opposed but complementary manner.