

ECM-anchored Tie2 is regulated. Downstream signaling of Ang1/Tie2 is dependent on the presence or absence of cell-cell contacts. Therefore, disruption of endothelial cell-cell adhesions may trigger the formation of ECM-anchored Tie2 and the subsequent induction of angiogenic signaling. VEGF not only promotes angiogenesis, but also induces hyperpermeability by disrupting the cell-cell adhesions (Weis and Cheresh, 2005; Gavard and Gutkind, 2006; Shibuya and Claesson-Welsh, 2006). Thus, it is possible that, when cell-cell adhesions are disrupted by VEGF, Ang1 induces formation of ECM-anchored Tie2 and accelerates angiogenesis cooperatively with VEGF (Fig. 3). Consistently, it has been reported that VEGF-induced angiogenesis is potentiated by Ang1 *in vivo* (Asahara et al., 1998). However, since Ang1 also counteracts VEGF-mediated disruption of cell-cell contacts, the synergistic or counteractive effect of VEGF and Ang1 may be determined by a balance between Ang1 and VEGF levels and the timing of cell-cell destruction (Fig. 3).

Ang2 may also play an important role in transition between *trans*-associated Tie2 and ECM-anchored Tie2. Ang2 is produced by endothelial cells under hypoxic conditions and antagonizes Ang1-induced Tie2 activation, thereby destabilizing the blood vessels (Fiedler and Augustin, 2006; Augustin et al., 2009). In

the destabilized vessels where endothelial cells lose the cell-cell contacts, activation of ECM-anchored Tie2 by Ang1 may promote angiogenesis cooperatively with VEGF produced from hypoxic tissues.

Conclusion

We summarized the recent findings on the mechanisms by which Ang1/Tie2 signal regulates vascular quiescence and angiogenesis. We and Alitalo's group have clearly demonstrated that the dual functions of Ang1/Tie2 signal are mediated through Ang1-assembled Tie2 signaling complexes at cell-cell and cell-ECM contacts (Fukuhara et al., 2008; Saharinen et al., 2008). These studies have, however, also raised a number of new questions. For instance, how does *trans*-associated Tie2 specifically activate the AKT signaling pathway responsible for vascular maintenance? Does *trans*-association of Tie2 physically enhance vascular integrity by acting as a cell-adhesion molecule? How does ECM-anchored Tie2 promote assembly of integrin-mediated cell adhesions and regulate angiogenesis? In addition, it has to be addressed whether Ang1/Tie2 signal utilizes these molecular mechanisms to regulate vascular quiescence and angiogenesis *in vivo*. Solving these questions will be useful for the development of

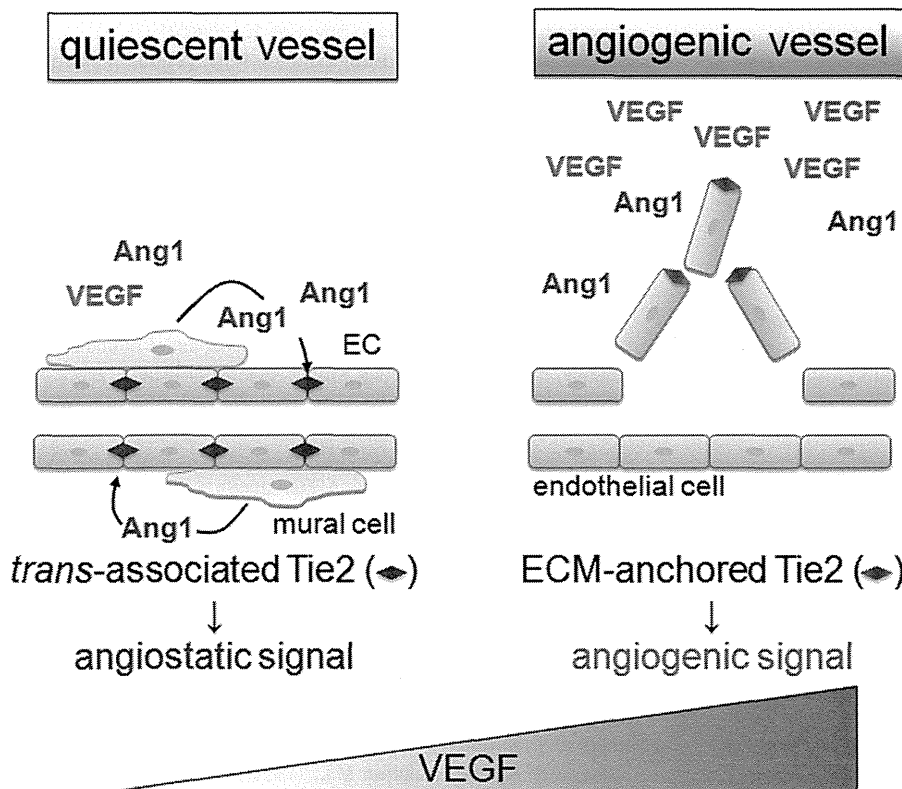


Fig. 3. Proposed model for the dual effects of angiopoietin-1/Tie2 signaling on vascular quiescence and angiogenesis (Left) In quiescent vessels, angiopoietin (Ang) 1 released from mural cells induces *trans*-association of Tie2 at endothelial cell-cell contacts, which activates the angiostatic signaling pathway to maintain vascular quiescence. Since only a limited amount of vascular endothelial growth factor (VEGF) exists in this condition, VEGF-induced angiogenesis is suppressed by the Ang1/Tie2 signal. (Right) However, once VEGF is released from ischemic tissues, detachment of mural cells from endothelial cells and disruption of inter-endothelial cell adhesions occur. In this situation, Tie2 is anchored to extracellular matrix-bound Ang1 and activates the angiogenic signaling pathway, thereby promoting angiogenesis cooperatively with VEGF.

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novel pro- and anti-angiogenic therapies.

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Roles of intrinsic angiogenesis inhibitor, vasohibin, in cervical carcinomas

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The aim of the present study is to clarify the critical roles of vasohibin in cervical carcinomas. We investigated the expression ratios of vasohibin and vascular endothelial growth factor (VEGF) receptor-2 on endothelium and microvessel density, lymphatic vessel density (LVD) by immunohistochemistry. Sixty-one squamous cell carcinoma (SCC), 18 mucinous adenocarcinoma (Adenocarcinoma), 38 carcinoma *in situ* (CIS), and 35 normal cervical epithelium were collected. We investigated the expression of vasohibin and compared it with the expression of VEGF receptor-2 (VEGFR-2, KDR/flk-1), and CD34 in the stromal endothelium. Expression of VEGF was counted using the histological score (H score). D2-40 was used as a marker for lymphatic endothelial cells to investigate LVD. The microvessel density of the normal cervical epithelium was significantly lower than that of CIS, SCC, and Adenocarcinoma ($P < 0.05$). The expression ratio of vasohibin in the normal cervical epithelium was significantly lower than that of SCC and Adenocarcinoma ($P < 0.05$). The expression ratio of VEGFR-2 of the normal cervical epithelium was significantly lower than that of SCC and Adenocarcinoma ($P < 0.05$). The LVD of the normal cervical epithelium was significantly lower than that of CIS, SCC, and Adenocarcinoma ($P < 0.05$). For normal cervical epithelium, CIS, and SCC, there was a moderate correlation between the expression percentage of vasohibin and the expression percentage of VEGFR-2 ($P < 0.05$, $r^2 = 0.3018$). This is the first study to elucidate the correlation between the expression of vasohibin in the stromal endothelial cells and the expression of VEGFR-2 in human cervical carcinomas. (*Cancer Sci* 2011; 102: 446–451)

Cervical carcinoma is a common gynecologic malignancy in women worldwide. In 2008 in the USA, an estimated 11 070 new cases of invasive cervical cancer were diagnosed and 3870 cancer-related deaths were expected to occur; this represents approximately 1% of cancer deaths in women.¹ The morbidity of cervical cancer, especially in women younger than 40 years old, is rapidly increasing and is the worst in Japan.² It is well recognized that angiogenesis (the process of forming new vessels) is required for tumor growth and enables the hematogenous spread of tumor cells throughout the cervical cancer. Several studies document the association between the MVD and/or the extent of endothelial proliferation, the pre-cancer and tumor stage, and the invasive disease of the cervical cancer.^(3–6) The expression of various angiogenesis stimulators, such as VEGFs, angiopoietins, and thymidine phosphorylase, has been reported in cervical cancer and dysplasia.^(7,8) Several published reports similarly report stromal LVD in cervical neoplasia.^(9–11) Angiogenesis is determined by the local balance between angiogenic stimulators and inhibitors. However, the significance of endogenous angiogenesis inhibitors in cervical cancer is poorly documented.

We isolated a novel angiogenesis inhibitor, vasohibin, which is specifically expressed in ECs. Its basal expression in quiescent

ECs is low; however, its production is induced by angiogenic stimuli such as VEGF-A and fibroblast growth factor-2, and it inhibits angiogenesis in an autocrine manner.^(12,13) We therefore propose that vasohibin acts as a negative feedback regulator to inhibit angiogenesis. VEGF-A is the most important factor for angiogenesis and most VEGF-A-mediated signals for angiogenesis are transduced through VEGF receptor-2 (VEGFR-2).⁽¹⁴⁾ We observed that the VEGF-A-mediated induction of vasohibin was preferentially mediated through the VEGFR-2 signaling pathway.⁽¹⁵⁾ In human cancer tissues, we have already reported a significant correlation between the expression ratio of vasohibin and VEGFR-2 in the vascular endothelial cells of endometrial cancer and breast cancer.^(16–18)

In the present study, we aimed to elucidate the significance of vasohibin in human cervical cancer and in the normal cervical squamous epithelium. We investigated the correlations between a tumor's biology (including vasohibin) and the clinical prognostic factors for cervical cancer (such as MVD, LVD, and the expression rate of vasohibin and VEGFR-2 in the endothelial cells). The histological type, lymph node metastasis, vessel involvement, and staging were the most important prognostic factors for cervical cancer. Our analysis revealed a significantly positive correlation between vasohibin and VEGFR-2 in cervical cancer. This is the first study to profile the expression of vasohibin as a negative feedback regulator of angiogenesis in human cervical cancer.

Materials and Methods

Tissue specimens and clinical data. There were 61 SCC, 18 mucinous adenocarcinoma (Adenocarcinoma), 38 CIS, and 35 normal cervical epithelium. Of the 61 SCC: 11 were stage IA (lymph node metastasis 0/11, vessel involvement 0/11, parametrium invasion 0/11); 29 were stage IB (lymph node metastasis 6/29, vessel involvement 11/29, parametrium invasion 0/29); 12 were stage IIA (lymph node metastasis 3/12, vessel involvement 7/12, parametrium invasion 0/12); and nine were stage IIB (lymph node metastasis 4/9, vessel involvement 6/9, parametrium invasion 8/9). Of the 18 mucinous adenocarcinomas: three were stage IA (lymph node metastasis 0/3, vessel involvement 1/3, parametrium invasion 0/3); 11 were stage IB (lymph node metastasis 1/11, vessel involvement 0/11, parametrium invasion 0/11); and four were stage IIB (lymph node metastasis 3/4, vessel involvement 4/4, parametrium invasion 3/4). We investigated the expression of vasohibin, and compared it with the expression of VEGFR-2 (KDR/flk-1) and CD34 (as a marker for vascular endothelial cells) in the stromal vascular endothelium. We used VEGF to indicate the presence of carcinoma cells and normal squamous epithelium. To investigate MVD, CD34

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was used as a marker for vascular endothelial cells. To investigate LVD, D2-40 was used as a marker for lymphatic endothelial cells. Immunohistochemical assessment was classified as negative or positive, based on the staining intensity.

Tissue specimens were retrieved from the surgical pathology files of Tohoku University Hospital (Sendai, Japan). The Ethics Committee at Tohoku University School of Medicine (Sendai, Japan) approved the protocol for this study.

The average age was 43.1 years in patients with SCC, 47.1 years old in patients with adenocarcinomas; and 41.0 years old in patients with CIS. Each patient provided written, informed consent before her surgery. None of the patients who were examined had received irradiation, hormonal therapy, or chemotherapy prior to surgery. The clinicopathological findings of the patients (including age, histology, stage, grade, and preoperative therapy) were retrieved by an extensive review of the charts. The standard primary treatment for cervical carcinoma at the Tohoku University Hospital was surgery, which consisted of abdominal radical hysterectomy and pelvic lymphadenectomy. The lesions were classified according to the Histological Typing of Female Genital Tract Tumors by the World Health Organization and staged in accordance with the International Federation of Gynecology and Obstetrics system.^(19,20) Patients with subtypes other than CIS, SCC, or mucinous adenocarcinoma, or patients who had second primary carcinoma, were excluded from this series.

All specimens were routinely processed (i.e., 10% formalin and fixed for 24–48 h), embedded in paraffin, and thin sectioned (3 μ m).

Immunohistochemical staining and the scoring of immunoreactivity. We carried out immunohistochemical staining for vasohibin, VEGFR-2, CD34 (as a marker for vascular endothelial cells), and D2-40 (as a lymphatic vessel marker). The presence of VEGF-A was assessed in normal squamous epithelium and in carcinoma cells. Paraffin-embedded tissue sections from human endometrial cancers were deparaffinized, rehydrated, and incubated with 3% H₂O₂ for 10 min to block endogenous peroxidase activity. The sections were incubated for 30 min at room temperature in a blocking solution of 10% goat serum (Nichirei Biosciences, Tokyo, Japan). They were then stained for 12 h at 4°C with primary antibodies, followed by staining for 30 min at room temperature with secondary antibodies. The primary antibodies were all mouse mAbs and were used as follows: 2 μ g/mL anti-human vasohibin mAb, anti-VEGFR-2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted at 1:100; anti-CD34 (Dako, Copenhagen, Denmark) diluted at 1:200; anti-D2-40 (Dako) diluted at 1:100; and anti-VEGF-A (Lab Vision, Fremont, CA, USA) diluted at 1:100. We previously described a mouse mAb against a synthetic peptide corresponding to the 286–299 amino acid sequence of vasohibin.⁽¹⁵⁾ The positive control slide for CD34 antigen was prepared from paraffin-fixed breast cancer tissue that contained a high microvessel density. Nuclei were counterstained with hematoxylin.

Three investigators (K.A., T.E., and K.Y) independently evaluated the immunohistochemical staining of the entire group of tissue sections. They were blinded to the clinical course of the patients. The average number counted by the three investigators was used for subsequent analysis.

We carefully examined the areas where cancer cells had come in contact with or had invaded the stroma. After first scanning the immunostained section at low magnification, microvessels having CD34-positive signals were counted. The areas with the greatest number of distinctly highlighted microvessels were selected. Any cell clusters with CD34-positive signals were regarded as a single countable microvessel, regardless of whether the lumen was visible. Unstained lumina were considered artifacts, even if they contained blood or tumor cells.

Microvessel density was assessed by light microscopy in areas of invasive tumor containing the highest number of capillaries and small venules per area (i.e., neovascular hot spots), in accordance with an original method.⁽²¹⁾ Lymphatic vessel density was investigated by D2-40-positive signals, using the same procedure described as above.

Immunostaining for vasohibin and VEGFR-2 was evaluated in the serial thin sections. Positive immunoreactive signals for vasohibin and VEGFR-2 in CD34-positive microvessels were counted; from this the positive ratios of vasohibin and VEGFR-2 in microvessels were calculated. Using an established antibody, immunohistochemistry detected the protein expression of a selected angiogenic factor (i.e., VEGF-A). For this marker, the intensity of cytoplasmic staining and the proportion of positive tumor cells were recorded. A staining index (with values 0–9) was calculated as the product of the staining intensity (0–3) and the area of positive staining (1, <10%; 2, 10–50%; 3, >50%).⁽²²⁾

Statistical analysis. Statistical analyses such as the Student's *t*-test and the Pearson's correlation coefficient test were carried out using StatView (version 4.5, Abacus Concepts Inc, Piscataway, NJ, USA). A *P*-value of <0.05 was considered significant.

Results

Figure 1 shows that the cells stained positively for vasohibin, VEGFR-2, CD34, and D2-40 on the normal cervix, CIS, SCC, and Adenocarcinoma. There were no significant differences between clinical factor (lymph node metastasis, vessel involvement, parametrium invasion) and MVD, LVD, the vasohibin expression ratio in the microvessels, the VEGFR-2 expression ratio in the microvessels, and the histology score of VEGF-A expression.

Microvessel density. CD34-positive MVD (number of vessels per mm²) were: 34.7 \pm 2.5 in normal cervical epithelium; 46.1 \pm 2.5 in CIS; 45.7 \pm 3.5 in SCC; and 73.85 \pm 8.4 in Adenocarcinoma (Fig. 2A). The MVD of Adenocarcinoma was significantly higher than that of the normal cervical epithelium, CIS and SCC. The MVD of normal cervical epithelium was significantly lower than that of CIS and SCC.

Lymphatic vessel density. D2-40-positive LVD (number of vessels per mm²) was: 3.7 \pm 0.8 in the normal cervical epithelium; 7.3 \pm 0.8 in CIS; 17.6 \pm 1.8 in SCC; and 34.6 \pm 6.5 in Adenocarcinoma (Fig. 2B). The LVD of Adenocarcinoma was significantly higher than that of the normal cervical epithelium, CIS, and SCC. The LVD of SCC was significantly higher than that of the normal cervical epithelium and CIS. The LVD of CIS was significantly higher than that of the normal cervical epithelium.

Vasohibin expression ratio in microvessels. The positive ratio of vasohibin in microvessels was 53.6 \pm 4.2% in normal cervical epithelium; 59.1 \pm 4.6% in CIS; 71.5 \pm 3.2% in SCC; and 79.3 \pm 4.0% in Adenocarcinoma (Fig. 2C). The vasohibin-immunopositivity of the Adenocarcinoma microvessels was significantly higher than that of the normal cervical epithelium, CIS, and SCC. The vasohibin-immunopositivity of the SCC microvessels was significantly higher than that of the normal cervical epithelium and CIS.

VEGFR-2 expression in microvessels. The positive ratio of VEGFR-2 in microvessels was 42.6 \pm 3.8% in the normal cervical epithelium; 42.1 \pm 4.3% in CIS; 52.6 \pm 2.6% in SCC; and 58.2 \pm 4.7% in adenocarcinoma (Fig. 2D). The VEGFR-2 immunopositivity in Adenocarcinoma microvessels was significantly higher than that of the normal cervical epithelium, CIS, and SCC. The VEGFR-2 immunopositivity of SCC microvessels was significantly higher than that of the normal cervical epithelium and CIS.

Histology score of VEGF-A expression. VEGF-A expression was present in the cytoplasm of epithelial cells. The VEGF-A

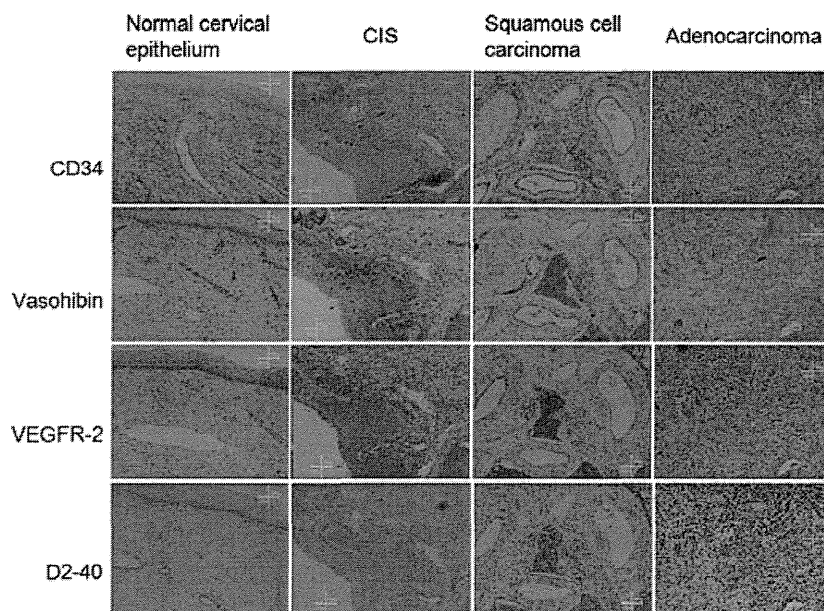


Fig. 1. Immunohistochemistry of the normal cervix with a normal epithelium, carcinoma *in situ* (CIS), squamous cell carcinoma, and adenocarcinoma. All sections are positively stained for: vasohibin (upper left, original magnification, $\times 200$); vascular endothelial growth factor receptor-2 (VEGFR-2; upper right, original magnification, $\times 200$); CD34 (lower left, original magnification, $\times 200$); and D2-40 (lower right, original magnification, $\times 200$).

positive ratio of cytoplasmic staining intensity was: 37.9 ± 7.3 in the normal squamous epithelium; 58.6 ± 9.1 in CIS; 88.5 ± 8.7 in SCC; and 52.8 ± 16.3 in Adenocarcinoma (Fig. 2E). The VEGF-A positive ratio of cytoplasmic staining intensity in SCC was significantly higher than that of the normal squamous epithelium, CIS, and Adenocarcinoma. The VEGF-A positive ratio of cytoplasmic staining intensity in CIS was significantly higher than that of the normal squamous epithelium.

Correlation between vasohibin and VEGFR-2 positive ratios in microvessels. We emphatically analyzed through normal cervix-CIS-SCC as the multi-step carcinogenesis model. A moderate positive correlation existed between vasohibin and VEGFR-2 positive ratios in the microvessels of the normal cervical epithelium, CIS, and SCC ($P < 0.05$, $r^2 = 0.301$) (Fig. 3).

Discussion

Using immunohistochemistry, we examined the vascular density of cervical cancer and compared it with that of the normal cervix. Our results with MVD and LVD in CIS and SCC were almost identical with some previous reports.^(3-5,23) Therefore, this proved the validity of our investigation. The major common perception of cervical cancer is that a CIS integrates the human papillomavirus and progresses to SCC in several years. Some reports indicate that the MVDs of CIS and SCC are greater than the MVD of the normal cervix. Therefore, we thought that it was important in our investigation to consider the vessel number in the normal cervix and to compare it with that of CIS and SCC. In accordance with our expectation, the MVD and LVD in the normal cervix were significantly lower than in CIS and SCC. These results suggested that hypervascularity is an important phenomenon of the progression of cervical squamous neoplasia. One published report showed that the VEGF intensity of SCC of the cervix increases in correlation with the clinical stage;⁽²⁴⁾ however, in the study there was no significance between the clinical features and the MVD, LVD, vasohibin expression ratio, VEGFR-2 expression ratio, and histological score (H score) of VEGF-A. In our study, we only found significant histological differences in the normal cervix, CIS, SCC, and Adenocarcinoma. Of course, it is important to investigate more cases of cervical neoplasia, and we suggest that peritumoral stromal angiogenesis is strongly ruled by the histological type of tumor. The VEGF-A positive ratio of cytoplasmic staining intensity in

the normal cervical epithelium was significantly lower than that in CIS and in SCC. This result clearly showed that the cytoplasmic expression level of VEGF-A in squamous epithelium cells and squamous neoplasia was increased in accordance with the progression.

It is generally accepted that, at the same clinical stage, Adenocarcinoma has a poorer prognosis than SCC.⁽²⁵⁻²⁹⁾ However, angiogenesis in Adenocarcinoma has been analyzed relatively little because of the minor frequency of Adenocarcinoma. In this study, we compared normal cervix, CIS, SCC, and Adenocarcinoma. We clearly showed that MVD and LVD were significantly higher in Adenocarcinoma than in SCC. This finding might contribute to understanding the poorer prognosis of adenocarcinoma of the cervix. To our regret, in this study we did not establish the significance between LVD and lymph node metastasis, because of insufficient number of cases. To clarify the issue, the same investigation should be repeated in a higher number of cases. However, the H score of VEGF-A of Adenocarcinoma was lower than that of SCC. We believe that it was difficult to detect VEGF-A in the cytoplasm of Adenocarcinoma because the cytoplasm was rich in mucus. In an *in vitro* study, there are many methods of measuring proteins in the cytoplasm, so we must examine measuring proteins in paraffin-embedded tissues except immunohistochemistry. Similar to MVD, LVD in this study was significantly increased in SCC and Adenocarcinoma. Some studies have reported the presence of peritumoral lymphatic vessels in cervical cancers and show that a high LVD is strongly associated with aggressive cervical cancer features such as lymph node metastasis, vessel involvement, parametrium invasion, and poor prognosis.^(30,31) Although we expected that the LVD would be correlated with lymph node metastasis, there was no significant correlation between the two.

We examined the expression of vasohibin, as described in our previous report.⁽¹⁶⁾ Vasohibin is induced in ECs by stimulation with VEGF.^(12,15) Indeed, the expression of vasohibin in tumor vessels was shown to be correlated with that of VEGF in human breast cancer and human non-small cell lung carcinoma.^(17,18) However, because of the difficulty of immunostaining VEGF-A in adenocarcinoma, we did not show such correlation in this study. VEGFR-2 receptor transduces most of the angiogenesis-related signals in ECs. The VEGF/VEGFR-2 signaling pathway is also important for the induction of vasohibin in ECs.⁽¹⁵⁾ We previously revealed that the VEGF-A-mediated induction of

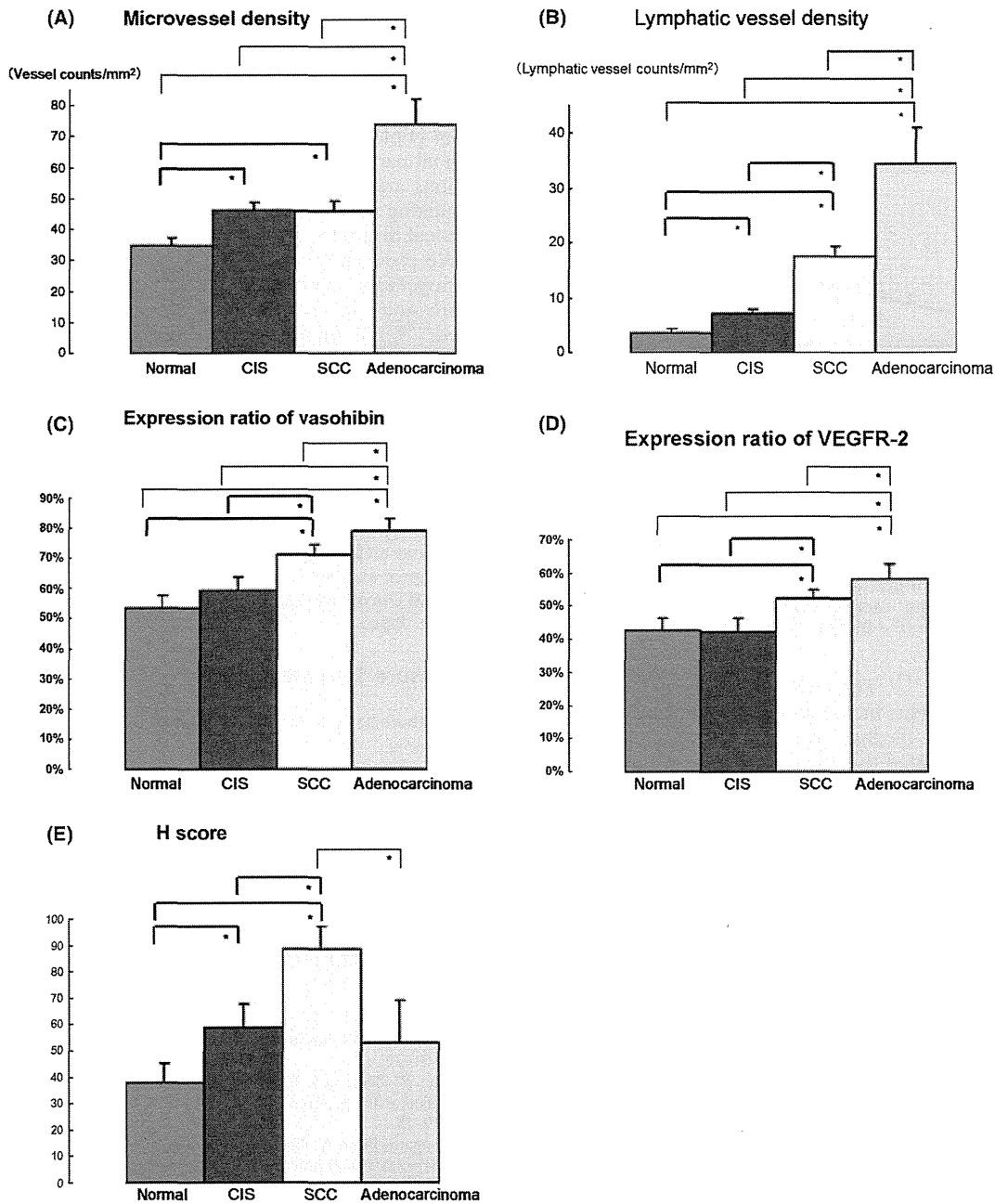


Fig. 2. (A) Microvessel density (MVD) of the normal cervix, carcinoma *in situ* (CIS), squamous cell carcinoma (SCC), and mucinous adenocarcinoma (Adenocarcinoma). The MVD of Adenocarcinoma is significantly higher than that of the normal cervix, CIS, and SCC. The MVD of the normal cervix is significantly lower than that of CIS and SCC. * $P < 0.05$. (B) Lymphatic vessel density (LVD) of the normal cervix, CIS, SCC, and Adenocarcinoma. The LVD of Adenocarcinoma is significantly higher than that of the normal cervix, CIS, and SCC. The LVD of SCC is significantly higher than that of the normal cervix and CIS. The LVD of CIS is significantly higher than that of the normal cervix. * $P < 0.05$. (C) The proportion of vasohibin/CD34-positive vessels in the normal cervix, CIS, SCC, and Adenocarcinoma. Vasohibin immunopositivity in microvessels in Adenocarcinoma is significantly higher than that in the normal cervix, CIS, and SCC. Vasohibin immunopositivity in the microvessels in SCC is significantly higher than that in the normal cervix and CIS. * $P < 0.05$. (D) Proportion of vascular endothelial growth factor receptor-2 (VEGFR-2)/CD34-positive vessels in the normal cervix, CIS, SCC, and Adenocarcinoma. VEGFR-2 immunopositivity in the microvessels in adenocarcinoma is significantly higher than in the normal cervix, CIS, and SCC. VEGFR-2 immunopositivity in the microvessels in SCC is significantly higher than in the normal cervix and CIS. * $P < 0.05$. (E) VEGF-A staining indexes of the cytoplasm of the tumor cell and normal cervical epithelium cells. The VEGF-A positive ratio of cytoplasmic staining intensity in SCC is significantly higher than that in the normal squamous epithelium, CIS, and Adenocarcinoma. The VEGF-A positive ratio of cytoplasmic staining intensity in CIS is significantly higher than in the normal squamous epithelium. H score, histological score. * $P < 0.05$.

vasohibin was preferentially mediated through the VEGFR-2 signaling pathway.⁽¹⁵⁾ In our results, the expression ratio of vasohibin in vascular endothelial cells increased as the tissue progressed from a normal cervix to SCC to Adenocarcinoma. In

addition, the expression ratio of VEGFR-2 in vascular endothelial cells increased as the tissue progressed from a normal cervix to SCC to Adenocarcinoma. This correlation of vasohibin and VEGFR-2 is also evident in endometrial adenocarcinoma⁽¹⁶⁾ and

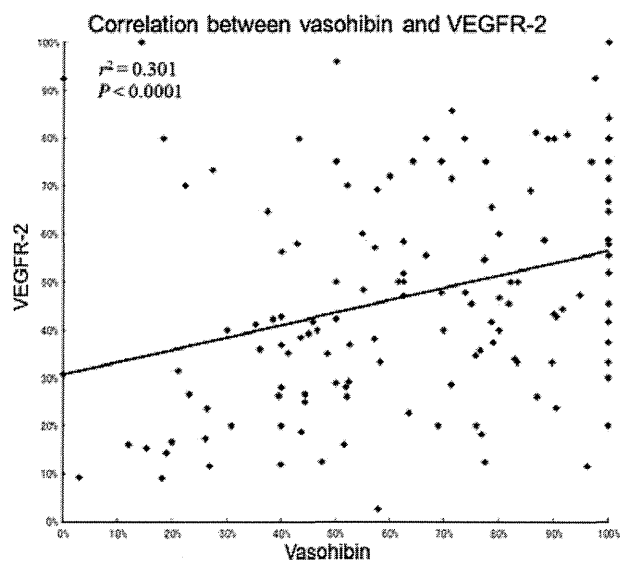


Fig. 3. Correlation between vasohibin and vascular endothelial growth factor receptor-2 (VEGFR-2). A moderate positive correlation exists between vasohibin-positive and VEGFR-2-positive ratios in the microvessels of the normal cervical epithelium, carcinoma *in situ*, and squamous cell carcinoma ($P < 0.0001$, $r^2 = 0.301$).

human breast cancer.⁽¹⁷⁾ Thus, the pathological studies from human cancer specimens, including our present one, support the theory on the negative feedback role of vasohibin in cancers. Lu *et al.*⁽³²⁾ recently reported that the increased expression of Zeste homolog 2 (EZH2) predicts poor prognosis of ovarian cancers. This poor prognosis was explained by EZH2-mediated silencing of vasohibin expression in tumor vessels. As the level of vasohibin expression is considerably variable (Fig. 3), it would be interesting to see whether lower expression of vasohibin in tumor vessels predicts poor prognosis.

It has been established that there is a significant relationship between cervical papillomavirus infection and cervical cancer.

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It is also clear that CIS progresses to SCC in several years. In our study, a moderate positive correlation existed between the positive ratios of vasohibin and VEGFR-2 expression in the normal cervix, CIS, and SCC. As the expression of vasohibin increased in correlation with the expression of VEGFR-2, vasohibin could be an important biomarker of angiogenesis in the normal cervix and in cervical cancer. However, further investigations are required to clarify the precise role of vasohibin in regulating anti-angiogenic activity in the normal cervix and in cervical disorders.

We previously reported the function of vasohibin as a novel angiogenesis inhibitor.^(12–15,18,33) The profiling of vasohibin expression in human cancer tissues is being accumulated.^(18,33,34) In the future, vasohibin could potentially have medical application as a novel angiogenesis inhibitor.

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Disclosure Statement

The authors have no conflict of interest to declare.

Abbreviations

CIS	carcinoma <i>in situ</i>
EC	endothelial cell
SCC	squamous cell carcinoma
MVD	microvessel density
LVD	lymphatic vessel density
VEGF	vascular endothelial growth factor
VEGFR-2	VEGF receptor-2

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Article

Mutual Balance between Vasohibin-1 and Soluble VEGFR-1 in Endothelial Cells

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Abstract: Vasohibin-1 (VASH1) is a VEGF-inducible gene of endothelial cells (ECs) that acts as a negative feedback regulator of angiogenesis. To further characterize the function of VASH1, we transfected human VASH1 gene into the mouse EC line MS1, established stable VASH1 expressing clones, and determined gene alteration by cDNA microarray analysis. Among the various angiogenesis-related genes, vascular endothelial growth factor type 1 receptor (VEGFR-1) and its alternative spliced form, soluble VEGFR1 (sVEGFR-1), were found to be the most significantly down-regulated genes. Transient overexpression of VASH1 in human umbilical vein endothelial cells confirmed the down-regulation of VEGFR-1 and sVEGFR-1. sVEGFR-1 is a decoy receptor for VEGF and inhibits angiogenesis. Interestingly, when sVEGFR-1 was overexpressed in ECs, it inhibited the expression of VASH1 in turn. These results suggest that VASH1 and sVEGFR-1, two angiogenesis inhibitors, mutually balance their expressions in ECs.

Keywords: angiogenesis inhibitor; endothelial cell; VASH1; sVEGFR-1

1. Introduction

Angiogenesis, the formation of neovessels, is involved in both physiological and pathological conditions. Angiogenesis is regulated by a local balance between stimulators and inhibitors. Angiogenesis stimulators include vascular endothelial growth factor (VEGF), whereas angiogenesis inhibitors include

thrombospondins, pigment epithelium derived factor, angiostatin, endostatin, and so forth [1].

The VEGF family consists of five members; VEGF-A, VEGF-B, VEGF-C, VEGF-D, and placenta growth factor (PlGF). There are also three VEGF receptor (VEGFR) tyrosine kinases; VEGFR-1, VEGFR-2 and VEGFR-3. Members of the VEGF family show different affinities for the receptors. VEGFR-1 is able to bind VEGF-A, VEGF-B and PlGF. VEGFR-2 is activated primarily by VEGF-A, but cleaved forms of VEGF-C and VEGF-D can also activate VEGFR-2. VEGFR-3 is activated by VEGF-C and VEGF-D. Vascular endothelial cells (ECs) express mainly VEGFR-1 and VEGFR-2, whereas lymphatic ECs mainly express VEGFR-3 in adults. VEGFR-2 is the major mediator of VEGF-A driven responses for angiogenesis. The binding-affinity of VEGFR-1 for VEGF-A is one order of magnitude higher than that of VEGFR-2, whereas the kinase activity of VEGFR-1 is about 10-fold weaker than that of VEGFR-2 [2]. *VEGFR-1* (-/-) mice died in utero because of overgrowth of ECs, but mice lacking the tyrosine kinase domain of VEGFR-1 remain healthy and have a normal vasculature [3,4]. Thus, the ligand binding domain of VEGFR-1 are sufficient for normal vascular development in embryo, most likely by sequestering VEGF-A from VEGFR-2.

We recently isolated vasohibin-1 (VASH1) from VEGF-A inducible genes in ECs that inhibits migration and proliferation of ECs in culture, and exhibits anti-angiogenic activity *in vivo* [5]. The expression of VASH1 in ECs is induced not only by VEGF-A but also by fibroblast growth factor 2 (FGF-2), another potent angiogenic factor [5,6]. Thus, VASH1 is thought to be a negative-feedback regulator of angiogenesis. Immunohistochemical analysis revealed that VASH1 protein is expressed selectively in ECs in the developing human or mouse embryo, is reduced in expression in the post-neonate, but is induced in ECs at the site of angiogenesis [7]. Analysis of the spatiotemporal expression and function of VASH1 during angiogenesis revealed that VASH1 is expressed not in ECs at the sprouting front but in ECs of newly formed blood vessels behind the sprouting front where angiogenesis is terminated [8]. The expression of VASH1 is evident in various pathological processes such as cancers [9-13], atherosclerosis [14], age-dependent macular degeneration (AMD) [15], diabetic retinopathy [16], and so forth. Moreover, when applied exogenously, VASH1 shows anti-angiogenic activity under various pathological conditions such as in tumors, arterial intimal thickening and retinal neovascularization [9,14,17]. However, the molecular mechanisms underlying angiogenesis inhibition by VASH1 remain to be characterized. Here we intended to characterize the target genes of VASH1 in ECs. Using cDNA microarray analysis of stable VASH1 expressing EC clones, we identified both full-length and soluble forms of VEGFR-1 as the target genes of VASH1 in ECs.

2. Materials and Methods

2.1. Cells

MS1, an immortalized cell line with a SV40 large T antigen from mouse pancreatic ECs [18], was purchased from American Type Culture Collection (Manassas, VA, USA). The cells were cultured in α MEM (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS, JRHBiosciences, San Antonio, TX, USA). Human umbilical vein endothelial cells (HUVECs) were obtained from KURABO (Osaka, Japan) and were cultured on type I collagen-coated dishes (IWAKI,

Tokyo, Japan) containing endothelial basal medium-2 (EBM-2; Clonetics Corp., San Diego, CA, USA) supplemented with EC growth supplements and 2% FBS.

2.2. Establishment of VASH1 Expressing MSI Clones

To improve the activity of transcription, we placed the CMV promoter of the pcDNA3.1/Hygro plasmid (Invitrogen) with the chicken β -actin promoter derived from pCALL2 [19]. This vector, pCALL2-pcDNA3.1/Hygro, was used for the transfection in this study. For the production of the VASH1 expression vector, the human VASH1 gene (5481 bp) containing the complete open reading frame (386 n.t.-1483 n.t.) [5] was cloned into the pCALL2-pcDNA3.1/Hygro vector at multiple cloning sites (Xho-I and Not-I). MSI cells were transfected with the VASH1 expression vector by using Effectene transfection reagent (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. After the transfection, the cells were selected by hygromycin (500 μ g/mL, Invitrogen). Following the selection, the cells were seeded at 0.3 cells per well in 96 well plates with 100 μ L of culture medium in each well. The cells were later expanded into larger wells.

2.3. Gene Transfer in HUVECs

A replication-defective adenovirus vector encoding the human VASH1 (AdvASH1) or the β -gal gene (AdLacZ) was prepared as described previously [5]. The replication-defective adenovirus vector encoding the human VEGFR-1 gene (AdVEGFR-1) was a generous gift from Masabumi Shibuya (Tokyo Medical and Dental University). The HUVECs were infected with the adenovirus vectors at a multiplicity of infection (MOI) of 10 to 100. After the infection, RNAs and proteins were extracted at 24 and 36 hours, respectively.

2.4. Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted by the acid guanidinium-phenol-chloroform method using ISOGEN (Nippon Gene, Toyama, Japan). RT-PCR was performed by using a one step RT-PCR kit (Invitrogen) according to the manufacturer's instructions. The following primer pairs were synthesized and used for amplification: the respective sense (S) and the antisense (AS) primer pairs used were as follows: mouse and human VASH1, 5'-ATGGACCTGGCCAAGGAAAT-3' and 5'-CATCCTTCTTCCGGTCTTG-3'; mouse VEGFR-1, 5'-GCGCATGACGGTCATAGAAG-3' and 5'-CAGGTGTGGCGCTCCGAAT-3'; human VEGFR-1, 5'-ATGGTCAGCTACTGGGACAC-3' and 5'-GAATGACGAGCTCCCTTCCTT-3'; mouse sVEGFR-1, 5'-ACTCTCAGACCCCTGGAATC-3' and 5'-GATCCGAGAGAAATGGCCT-3'; human sVEGFR-1, 5'-CATCACTCAGCGCATGGCAA-3' and 5'-CAGCCTTTTTGTTGCGTGC-3'; mouse and human G3PDH, 5'-ACCACAGTCCATGCCATCAC-3' and 5'-TCCACCACCCTGTTGCTGTA-3'. The PCR consisted of 27 cycles of 94 °C for 15 s, at 58 °C for 30 s, and finally at 68 °C for 30 s. The PCR products were electrophoresed through a 2% agarose gel containing 0.5 mg/mL ethidium bromide and visualized.

2.5. Western Blot Analysis

Western blot analysis was performed as described previously [20]. Briefly, extracted protein was separated by SDS-PAGE on a 7.5-10% separating gel and transferred to a Hybond-ECL membrane

(Amersham, Buckinghamshire, UK). The membrane was incubated with anti-human VASH1 monoclonal antibody (Ab) [5], anti-VEGFR-1 Ab (Santa Cruz Biotechnology, Inc., CA, USA), anti-sVEGFR-1 Ab (Zymed Lab., San Francisco, CA, USA), or anti- β -actin Ab (Sigma, St. Louis, MO, USA) as the primary antibody according to the manufacturer's instructions. The signal was visualized by using horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence (Immobilon Western, Millipore, Billerica, MA, USA) with a LAS-1000 image analyzer (Fuji Film, Tokyo, Japan).

2.6. Cell Proliferation

Cells were inoculated at a density of 1×10^4 per well into 100 mm dishes, and cultured in 10% FCS/ α MEM. After incubation for the desired period, the cells were counted with a hemocytometer.

2.7. Cell Migration

Cells were harvested with trypsin-EDTA, resuspended in 10% FCS/ α MEM in a final volume of 100 μ L, loaded (5×10^4 cells per well) into the upper chamber of a Transwell Polycarbonate Membrane (pore size: 8 μ m; Costar, Cambridge, MA, USA) containing 600 μ L of 10% FCS/ α MEM in the lower chamber, and incubated at 37 °C for 4 hours. Cells on the lower surface were stained with the reagents from a Diff Quick kit (International Reagents, Kobe, Japan) and counted.

2.8. cDNA Microarray Analysis

Total RNA was isolated from the mock control and clone 4 by ISOGEN according to the manufacturer's instructions. The RNAs were reverse-transcribed in the presence of Cy3 or Cy5-labeled CTP, respectively. The labeled probes were hybridized to a Filgen Array Mouse 32K (Oxford Gene Technology, Oxford, UK) containing 31,769 genes, and the signals were detected by use of a GenePix 4000B (Olympus, Tokyo, Japan). Genes with a Cy5 signal/Cy3 signal ratio >2.0 or <0.5 were considered to have changed in activity.

2.9. Blood Pressure and Urinary Albumin Excretion of Mice

Animal studies were reviewed and approved by the committee for animal study at our institute in accord with established standards of humane handling. AdVASH1, AdVEGFR-1 or AdLacZ (1×10^9 plaque-forming units [pfu]) was intravenously injected into a tail vein of BALB/c mice (Charles River Laboratories Japan, INC.). Before and seven days after the viral injection, mean blood pressure of conscious mice was measured by the tail cuff method (BP-98A; Softron Co. Ltd., Tokyo, Japan) according to the manufacture's instruction. Seven days after the viral injection, mice were put in mouse metabolic cages (Metabolica type MM; Sugiyama-Gen Iriki Co. Ltd., Tokyo, Japan) and urine was collected for successive eight hour periods. Urine was centrifuged at $2,000 \times g$ and the urinary albumin level was determined using an ELISA kit (Albuwell M; Exocell, Philadelphia, PA). Urinary creatinine levels were also measured by an ELISA kit (The Creatinine Companion; Exocell).

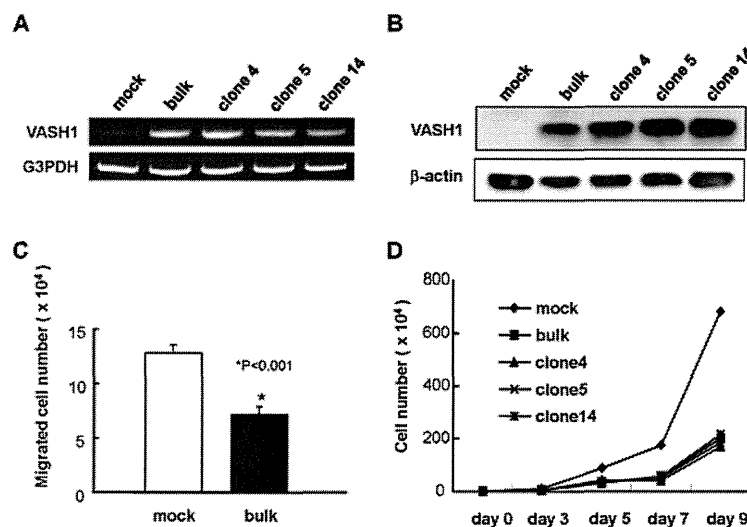
2.10. Calculations and Statistical Analysis

The statistical significance of differences in the data was evaluated by use of unpaired analysis of variance, and P values were calculated by the unpaired Student t test. $P < 0.05$ was accepted as statistically significant.

3. Results

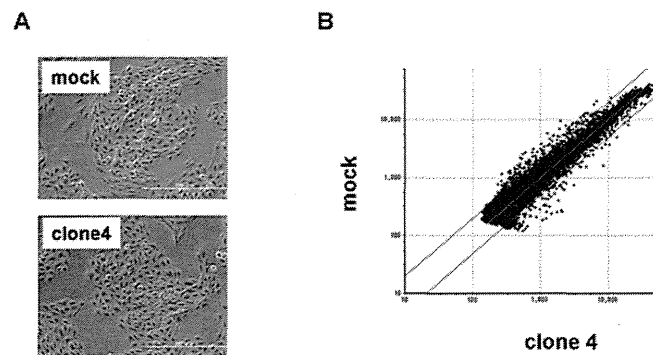
We introduced the human VASH1 gene into MS1 cells, established bulk transfectants, and thereafter isolated 3 clones. First the expression of VASH1 mRNA and protein was determined (Figure 1A and B). We then examined the properties of the VASH1 expressing mouse endothelial clones. As expected, migration and proliferation were significantly decreased in these VASH1 transfectants (Figures 1C and D).

Figure 1. Establishment of VASH1 overexpressing MS1 clones.



Establishment of VASH1 overexpressing MS1 bulk and clones was described in Materials and Methods. Total RNAs and proteins were extracted from each clone. RT-PCR (A) and Western (B) blot analysis for VASH1 was performed. (C) Migration was compared between mock and VASH1 transfectants. Means and SDs are shown. (D) Proliferation was compared between mock and VASH1 transfectants.

We used clone 4 (Figure 2A) for the following analysis, as it showed the highest expression of VASH1 (Figure 1A). Total RNA was isolated and the gene expression profile was compared with the mock control by cDNA microarray analysis (Figure 2B). The scatter plot of the mock control versus clone 4 is shown in Figure 2B. Among 31,769 mouse genes, 170 genes were increased by more than 200%, whereas 178 genes were decreased by less than 50% in clone 4. The top 20 up-regulated and down-regulated genes are listed in Table 1 and Table 2, respectively. We further evaluated the known angiogenesis-related genes. It was revealed that the expression of 7 genes (CCL2, CCL5, TIMP-1, COX-2, ARNT, CXCL1, and Jagged1) was augmented by more than 200%, whereas that of 2 genes (VEGFR-1 and Ets-1) was down-regulated by less than 50% (Table 3).

Figure 2. Scatter plot of mock vs. VASH1 over expression clone 4.

(A) Morphology of mock and VASH1 expressing clone 4 is shown. (B) RNAs were extracted from the mock and clone 4, and cDNA microarray analysis was performed as described in Materials and Method. Two lines indicate a 1:2 (lower) or 2:1 (upper) ratio between mock vs. VASH1 overexpressing clone 4.

Table 1. Top 20 up-regulated genes in the VASH1 stable transfectant.

Fold induction	Gene name	Accession No.
8.07	Estradiol 17 beta-dehydrogenase 5	NM_030611
7.65	T-cell specific GTPase	NM_011579
6.16	Dematin	NM_013514
5.43	interferon-induced protein with tetratricopeptide repeats 3	NM_010501
4.91	Osteoactivin	NM_053110
4.63	BTB/POZ domain containing protein 9	NM_172618
4.29	interferon, alpha-inducible protein	NM_172618
4.26	Galectin-3	NM_010705
4.19	ADP-ribosylation factor-like 2 binding protein	NM_024191
3.79	potassium channel interacting protein 4	NM_030265
3.79	CCL2	NM_011333
3.78	olfactory receptor 576	NM_001001805
3.59	KELCH-like protein 4	NM_172781
3.41	interferon-induced protein 44	NM_133871
3.39	interferon, alpha-inducible protein 27	NM_029803
3.12	acyl-Coenzyme A thioesterase 2	NM_019736
3.28	stefin A2 like 1	NM_173869
3.11	guanylate nucleotide binding protein 3	NM_018734
3.09	2'-5'-oligoadenylate synthetase 1A	NM_145211
3.00	Male-specific lethal 3-like 1	NM_010832

Table 2. Top 20 down-regulated genes in the VASH1 stable transfectant.

Fold induction	Gene name	Accession No.
0.22	Phospholipid transfer protein	NM_011125
0.22	VEGFR1	NM_010228
0.28	Oligodendrocyte transcription factor 1	NM_016968
0.28	olfactory receptor 635	NM_147118

Table 2. *Cont.*

0.28	PHD finger protein 19	NM_028716
0.28	Trace amine receptor 1	NM_053205
0.29	Gastrokine 1	NM_025466
0.29	Mast cell carboxypeptidase A	NM_007753
0.30	poliovirus receptor-related 2	NM_008990
0.30	Versican core protein	XM_488510
0.30	Janus kinase 3	NM_010589
0.30	thrombopoietin	NM_009379
0.31	nudix	NM_025539
0.31	Anaplastic lymphoma kinase	NM_007439
0.33	Lymphocyte antigen 86	NM_010745
0.33	Fc receptor-like 3	NM_144559
0.34	Serine/threonine-protein kinase ULK1	NM_009469
0.34	Runt-related transcription factor 3	NM_019732
0.34	RAB GTPase activating protein 1	AK_044346
0.35	kidney expressed gene 1	NM_029550

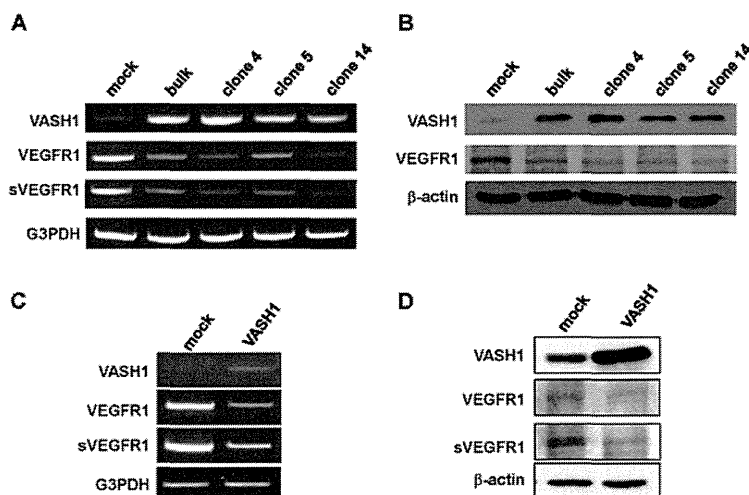
Table 3. Altered expression of angiogenesis-related genes in the VASH1 transfectant.

Gene name	Fold induction
VEGFR1	0.22
Ets-1	0.45
Jagged1	2.06
CXCL1	2.17
ARNT	2.18
COX-2	2.38
TIMP-1	2.39
CCL5	2.45
CCL2	3.79

Here we focused our attention on VEGFR-1, the most down-regulated gene. The characteristic feature of the VEGFR-1 gene is that it encodes not only a full-length membrane receptor but also a soluble form (sVEGFR-1) carrying the VEGF-binding domain as well as a 31-amino-acid stretch derived from an intron [22]. We therefore analyzed the expression of sVEGFR-1 as well. RT-PCR and Western blot analyses showed decrease expression of VEGFR-1 and sVEGFR-1 in all VASH1 expressing clones (Figures 3A and 3B). To further confirm these results, we transiently overexpressed VASH1 in HUVECs. RT-PCR and Western blot analyses also showed this decrease in the levels of VEGFR-1 and sVEGFR-1 (Figures 3C and D).

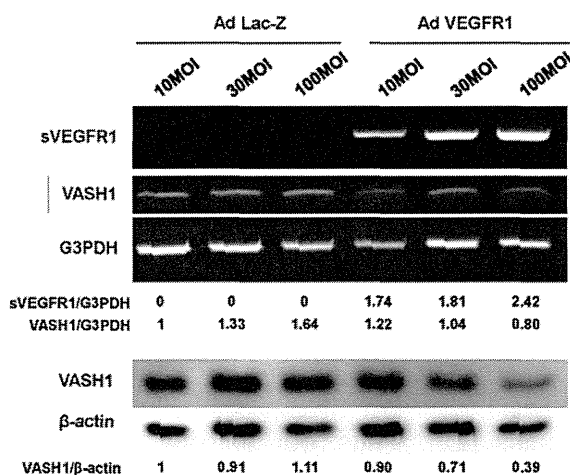
sVEGFR-1 is a decoy receptor and inhibits VEGF mediated signals. VASH1 is a VEGF-inducible angiogenesis inhibitor expressed in ECs. We therefore examined whether sVEGFR-1 affects the expression of VASH1 in ECs. To do so, we transiently overexpressed the sVEGFR-1 gene in HUVECs. RT-PCR and Western blot analyses demonstrated that sVEGFR-1 down-regulated the expression of VASH1 in HUVECs (Figure 4).

Figure 3. VASH1 inhibits VEGFR-1 and sVEGFR-1 expression in ECs.



Total RNAs and proteins were extracted from mock cells and from each VASH1 overexpressing clone, and RT-PCR (A) and Western blot analysis (B) for VEGFR-1 and sVEGFR-1 were performed as described in Materials and Method. The HUVECs were infected with the Lac-Z or VASH1 adenovirus vector at a multiplicity of infection (MOI) of 30. After the infection, total RNAs and proteins were extracted from vasohibin-1 over expression HUVECs. RT-PCR (C) and Western (D) blot analysis for VEGFR-1 and sVEGFR-1 was performed.

Figure 4. sVEGFR-1 inhibits VASH1 expression in ECs.

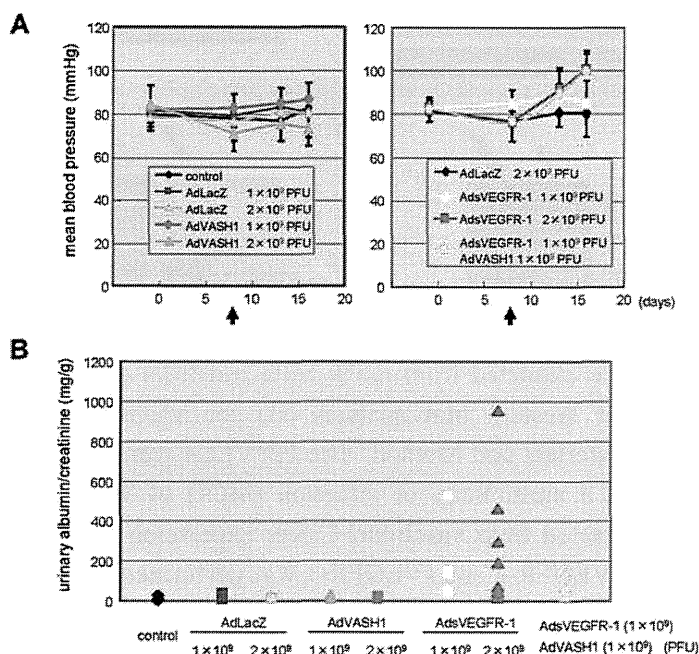


The HUVECs were infected with the Ad sVEGFR-1 at the indicated a multiplicity of infection (MOIs). After the infection, total RNAs and proteins were extracted from sVEGFR-1 overexpressing HUVECs. RT-PCR (upper panel) and Western blot analysis (lower panel) for VASH1 were performed. Intensity of bands was quantified, and standardized values are shown.

The blockade of VEGF mediated signals causes regression of normal quiescent vessels, hypertension and proteinuria [21]. Here we examined whether VASH1 caused hypertension and proteinuria. Ad sVEGFR-1 increased mean blood pressure, but Ad VASH1 did not (Figure 5A). Similarly Ad sVEGFR-1 increased the urinary albumin excretion, but Ad VASH1 did not (Figure 5B).

AdVASH1 exhibited little effect on the increased mean blood pressure or urinary albumin excretion induced by AdsVEGFR-1 (Figure 5 A and B).

Figure 5. Different effects of sVEGFR-1 and VASH1 on blood pressure and urinary albumin excretion.



A: Indicated PFU of adenovirus vectors were injected *via* a tail vein on day 8. Arrows indicate the timing of adenovirus injection. Mean blood pressure was assessed on day 0, 7, 12, 16; means and SDs are shown. **B:** Indicated PFU of adenovirus vectors were injected. Twenty-four-hour urinary albumin excretion was quantified on day 7 after the adenovirus injection.

4. Discussion

Here we analyzed the target genes of VASH1 in ECs, and revealed for the first time that VASH1 down-regulated the expression of both full-length and soluble form of VEGFR-1 in ECs. Interestingly, sVEGFR-1, a decoy receptor that blocks VEGF mediated signals, down-regulated the expression of VASH1 in return. Endogenous sVEGFR-1 is thought to inhibit angiogenesis by reducing VEGF-mediated angiogenic signals [22]. Thus, our present study indicates that these two factors mutually regulate their expression in ECs. We propose that VASH1 and sVEGFR-1 interact with each other within ECs for the fine tuning of angiogenesis.

The expression of VASH1 in ECs is known to be induced by VEGF-VEGFR2 and its downstream PKC- δ mediated signaling pathway [6]. We therefore think it reasonable that sVEGFR-1 would inhibit the expression of VASH1 in ECs. In contrast, the regulation of the expression of full-length and soluble form of VEGFR-1 is not well characterized. Further study is required to elucidate the mechanism as to how VASH1 down-regulates the expression of VEGFR-1 in ECs.

From the clinical experience of anti-angiogenic cancer treatment, it is now well recognized that the *in vivo* blockade of VEGF-mediated signals causes vascular complications including hypertension and proteinuria [21]. Indeed, the tail vein injection of AdsVEGFR-1 increased blood pressure and induced