*J. Biochem.* 2009;145(5)653–659
doi:10.1093/jb/mvp021

# Induction and Expression of Anti-Angiogenic Vasohibins in the Hematopoietic Stem/Progenitor Cell Population

**Hisamichi Naito[1], Hiroyasu Kidoya[1], Yasufumi Sato[2] and Nobuyuki Takakura[1,*]**

*[1]Department of Signal Transduction, Research Institute for Microbial Diseases, Osaka University, 3-1 Yamada-oka, Suita-shi, Osaka 565-0871, Japan; and [2]Department of Vascular Biology, Institute of Development, Aging, and Cancer, Tohoku University, 4-1 Seiryo-machi, Aoba-ku, Sendai 980-8575, Japan*

Received January 8, 2009; accepted January 23, 2009; published online January 29, 2009

**Haematopoiesis and blood vessel formation are closely associated, with several molecules employed by both systems. Recently, vasohibin-1 (VASH1), an endothelium-derived negative feedback regulator of angiogenesis, has been isolated and characterized. VASH1 is induced by VEGF or bFGF in endothelial cells (ECs) and inhibits their proliferation and migration. However, there are no data on the induction and expression of VASH1 in haematopoietic cells (HCs). Here, we show that the haematopoietic stem cell (HSC) population, but not haematopoietic progenitors (HPs) or mature HCs from adult bone marrow (BM) constitutively express VASH1. However, HPs, but not HSCs, can be induced to express VASH1 after BM suppression by 5-FU. Knock-down of the *VASH1* gene in VASH1$^+$ leukaemia cells induced cell proliferation. These results suggest a role for VASH1 in negative feedback regulation of HP proliferation during recovery following BM ablation.**

**Key words: 5-FU, bone marrow ablation, haematopoietic progenitor cells, haematopoietic stem cell, vasohibin.**

Abbreviations: bFGF, basic fibroblast growth factor; BM, bone marrow; EC, endothelial cell; HC, haematopoietic cell; HP, haematopoietic progenitor; HSC, haematopoietic stem cell; VASH1, vasohibin-1; VEGF, vascular endothelial growth factor.

The haematopoietic and vascular systems are closely related in several respects. It has been suggested that haematopoietic cells (HCs) and endothelial cells (ECs) arise from a common progenitor during development, the so-called haemangioblast (*1*) or hemogenic angioblast (*2*), which originates from mesodermal cells. In addition, after the development of haematopoietic stem cells (HSCs) and ECs, the latter supports the differentiation, proliferation and survival of the former, which themselves support angiogenesis (*3–10*). Moreover, it has been reported that erythropoietin, originally identified as a haematopoietic cytokine, also induces proliferation of ECs (*11*), suggesting that there are several factors commonly utilized in vascular development and haematopoiesis.

Recently, a novel anti-angiogenic factor, vasohibin-1 (VASH1), has been isolated from human umbilical vein endothelial cells (HUVECs) (*12*). VASH1 is upregulated by vascular endothelial growth factor (VEGF) in HUVECs and has been suggested to act as a negative feedback regulator of VEGF and basic fibroblast growth factor (bFGF) signalling in HUVECs. VASH1 is widely conserved among species (*13*) and is present in multiple processing forms (*14*), and it has been reported that alternative splicing of the VASH1 pre-mRNA transcript generates a potent anti-angiogenic protein (*15*). However, in mice, alternatively spliced forms of VASH1 have not been isolated. One VASH1 paralogue, termed vasohibin-2 (VASH2), with anti-angiogenic activity in mammals, has also been isolated recently (*16*). VASH1 is upregulated in retina upon stimulation with VEGF and suppresses retinal neovascularization in mice with ischemic retinopathy (*17*). VASH1 expression has been observed in ECs of adventitial microvessels in atherosclerotic lesions, where it inhibits adventitial angiogenesis and neointimal formation after cuff placement on the mouse femoral artery (*18*). Moreover, VASH1 is selectively expressed on vascular EC in both cyclic endometria and endometrial carcinomas and suppresses tumour growth and angiogenesis in a mouse xenograft tumour model (*19*). Taken together, these data suggest that VASH1 is a candidate target molecule for manipulating tumour angiogenesis.

While the unique function of VASH1 as a negative feedback regulator of angiogenesis has been extensively studied, its expression in cell lineages other than ECs has not been documented. On the basis of the hypothesis that the haematopoietic and vascular systems utilize similar molecules, here, we investigated the expression of VASH1 in several fractions of normal HCs in the bone marrow (BM) as well as in leukaemic cells and assessed the mechanisms regulating HC VASH1 expression.

## MATERIALS AND METHODS

*Mice*—C57BL/6 mice were purchased from SLC (Shizuoka, Japan). All animal studies were approved by

*To whom correspondence should be addressed. Tel: +81-6-6879-8316, Fax: +81-6-6879-8314,
E-mail: ntakaku@biken.osaka-u.ac.jp

© The Authors 2009. Published by Oxford University Press on behalf of the Japanese Biochemical Society. All rights reserved.

Downloaded from http://jb.oxfordjournals.org/ at Islamic Azad University on September 28, 2012

the Animal Care Committee of Osaka University. For BM ablation studies, 10-week-old mice were treated with a single-tail vein injection of 5-FU (Kyowa Hakko Kogyo Co., Ltd, Tokyo, Japan; 150 mg/kg body weight). The mouse model of hind limb ischemia was as described previously (*3*).

*Cell Preparation and Flow Cytometry*—Cell preparation from the hind limb, BM and peripheral blood was carried out as previously reported (*3*). The cell-staining procedure for flow cytometry was as described previously (*3*) using anti-CD31, -CD45, -c-kit, -Sca-1 and anti-lineage (a mixture of ter119, Gr-1, Mac-1, B220, CD4 and CD8) monoclonal antibodies (mAbs; all from Pharmingen). All mAbs were purified and conjugated with either FITC or PE (phycoerythrin) or biotin. Biotinylated antibodies were visualized with PE-conjugated streptavidin or APC-conjugated streptavidin (Pharmingen). The stained cells were analysed and sorted by JSAN (Bay Bioscience, Kobe, Japan). For the procedures involving SP cells, Hoechst dye was used as previously described (*20*, *21*). BMMNCs were resuspended at $1 \times 10^6$ cells/ml and incubated with Hoechst 33342 (5 μg/ml) for 90 min at 37°C. Cells were then washed and analysed by JSAN (Bay Bioscience).

*Quantitative Real-Time Reverse Transcription PCR (qRT–PCR) Analysis*—Extraction of total RNA and qRT–PCR was performed as previously reported (*22*). Levels of specific amplified cDNA were normalized to *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) housekeeping gene levels. Primers used in this experiment were as follows: mouse *VASH1* (corresponding to human *VASH1A*, sense 5′-CAT CAG GGA GCT GCA GTA CA-3′, anti-sense 5′-GAT CAC AGC TTC CAG GCA TT-3′), human *VASH1A* (sense 5′-GCT GCA GTA CAA TCA CAC AGG-3′, anti-sense 5′-AGG TAA ATT CCC AGG ATC ACG-3′), human *VASH1B* (sense 5′-AAG CTG TGC AGC GTC ACA TC-3′, anti-sense 5′-ACT TTC AGA GCA GGA AGC TGA-3′) (*15*), mouse *GAPDH* (sense 5′-TGG CAA AGT GGA GAT TGT TGC C-3′, anti-sense 5′-AAG ATG GTG ATG GGC TTC CCG-3′), human *GAPDH* (sense 5′-GAA GGT GAA GGT CGG AGT C-3′ and anti-sense 5′-GAA GAT GGT GAT GGG ATT TC-3′).

*Western Blotting Analysis*—Methods for western blotting were previously described (*22*). Antibodies used in this experiment were anti-VASH1 (*12*) and anti-GAPDH (Chemicon, Temecula, CA).

*Cell Lines*—HUVECs were purchased from Kurabo (Osaka, Japan) and cultured in Humedia EG2 (Kurabo). For the induction analysis of VASH1A, HUVECs were starved for 12 h and stimulated with VEGF-$A_{165}$ (10 ng/ml, PeproTech, Rocky Hill, NJ) for 12 h.

Human leukaemia cell lines (KG1a, HL60, THP-1, MOLT-4, SKW3, BALL-1 and NALM6) as indicated in Fig. 4A were provided by the Riken Bioresource Center (Tsukuba, Japan) and the Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University.

*RNAi and Transfection*—Two Stealth™ RNAi duplexes were synthesized commercially by Invitrogen. Stealth RNAi duplexes with GC content similar to that of each test duplex were used as a negative control. Stealth™ RNAi #1 (5′-UCU GAU AUA GCG CUG CAC AGC UUC C-3′), Stealth™ RNAi #2 and Stealth™ RNAi #2 (5′-UUC CCU GAG AAG UAG GUC UUG AAG C-3′) were designed to target different coding regions of the human *Vash1* mRNA sequence. A BLAST (NCBI database) search was carried out to confirm that the targets of the two Stealth™ RNAi duplexes were exclusively in *Vash1*.

THP1 cells were seeded at $1 \times 10^5$ cells/ml, and transfection was accomplished using lipofectamine 2000 according to the manufacturer's instructions. For selecting transfected cells, each Stealth RNAi duplex was co-transfected with Block-iT Alexa Fluor Red Flurescent Oligo (Invitrogen) and 24 h later, positive cells were sorted by JSAN (Bay Bioscience) and cultured for growth assessment.

*Statistical Analysis*—All data are presented as mean ± SD. For statistical analysis, the statcel2 software package (OMS) was used with analysis of variance (ANOVA) performed on all data followed by Tukey–Kramer multiple comparison testing.

## RESULTS

*Expression of VASH1 in the HSC Population of Adult BM*—Adult bone marrow (BM) cells were fractionated into a $Lin^-c\text{-}Kit^+Sca\text{-}1^+$ HSC-enriched population (KSL cells), a $Lin^-$ haematopoietic progenitor (HP)-enriched population ($Lin^-$ cells) and a differentiated HC ($Lin^+$ cells) population (Fig. 1A). qRT–PCR analysis (Fig. 1C) indicated that KSL cells express *VASH1* at higher levels than either $Lin^-$ or $Lin^+$ cells. To further confirm the high level of *VASH1* expression in the HSC-enriched population, we identified HSCs by their ability to efflux Hoechst 33342 dye. This method defines an extremely small and haematopoietically potent subset of cells known as the side population (SP) (*20*, *21*). As shown in Fig. 1B, ~0.1% of BM cells are in the SP cell fraction, as previously reported (*20*). Treatment with verapamil, an inhibitor of ATP-binding cassette transporter superfamily pumps, resulted in the complete disappearance of this population. qRT–PCR analysis (Fig. 1D) indicated that SP cells express *VASH1* at much higher levels than cells from the main population (MP) or from the S and G2/M stages of the cell cycle. $Lin^+$ cells seemed not to express *VASH1* (Fig. 1C); also lymphocytes, myeloid cells and erythroid cells from BM and mononuclear cells (MNCs) from peripheral blood do not express *VASH1* (Fig. 1F). Although ECs derived from hind limb muscle of adult mice do express *VASH1* at levels 5-fold those of the HSC population in BM (Fig. 1E); nonetheless, we concluded that among HCs, *VASH1* is preferentially expressed in the HSC population and not in HPs or mature HCs in the BM in the steady state. Since it has been reported that VASH1 expression was upregulated upon the stimulation with VEGF or bFGF using HUVECs (*12*), we tried to observe whether the expression of VASH1 is upregulated in freshly isolated ECs from hind limb muscle of adult mice as used in Fig. 1E. Perhaps, by the technical limitation using primary ECs, we could not observe the upregulation of *VASH1* on primary ECs under stimulation with VEGF or bFGF.



Downloaded from http://jb.oxfordjournals.org/ at Islamic Azad University on September 28, 2012

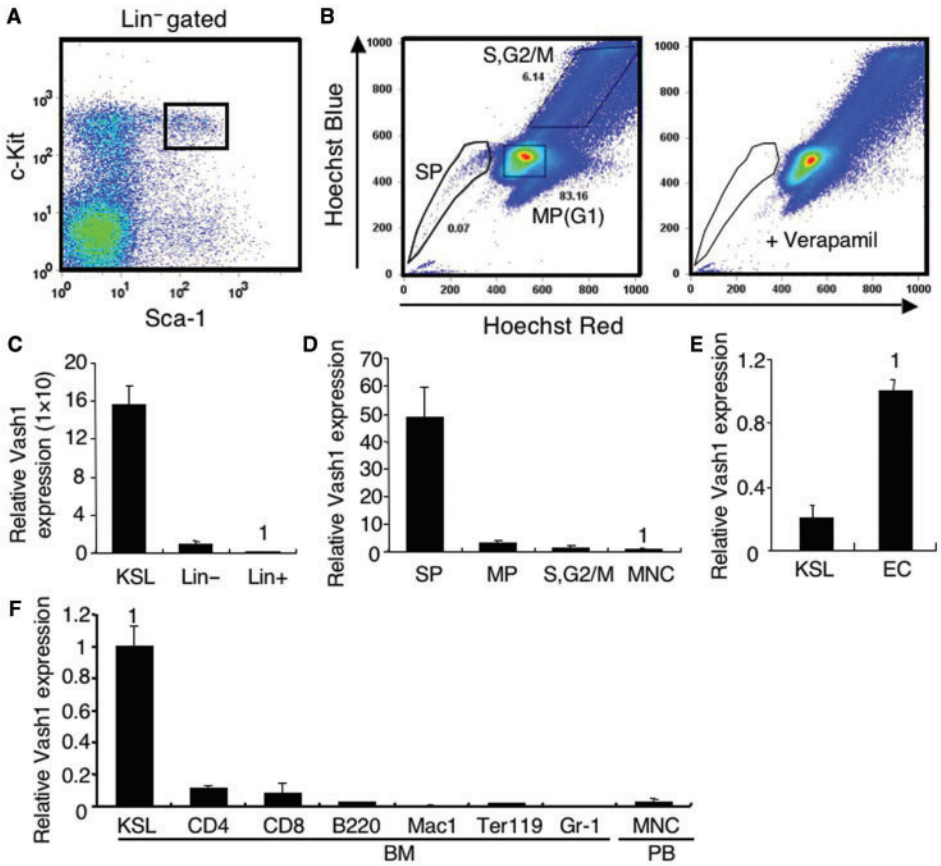


Fig. 1. **VASH1 expression in different haematopoietic lineages.** (A) Flow cytometric analysis of the HSC population. BM cells from 8-week-old mice were tested for the expression of Lin markers, c-Kit, Sca-1. Box indicates HSC population as $Lin^{-}$c-$Kit^{+}Sca\text{-}1^{+}$ cells. (B) Analysis of side population (SP) cells, main population (MP) cells and cells in the S, G2/M phase of the cell cycle from the BM of 8-week-old mice. The identification of SP cells was confirmed by their disappearance in the presence of Verapamil (right panel). (C–F) qRT–PCR for *VASH1* in several cells as indicated. Results (mean ± SD) are from three independently sorted sets of populations. (C) KSL HSC population, $Lin^{-}$ haematopoietic progenitor-enriched population and $Lin^{+}$ mature haematopoietic cells from BM of 8-week-old mice. (D) SP cells, MP cells and cells in S,G2/M from adult BM. Mononuclear cells (MNCs) from whole BM were also used for comparison. (E) Comparison of *VASH1* expression by KSL cells as indicated in (A) and ECs from hind limb muscle of 8-week-old mice defined as $CD31^{+}CD45^{-}$ cells. (F) Comparison of *VASH1* expression in KSL cells with adult BM cells positive for several lineage markers as indicated. MNCs from peripheral blood (PB) from the same mice were also used for comparison.

*Ischaemia Does Not Induce VASH1 in HCs*—In the murine femoral artery occlusion hind limb ischaemia model, the expression of VEGF and bFGF is increased (*23*). It has been reported that VASH1 expression is induced by VEGF or bFGF in ECs (*12*) and that several HCs including the HSC population migrate into ischemic tissue from BM to support angiogenesis (*8*). We reasoned that if regulatory mechanisms for VASH1 expression are similar in ECs and HCs, the latter may also express VASH1 in ischemic tissues. To test this, $CD31^{+}CD45^{-}$ ECs, $CD31^{-}CD45^{+}$ HCs and $CD31^{-}CD45^{-}$ non-EC/non-HCs were isolated from hind limb muscle in the normoxic or hypoxic condition and *VASH1* expression was examined (Fig. 2A). Although we could not succeed to induce *VASH1* expression in the culture of primary ECs as described earlier, we found that hypoxia-induced *VASH1* expression in ECs, but neither in HCs nor non-ECs/non-HCs (Fig. 2B). Moreover, ischaemia in the hind limb did not affect *VASH1* expression by HCs residing in the BM (data not shown). These findings suggested that the regulatory mechanisms controlling *VASH1* expression in HCs and ECs are different.

*VASH1 Expression is Induced in HPs After BM Ablation by 5-FU*—5-FU treatment in mice induces BM ablation as a result of killing the cycling HSCs and HPs. However, surviving HSCs and HPs undergo acute expansion to produce a number of mature HCs. We therefore analysed whether BM suppression with 5-FU affects *VASH1* expression. It is well known that although the HSC population is decreased in mice during the first few days after 5-FU (150 mg/kg) injection, the population of cycling HSCs and HPs increases dramatically 4–6 days thereafter (*24*). We therefore sorted KSL cells, $Lin^{-}$ cells, and $Lin^{+}$ cells from adult BM on day 7 after treatment with 5-FU (Fig. 2C and D). We found that *VASH1* expression in KSL cells and $Lin^{+}$ cells was not affected by 5-FU injection, but that it was now induced in $Lin^{-}$ HPs to a similar extent as present in KSL cells (Fig. 2D). This was also confirmed at the protein level (Fig. 2E).



Downloaded from http://jb.oxfordjournals.org/ at Islamic Azad University on September 28, 2012

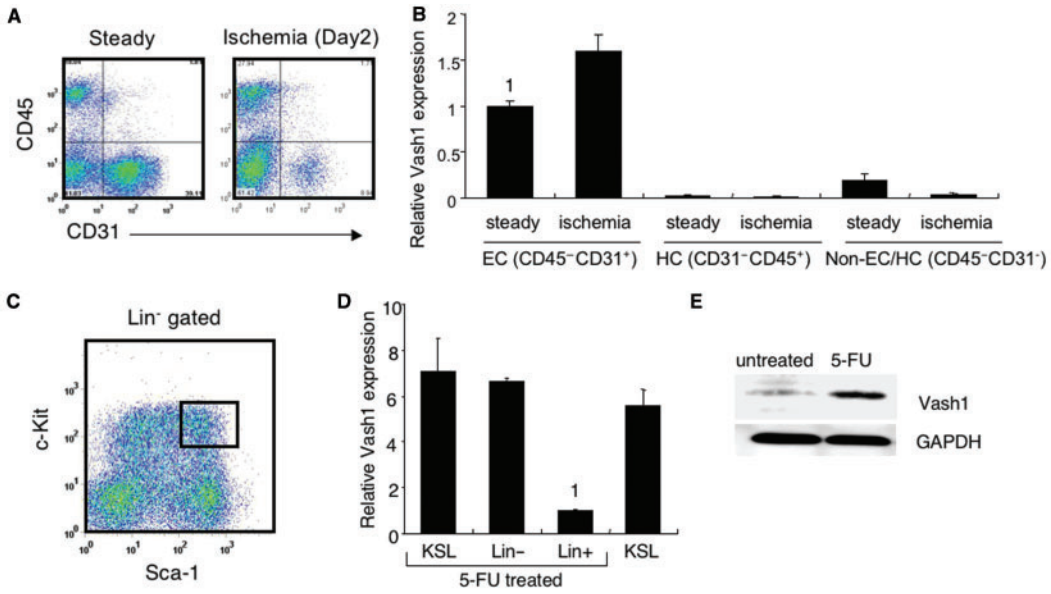


Fig. 2. **VASH1 expression on HCs in tissues under stress.** (A) Flow cytometric analysis of cells from hind limb muscle in the steady state and ischaemic state on the second day after femoral artery ligation. Cells were stained with CD31, an EC marker, and CD45, an HC marker. (B) qRT–PCR for *VASH1* expression in different cells sorted as shown in (A). Results (mean ± SD) are from three independently sorted sets of populations. C: BM cells from 10-week-old mice were isolated 7 days after systemic administration of 5-FU and tested for the expression of Lin markers, c-Kit, Sca-1 by flow cytometry. (D) qRT–PCR for levels of *VASH1* in KSL cells as shown in (C), Lin$^-$ cells, and Lin$^+$ cells. KSL cells before 5-FU treatment are also used for comparison. (E) Western blotting for VASH1 expression. Lin$^-$ haematopoietic progenitors from 10-week-old mice with or without 5-FU treatment were sorted and used for this analysis. GAPDH was used as an internal control.

*VASH1 and VEGFR2 Expression in HPs After BM Ablation by 5-FU*—It has been reported that HSCs and HPs express VEGFR2/Flk1, a receptor for VEGF, and that VEGF induces the expansion of VEGFR2$^+$ HPs (*25*). VEGF is upregulated after BM suppression (*26*). Moreover, knockdown of *VASH1* mRNA suggested that attenuation of VASH1 expression leads to a significant elevation in the level of *VEGFR2* mRNA in ECs (*17*). Therefore, it is possible that upregulated VASH1 in HPs suppresses VEGFR2 expression. Therefore, Lin$^-$ BM HPs in the steady state and after 5-FU treatment were sorted into VEGFR2$^+$ and VEGFR2$^-$ populations (Fig. 3A) and their *VASH1* expression quantified. It was found that VEGFR2$^+$ cells expressed *VASH1* at higher levels than VEGFR2$^-$ cells in the steady state as well as after treatment with 5-FU (Fig. 3B). However, VEGFR2$^-$ cells from 5-FU-treated animals expressed more *VASH1* than those from controls (Fig. 3B). Moreover, cells very strongly positive for VEGFR2, which were present in controls, disappeared after 5-FU treatment (Fig. 3A, box in left panel). This suggests that VASH1 induction in VEGFR2$^{high}$ HPs after 5-FU treatment may attenuate the expression of VEGFR2, which may shift VEGFR2$^{high}$ cells to VEGFR2$^{low/-}$ cells.

*Attenuation of Vash1 Expression Induces Proliferation of Leukaemia Cells*—To seek models for understanding the role of VASH1 induction in HPs after BM ablation, we searched for VASH1-expressing HC lines. Of three human acute myeloblastic leukaemia (AML) cell lines, two (KG1a and THP1) strongly expressed *VASH1A*, but none of four acute lymphoid leukaemia (ALL) cell lines did so (Fig. 4A and B). In this experiment, we used

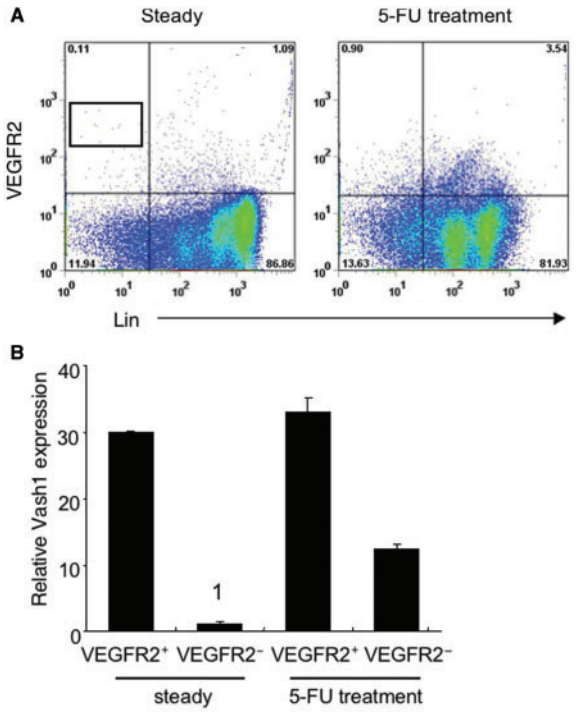


Fig. 3. **VEGFR2 expression by haematopoietic progenitors after treatment with 5-FU.** (A) BM cells from 10-week-old mice before and after (7 days) treatment with 5-FU were stained for lineage markers and antibody against VEGFR2. Note that Lin$^-$VEGFR2$^{high}$ cells indicated by the box disappeared after treatment with 5-FU. (B) qRT–PCR for *VASH1* in different cell populations from adult BM before and after treatment with 5-FU as shown in (A).



Downloaded from http://jb.oxfordjournals.org/ at Islamic Azad University on September 28, 2012

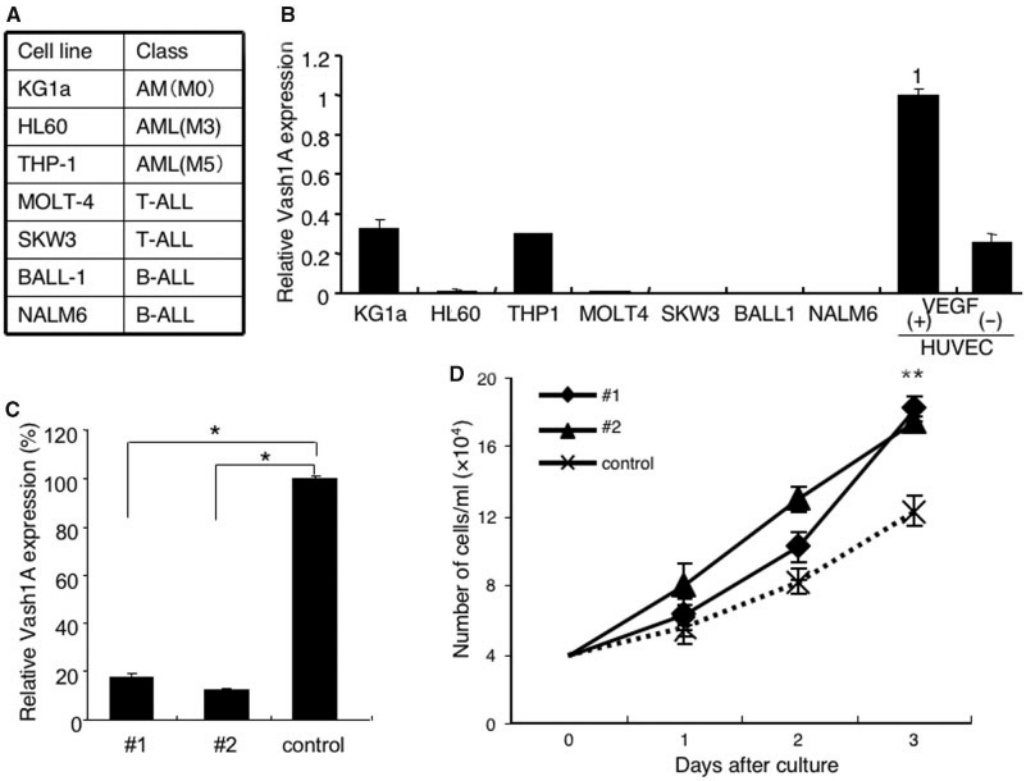


Fig. 4. **Expression of VASH1A in leukaemic cell lines.** (A) Leukaemic cell lines used in this experiment. (B) qRT–PCR for *VASH1A* in different leukaemic cells. HUVECs stimulated with (+) or without (−) VEGF were used as positive controls. (C) qRT-PCR for *VASH1A* after silencing by two RNAis (#1 and #2) or a control RNAi (control). $^{*}P < 0.05$ ($n = 3$, mean ± SD). (D) Cell growth after introduction of RNAi as described in (C). $^{**}P < 0.05$ ($n = 3$, mean ± SD).

HUVECs as a positive control and confirmed that VEGF induces *VASH1A* expression in HUVECs as previously reported (*12*) (Fig. 4B).

As it has been suggested the existence of alternatively splicing short forms of *VASH1A* termed *VASH1B*, we detected *VASH1B* in these two leukaemia cell line (KG1a and THP1) at a similar extent with *VASH1A* (data not shown).

Using RNAi methodology targeting two different cording regions of the human *Vash1* mRNA sequence, we then tested the effect of blocking VASH1 on cell growth. For reasons that remain unclear, this approach was not successful technically with KG1a cells, but *VASH1A* expression in THP1 cells could be greatly attenuated in this way (Fig. 4C). Proliferation of cells in which *VASH1A* had been knocked down was significantly greater than in controls, suggesting that attenuation of VASH1A expression enhances cell proliferation (Fig. 4D).

## DISCUSSION

It has been reported that VASH1 expression is induced in ECs after stimulation with VEGF or bFGF and that this factor then inhibits their proliferation and migration. Therefore, it has been suggested that VASH1 acts as a negative feedback regulator for angiogenesis to inhibit overgrowth of blood vessels. In the BM, SP cells are suggested to be the most immature HSC population that can be serially transplantable into lethally irradiated mice. As most SP cells are dormant and most likely adhere to osteoblasts in the BM (*21*), it is possible that VASH1 inhibits SP cell-cycle progression in such BM niches. To address this, an HSC-specific conditional knock-out of the *VASH1* gene will be required. However, thus far molecules specifically expressed on SP cells have not been well documented, with the exception of ABCG2, an ABC transporter. Therefore, we propose that our present data identifying a novel molecule expressed on SP cells will be useful at least as a marker.

It is of note that VASH1 expression was induced in HPs, but not HSCs, during recovery from BM ablation. It had been considered that surviving HSCs in the BM start to self-renew and subsequently HPs derived from these HSCs acutely proliferate. However, in HSC division, it is not clear whether a single HSC gives rise to two HSC by symmetrical cell division or whether one HSC and one progenitor are produced by asymmetric cell division. Of course, it is possible that both types of cell division occur, but the mechanisms responsible for controlling when HSCs stop dividing remain obscure. If VASH1 has a role in maintaining HSC pool size in the BM by inhibiting cell growth, it would be expected that VASH1 expression should be induced in this population during recovery after BM ablation. However, VASH1 expression in HSCs was not affected by treatment



Downloaded from http://jb.oxfordjournals.org/ at Islamic Azad University on September 28, 2012

with 5-FU, suggesting that their cell division occurs very early after ablation of the BM and stops soon after giving rise to daughter progenitor cells.

During the recovery stage, HPs proliferate acutely; however, their cell division needs to be downregulated again after sufficient mature HCs have been generated. The mechanism responsible for this negative feedback regulation has thus far eluded identification. Our results presented here suggest that VASH1 might be a one of the negative regulators active at the final stage of acute recovery following BM ablation, because knockdown of the *VASH1A* gene-enhanced proliferation of VASH1A$^+$ cells from leukaemic lines.

Currently, it is thought that the expression of VASH1 and VEGFR2 is reciprocally cross-regulated in ECs (*17*). Attenuation of VEGFR2 expression by VASH1 may reduce responsiveness to VEGF, resulting in inhibition of angiogenesis. In the present study, HPs highly expressing VEGFR2 disappeared during recovery from BM ablation. It has been reported that VEGF promotes proliferation of HPs (*25*). Therefore, upregulation of VASH1 on HPs may reduce the expression of VEGFR2 as a means of negative feedback regulation of HP proliferation. Regulation of VEGFR2 expression by HPs may be part of the mechanism controlling the function of VASH1 in haematopoiesis. However, VEGFR2$^+$ HPs represent a very minor population among HPs. Therefore, other molecules must be involved in the negative regulation of cell growth in haematopoiesis. To understand the precise mechanism, targeted disruption of the VASH1 gene in HPs is required. This would enable the determination of the precise function of VASH1 in HP proliferation and negative feedback regulation to maintain HP pool size.

ACKNOWLEDGEMENT

We thank Mrs K. Fukuhara and N. Fujimoto for technical assistance.

FUNDING

This work was partly supported by the Japanese Ministry of Education, Culture, Sports, Science and Technology and the Japan Society for Promotion of Science.

CONFLICT OF INTEREST

None declared.

REFERENCES

1. Choi, K., Kennedy, M., Kazarov, A., Papadimitriou, J.C., and Keller, G. (1998) A common precursor for hematopoietic and endothelial cells. *Development* **125**, 725–732
2. Nishikawa, S.I., Nishikawa, S., Kawamoto, H., Yoshida, H., Kizumoto, M., Kataoka, H., and Katsura, Y. (1998) *In vitro* generation of lymphohematopoietic cells from endothelial cells purified from murine embryos. *Immunity* **8**, 761–769
3. Yamada, Y. and Takakura, N. (2006) Physiological pathway of differentiation of hematopoietic stem cell population into mural cells. *J. Exp. Med.* **203**, 1055–1065
4. Sata, M., Saiura, A., Kunisato, A., Tojo, A., Okada, S., Tokuhisa, T., Hirai, H., Makuuchi, M., Hirata, Y., and Nagai, R. (2002) Hematopoietic stem cells differentiate into vascular cells that participate in the pathogenesis of atherosclerosis. *Nat. Med.* **8**, 403–409
5. Sugiyama, T., Kohara, H., Noda, M., and Nagasawa, T. (2006) Maintenance of the hematopoietic stem cell pool by CXCL12-CXCR4 chemokine signaling in bone marrow stromal cell niches. *Immunity* **25**, 977–988
6. Avecilla, S.T., Hattori, K., Heissig, B., Tejada, R., Liao, F., Shido, K., Jin, D.K., Dias, S., Zhang, F., Hartman, T.E., Hackett, N.R., Crystal, R.G., Witte, L., Hicklin, D.J., Bohlen, P., Eaton, D., Lyden, D., de Sauvage, F., and Rafii, S. (2004) Chemokine-mediated interaction of hematopoietic progenitors with the bone marrow vascular niche is required for thrombopoiesis. *Nat. Med.* **10**, 64–71
7. Takakura, N., Watanabe, T., Suenobu, S., Yamada, Y., Noda, T., Ito, Y., Satake, M., and Suda, T. (2000) A role for hematopoietic stem cells in promoting angiogenesis. *Cell* **102**, 199–209
8. Takakura, N. (2006) Role of hematopoietic lineage cells as accessory components in blood vessel formation. *Cancer Sci.* **97**, 568–574
9. Coussens, L.M., Raymond, W.W., Bergers, G., Laig-Webster, M., Behrendtsen, O., Werb, Z., Caughey, G.H., and Hanahan, D. (1999) Inflammatory mast cells up-regulate angiogenesis during squamous epithelial carcinogenesis. *Genes Dev.* **13**, 1382–1397
10. Hiratsuka, S., Nakamura, K., Iwai, S., Murakami, M., Itoh, T., Kijima, H., Shipley, J.M., Senior, R.M., and Shibuya, M. (2002) MMP9 induction by vascular endothelial growth factor receptor-1 is involved in lung-specific metastasis. *Cancer Cell* **2**, 289–300
11. Ribatti, D., Presta, M., Vacca, A., Ria, R., Giuliani, R., Dell'Era, P., Nico, B., Roncali, L., and Dammacco, F. (1999) Human erythropoietin induces a pro-angiogenic phenotype in cultured endothelial cells and stimulates neovascularization in vivo. *Blood* **93**, 2627–2636
12. Watanabe, K., Hasegawa, Y., Yamashita, H., Shimizu, K., Ding, Y., Abe, M., Ohta, H., Imagawa, K., Hojo, K., Maki, H., Sonoda, H., and Sato, Y. (2004) Vasohibin as an endothelium-derived negative feedback regulator of angiogenesis. *J. Clin. Invest.* **114**, 898–907
13. Nimmagadda, S., Geetha-Loganathan, P., Pröls, F., Scaal, M., Christ, B., and Huang, R. (2007) Expression pattern of Vasohibin during chick development. *Dev. Dyn.* **236**, 1385–1362
14. Sonoda, H., Ohta, H., Watanabe, K., Yamashita, H., Kimura, H., and Sato, Y. (2006) Multiple processing forms and their biological activities of a novel angiogenesis inhibitor vasohibin. *Biochem. Biophys. Res. Commun.* **342**, 640–646
15. Kern, J., Bauer, M., Rychli, K., Wojta, J., Ritsch, A., Gastl, G., Gunsilius, E., and Untergasser, G. (2008) Alternative splicing of vasohibin-1 generates an inhibitor of endothelial cell proliferation, migration, and capillary tube formation. *Arterioscler. Thromb. Vasc. Biol.* **28**, 478–484
16. Shibuya, T., Watanabe, K., Yamashita, H., Shimizu, K., Miyashita, H., Abe, M., Moriya, T., Ohta, H., Sonoda, H., Shimosegawa, T., Tabayashi, K., and Sato, Y. (2006) Isolation and characterization of vasohibin-2 as a homologue of VEGF-inducible endothelium-derived angiogenesis inhibitor vasohibin. *Arterioscler. Thromb. Vasc. Biol.* **26**, 1051–1057
17. Shen, J., Yang, X., Xiao, W.H., Hackett, Y., Sato, Y., and Campochiaro, P.A. (2006) Vasohibin is up-regulated by VEGF in the retina and suppresses VEGF receptor 2 and retinal neovascularization. *FASEB J.* **20**, 723–725
18. Yamashita, H., Abe, M., Watanabe, K., Shimizu, K., Moriya, T., Sato, A., Satomi, S., Ohta, H., Sonoda, H., and Sato, Y. (2006) Vasohibin prevents arterial neointimal formation through angiogenesis inhibition. *Biochem. Biophys. Res. Commun.* **345**, 919–925

Downloaded from http://jb.oxfordjournals.org/ at Islamic Azad University on September 28, 2012

19. Yoshinaga, K., Ito, K., Moriya, T., Nagase, S., Takano, T., Niikura, H., Yaegashi, N., and Sato, Y. (2008) Expression of vasohibin as a novel endothelium-derived angiogenesis inhibitor in endometrial cancer. *Cancer Sci.* **99**, 914–919
20. Goodell, M.A., Brose, K., Paradis, G., Conner, A.S., and Mulligan, R.C. (1996) Isolation and functional properties of murine hematopoietic stem cells that are replicating *in vivo*. *J. Exp. Med.* **183**, 1797–1806
21. Arai, F., Hirao, A., Ohmura, M., Sato, H., Matsuoka, S., Takubo, K., Ito, K., Koh, G.Y., and Suda, T. (2004) Tie2/angiopoietin-1 signaling regulates hematopoietic stem cell quiescence in the bone marrow niche. *Cell* **118**, 149–161
22. Kidoya, H., Ueno, M., Yamada, Y., Mochizuki, N., Nakata, M., Yano, T., Fujii, R., and Takakura, N. (2008) Spatial and temporal role of the apelin/APJ system in the caliber size regulation of blood vessels during angiogenesis. *EMBO J.* **27**, 522–534
23. Kinnaird, T., Stabile, E., Burnett, M.S., and Epstein, S.E. (2004) Bone-marrow-derived cells for enhancing collateral development: mechanisms, animal data, and initial clinical experiences. *Circ. Res.* **95**, 354–363
24. Darnowski, J.W. and Handschumacher, R.E. (1985) Tissue-specific enhancement of uridine utilization and 5-fluorouracil therapy in mice by benzylacyclouridine. *Cancer Res.* **45**, 5364–5368
25. Smith, S.L., Agbemadzo, B., Reems, J.A., Tyler, T., Kiss, J., and Moldwin, R.L. (2000) Expansion of CD34 + KDR + cells in cord blood after culture with TPO, FLT-3L, SCF, and VEGF. *Exp. Hematol.* **28**, 94
26. Rafii, S., Avecilla, S., Shmelkov, S., Shido, K., Tejada, R., Moore, M.A., Heissig, B., and Hattori, K. (2003) Angiogenic factors reconstitute hematopoiesis by recruiting stem cells from bone marrow microenvironment. *Ann. NY Acad. Sci.* **996**, 49–60

Downloaded from http://jb.oxfordjournals.org/ at Islamic Azad University on September 28, 2012