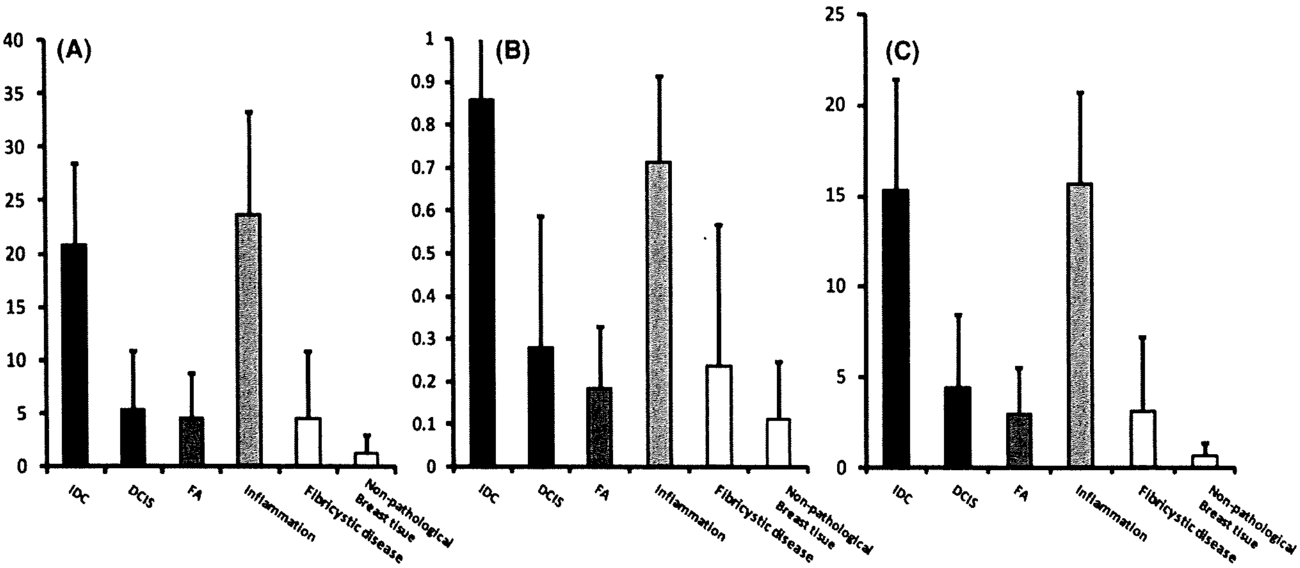


IDC	20.9 ± 7.7
DCIS	5.3 ± 5.5
FA	4.6 ± 4.1
Inflammation	23.7 ± 9.7
Fibrocystic disease	4.6 ± 4.1
Non-pathological breast tissue	1.3 ± 1.8

IDC	0.857 ± 0.193
DCIS	0.279 ± 0.308
FA	0.183 ± 0.146
Inflammation	0.713 ± 0.200
Fibrocystic disease	0.237 ± 0.332
Non-pathological breast tissue	0.112 ± 0.136

IDC	15.3 ± 6.1
DCIS	4.4 ± 4.1
FA	2.9 ± 2.6
Inflammation	15.7 ± 5.0
Fibrocystic disease	3.1 ± 4.1
Non-pathological breast tissue	0.7 ± 0.7

Fig. 2. Analysis of vasohibin-1 immunohistochemistry according to histological subtypes. (A) Number of vasohibin-1-positive vessels in the 'hotspot'. (B) Vasohibin-1-positive ratio defined as the vasohibin-1-positive vessels/CD31-positive vessels. (C) Average of vasohibin-1-positive vessels in 10 different fields. The lower boxes are the statistical analysis compared with invasive ductal carcinoma (IDC) cases.

DCIS, 2.9 ± 2.6 in FA, 15.7 ± 5.0 in inflammatory lesions, 3.1 ± 4.1 in fibrocystic change and 0.7 ± 0.7 in non-pathological breast tissue. There were also statistically significant differences between IDC and four histological types (DCIS, FA, fibrocystic change and non-pathological breast tissue, $P < 0.001$). No significant differences were detected between IDC and inflammatory lesions ($P = 0.781$) (Fig. 2C).

Correlation between vasohibin-1-positive vessels and Ki-67 labeling index in carcinoma cells. A significant positive correlation was detected between the number of vasohibin-1-positive vessels and Ki-67 labeling index in breast tumor cells ($P < 0.001$).

Correlation between vasohibin-1-positive vessels and VEGF-A status in carcinoma cells. The number of vasohibin-1-positive vessels was 5.8 ± 5.5 in VEGF-A of score 0, 11.0 ± 9.4 of score 2, 15.1 ± 10.0 of score 3, 17.5 ± 10.1 of score 4, 22.1 ± 8.9 of score 5 and 22.7 ± 5.7 of score 6. There was a statistically significant association between vasohibin-1 in the vessels and VEGF-A scores in carcinoma cells ($P < 0.001$) (Fig. 3A).

Correlation between vasohibin-1-positive vessels and FGF-2 in carcinoma cells. The number of vasohibin-1-positive vessels was 6.3 ± 6.1 in FGF-2 of score 0, 19.1 ± 6.5 of score 1, 21.9 ± 7.2 of score 2 and 26.8 ± 8.4 of score 3. A statistically significant association was detected between vasohibin-1 immunoreactivity in the vessels and FGF-2 scores in carcinoma cells ($P < 0.001$) (Fig. 3B).

Correlation between vasohibin-1 and Flk-1 in microvessels in breast carcinoma. A significantly positive correlation was detected between vasohibin-1 and Flk-1 positive ratios in microvessels ($P < 0.001$) (Fig. 3C).

Correlation between vasohibin-1 and clinical stage of breast carcinoma cases. The number of vasohibin-1-positive vessels was 5.3 ± 5.5 in TNM Stage 0, 19.6 ± 6.7 in Stage I, 18.7 ± 8.6 in Stage II A, 22.1 ± 8.3 in Stage II B, 23.8 ± 5.8 in Stage III A, 28.7 ± 7.5 in Stage III B, 23.0 ± 7.5 in Stage III C and 21.2 ± 5.6 in Stage IV. Statistically significant differences were detected only between IDC and DCIS ($P < 0.001$) with no significant differences among the different stages of IDC.

Correlation between vasohibin-1 and histological grades of breast carcinoma cells. The number of vasohibin-1-positive vessels among different groups of carcinoma cases and histological grade was 18.4 ± 7.5 in grade I, 20.8 ± 7.0 in grade II and 28.0 ± 8.0 in grade III. There were statistically significant differences of vasohibin-1 density between grade I and III, and grade II and III cases ($P < 0.001$) with no significant difference between grade I and II cases ($P = 0.14684$).

Correlation between vasohibin-1 and overall survival or DFS in breast carcinoma patients. Patients were tentatively classified into two different groups according to the number of vasohibin-1-positive vessels: 0–20 and 21 or more. The 10-year overall survival rates were 0.932203 and 0.72549 among these two groups, respectively. (The total 10-year overall survival rate in this cohort of patients was 0.838836.) Statistically significant differences in the 0–20 and 21 or more groups was $P = 0.004$ (Fig. 4A). The 10-year DFS were 0.92736 and 0.708333, respectively, in these two groups. Statistically significant differences were also detected in the 0–20 and 21 or more groups was at $P \leq 0.001$. (The total 10-year DFS rate was 0.81777; Fig. 4B.) The following variables were included in the multivariate analysis of OS: vasohibin-1, MVD, VEGF-A and Ki-67. This multivariate analysis demonstrated that vasohibin-1 was associated with VEGF-A ($P = 0.038$) and Ki-67 ($P < 0.001$), but was not associated with MVD ($P = 0.083$). The multivariate analysis of

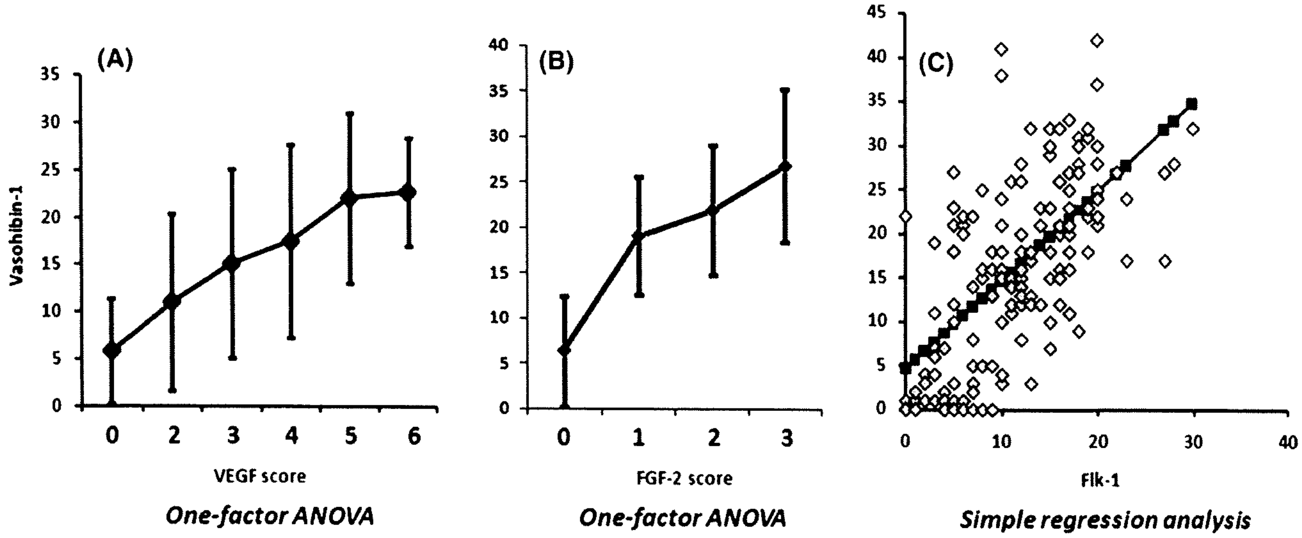
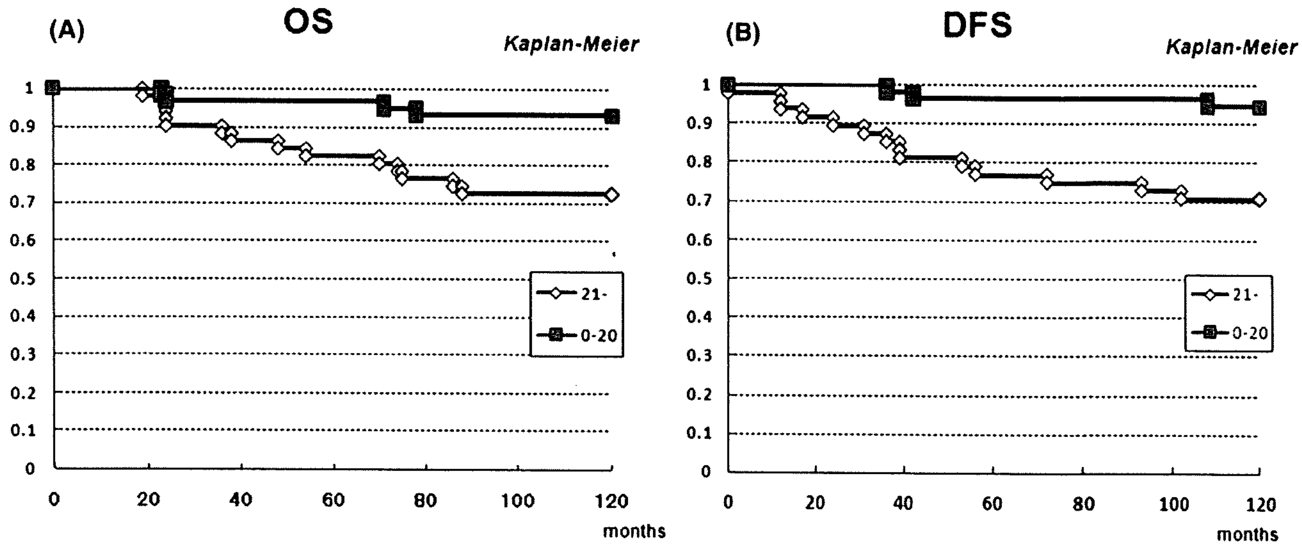


Fig. 3. (A) Result of the correlation between vasohibin-1-positive vessels and vascular endothelial growth factor (VEGF)-A expression in the tumor cells. (B) Result of the correlation between vasohibin-1-positive vessels and fibroblastic growth factor (FGF)-2 expression in the tumor cells. (C) Correlation between vasohibin-1 and Flk-1 in the 'hot spot'.



Vasohibin-1 positive vessels	10 year OS
0-20	0.932203
21-	0.72549

Vasohibin-1 positive vessels	10 year DFS
0-20	0.927356
21-	0.708333

Fig. 4. Summary of analysis of (A) overall survival and (B) disease free survival in relation to the status of vasohibin-1 expression. Patients were tentatively classified into two different groups according to the number of vasohibin-1-positive vessels: 0–20 and 21 or more.

DFS also revealed that vasohibin-1 was associated with VEGF-A (P = 0.004) and Ki-67 (P < 0.001), but was not associated with MVD (P = 0.081).

Double immunostaining with Ki-67 in microvessels. Ki-67/vasohibin-1 double immunostaining analysis demonstrated that Ki-67 labeling index of vasohibin-1-positive vessels was 46.5% (33.3–62.5%), whereas that of CD31-positive vessels was 23.5% (12.7–37.5%) (Fig. 5A,B).

Discussion

One of the most important functions of vasculature in general is to supply nutrients the distal organs. Three major types of regulation occur in the maintenance of vasculature: (i) vasodilation; (ii) changes in capillary permeability; and (iii) growth and development of new vessels, also known as angiogenesis.[24–26] Angiogenesis is a pivotal event in various biological processes

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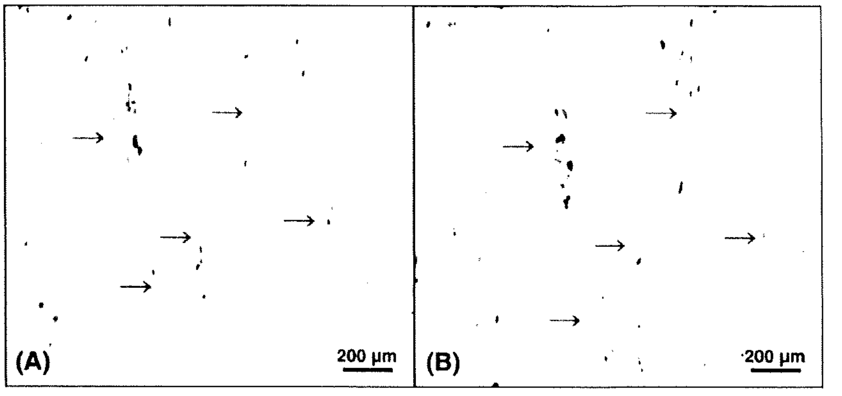

Fig. 5. Representative illustrations of double immunostaining for determining proliferating endothelial cells. (A) CD31/Ki-67 double staining; (B) vasohibin-1/Ki-67 double staining (arrow). (A) CD31 and (B) vasohibin-1 were colored blue, and Ki-67 was colored brown.

under both physiological and pathological conditions. Physiological conditions include embryonic development, reproduction and wound healing, and pathological conditions include cancers and inflammatory conditions.[2] *In situ* balance between angiogenesis stimulators such as VEGF and bFGF and inhibitors such as thrombospondin-1 (TSP-1) and pigment epithelium derived factor (PEDF) is generally considered to regulate the process of angiogenesis.[1] Negative feedback regulation is considered one of the most important physiological mechanisms with which bodies are endowed, and has been demonstrated to be involved in a wide range of biological phenomena.[27] This regulation is most effectively performed through the factors produced in endothelial cells but the endothelium-derived negative feedback regulators of angiogenesis have not been elucidated. Vasohibin-1 is therefore the first secretory anti-angiogenic factor from endothelial cells themselves induced by VEGF in EC.[2–4,28] The other anti-angiogenic regulator has been very recently identified and termed vasohibin-2 but this factor lacks the property of VEGF-A or bFGF inducibility in contrast to vasohibin-1.[28] Vasohibin-1 immunoreactivity was exclusively detected in endothelial cells in the present study, which is also consistent with results of previous studies of endometrial carcinoma[9] in lung carcinoma[3] and ischemic retina.[29] This is the first study to examine the status of vasohibin-1 in human breast disease in which angiogenesis also plays important roles in both physiological and pathological conditions.

Breast cancer has also been considered an angiogenic-dependent disease as in other human malignancies and angiogenesis has been demonstrated to play an essential role in breast cancer development, invasion and metastasis.[30–32] MVD assessed by CD31, CD34 and Factor VIII is generally considered as a gold-standard surrogate marker of tumor angiogenesis and has been also proposed by some investigators to identify patients at high risk of recurrence more precisely than classical indicators.[10,11]

In this study, we first examined how the vasohibin-1 expression was correlated to the MVD status. Vasohibin-1 immunodensity tended to be concordant with MVD in human breast tissues but they were not always parallel. The vasohibin-1 immunodensity was significantly higher in IDC than in DCIS but there was no difference of MVD between these two lesions. In addition, results of double immunostaining analysis which could simultaneously demonstrate two different proteins in the same cells, demonstrated the significant positive correlation between Ki-67-positive proliferating vascular endothelial cells, which may represent neovascular formation[16,17] and vasohibin-1-positive endothelial cells. Indeed, the Ki-67 labeling index among vasohibin-1-positive endothelial cells was significantly higher than Ki-67 in all CD31-positive endothelial cells. These results will clearly indicate that vasohibin-1 is considered a more appropriate biomarker for intratumoral neovascularization compared to CD31, which may detect all the vasculature including both resting and proliferating endothelial cells.

Results of our study also demonstrated the positive correlation between vasohibin-1 and VEGF-A or bFGF in carcinoma cells or Flk-1 in intratumoral endothelial cells, which also suggest that the vasohibin-1 in vasculature in human breast carcinoma is induced by VEGF-A, bFGF/Flk-1 signaling pathway. PKCδ was reported to play an important role in an induction of vasohibin-1 in endothelial cells.[4] Therefore, vasohibin-1 is supposed to be induced in the downstream of VEGF-A, bFGF/Flk-1 signaling pathway. Further investigations are necessary to reach the final conclusion.

The expression of vasohibin-1 in EC was proposed to be regulated either positively or negatively by certain factors at the transcriptional level, and this may influence the process of angiogenesis.[4] Another *in vivo* study also demonstrated the significantly positive correlation between vasohibin-1 and Flk-1 expression in vasculature of human endometrial carcinoma.[9] Significantly higher vasohibin-1 immunodensity in IDC than DCIS in our present study of human breast also indicate that the anti-angiogenic compensatory mechanism may be operational in invasive breast carcinoma, possibly in response to induction of angiogenesis by various factors related to carcinoma invasion into the surrounding stroma.

Results of several recent studies demonstrated the possible correlation between VEGF status in carcinoma cells and clinical outcome in breast cancer patients. VEGF was proposed to be correlated with worse DFS and overall survival rates especially in the patients with early-stage breast cancer.[33] VEGF expression in carcinoma cells was also reported as an independent prognostic marker in both node-positive and node-negative breast cancers.[34] Many previous immunohistochemical studies of MVD assessed by CD31, CD34 or Factor VIII antigen in human breast cancer demonstrated that high MVD in invasive ductal carcinoma is usually correlated with a greater likelihood of metastatic disease,[10] shorter relapse-free intervals and reduced overall survival in patients with node-negative breast cancer.[11] We therefore examined whether vasohibin-1 immunoreactivity is correlated with OS and DFS of the patients. Results of our study demonstrated that the cases with a higher number of vasohibin-1-positive vessels tended to be associated with better and statistically significant OS. In addition, a statistically negative or inverse correlation was detected between vasohibin-1 immunodensity and DFS. These results all suggest that an evaluation of the number of vasohibin-1-positive vessels may become one of the prognostic markers for metastasis and prognosis but it awaits further investigations to establish this approach as a surrogate marker such as MVD.

Recently, newer targeted therapies toward the control of tumor neovascularization such as anti-VEGF therapy have been developed in phase II and III clinical trials and demonstrated the clinical effects such as reduction of tumor angiogenesis and inhibition of solid tumors proliferation, either alone or in combination with chemotherapy.[35–38] In our present study, vasohibin-1 immunohistochemical staining was demonstrated to reasonably reflect the status of angiogenesis, and vasohibin-1 itself may be considered for anti-VEGF and anti-angiogenesis drugs to control tumor angiogenesis in future.

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ORIGINAL ARTICLE

Transcriptional silencing of ETS-1 efficiently suppresses angiogenesis of pancreatic cancer

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In this study, we addressed the hypothesis that transcriptional suppression of erythroblastosis virus E26 oncogene homolog 1 (ETS-1) is an efficient therapeutic approach to pancreatic adenocarcinoma by investigating the effect of ETS-1 suppression in human pancreatic cancer cells. We accomplished this by using an adenoviral vector encoding only the DNA-binding domain of wild-type ETS-1 (ETS-1 dominant negative, ETS-1-DN). ETS-1-DN decreases ETS-1-binding by competing for its binding to DNA. Adenoviral-mediated transfer of ETS-1-DN (adenoviral ETS-1-DN construct, AdETS-1-DN) into pancreatic tumor cell lines did not affect their proliferation rate *in vitro* but did significantly inhibit their *in vivo* growth in nude mice. Furthermore, to test the efficacy of ETS-1-DN *in vivo*, we injected the AdETS-1-DN into established human pancreatic adenocarcinomas grown in nude mice. This treatment significantly reduced tumor size as compared to saline injection, without any detectable side effects. Microvessel density in mouse xenografts displayed significantly lower values in tumors in which ETS-1 was downregulated. In addition, expression of the ETS-1-DN in the pancreatic cancer cells resulted in downregulation of urokinase-type plasminogen activator (u-PA) and metalloproteinase-1 (MMP-1) expression. Taken together, these data suggest that transcriptional inactivation of ETS-1 is able to significantly affect angiogenesis and growth of pancreatic cancer. This effect may be due in part to downregulation of MMP-1 and u-PA expression. Our results suggest that ETS-1-DN is a promising candidate for antiangiogenic gene therapy in pancreatic cancer.
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Keywords: ETS-1; angiogenesis; pancreatic cancer

Introduction

Although pancreatic ductal adenocarcinoma constitutes less than 2% of new cancer cases in the United States, it is the fifth leading cause of cancer-related deaths.[1] Considered by many to be one of the deadliest cancers, it is among the most studied malignancies and associated with a mean worldwide survival rate below 5%.[1,2] Although significant progress has been achieved with standard therapeutic approaches such as surgery, chemotherapy, or radiation, these approaches do not significantly improve the overall survival rate.[3] New vessel formation (angiogenesis) is a critical step in cancer progression. Clinical data have demonstrated that anti-vascular endothelial growth factor (VEGF) therapy with bevacizumab (an anti-VEGF specific antibody) is efficacious when combined with chemotherapy in colorectal and lung cancer.[4,5] Therefore, antiangiogenesis-based approaches aimed at preventing tumor vessel growth should be introduced to improve survival rates.[6]

However, a recent randomized phase 3 clinical study showed no benefit from bevacizumab in patients with pancreatic cancer. Thus, new antiangiogenic approaches are needed to treat this disease.

Controlled destruction of the principal barriers to tumor development and spread, that is, the basement membrane and extracellular matrix (ECM) compartments, is mostly influenced by the proteolytic activity surrounding a tumor mass. Although many factors regulate malignant tumor growth and spread, it is the protein profile within and interactions formed between a tumor and its microenvironment that are important for each step of tumor progression. The matrix metalloproteinases (MMPs) have recently been implicated in primary and metastatic tumor growth and angiogenesis;

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they also be involved in tumor promotion.[7] The *MMP* genes are transcriptionally responsive to a wide variety of oncogenes, growth factors, cytokines and hormones.[8] Notably, several recent studies have highlighted the erythroblastosis virus E26 oncogene homolog 1 (ETS-1) protein, which interacts with the urokinase-type plasminogen activator (*u-PA*) gene enhancer and with the promoters of the stromelysin-1 and *MMP* genes. The proto-oncogene ETS-1 is the cellular progenitor of v-ETS, a viral oncogene found in the genome of the E26 acute leukemia retrovirus.[9,10]

ETS-1 is the original member of a growing family of transcription factors that now includes over 50 members, each characterized by a conserved DNA-binding domain (EBD). The EBD binds to a consensus DNA sequence centered on a core GGAA/T motif, which has been designated as the ETS-binding site.[11] The genes for several proteins, including u-PA, MMP-1, MMP-3, MMP-9, integrin β-3, and VE-cadherin, have been reported to be downstream targets of ETS-1 in endothelial cell.[12,13] In addition to their importance in normal cellular function,[14] ETS products have also been implicated in several malignant and genetic disorders, based on their particular target genes. It has been reported that ETS-1 activity is correlated with the tumorigenic progression of carcinoma cells of the stomach,[15] thyroid,[16] and pancreas.[17] These findings have focused our attention on the potential role of ETS-1 as a multifunctional target for antiangiogenic gene therapy. Several studies have used EBD to downregulate ETS-1 activity in capillary endothelial cells or glioma cells. ETS-1 positively regulates angiogenesis, and the elimination of ETS-1 activity by a dominant-negative molecule inhibits angiogenesis *in vivo*.[18,19] Using a similar assay, downregulation of ETS-1 interfered with the expression of integrin α5, which is known to accelerate invasive events such as migration and adhesion in glioma cells.[20]

Our hypothesis in undertaking this study was that transcriptional suppression of ETS-1 is an efficient therapeutic approach for pancreatic adenocarcinoma.

In the present study, we have examined the role of ETS-1 downregulation using a gene therapy approach in an animal model in which transductions were carried out either *ex vivo* or *in vivo*. We report here the data demonstrating the efficacy of this approach for blocking angiogenesis, and we concomitantly gained insights into the mechanisms involved. These results suggest that targeting ETS-1 may be a valid approach for treating pancreatic cancer.

Materials and methods

Cell lines

The pancreatic cancer cell lines used were: BxPC3, Panc-1 (American Type Culture Collection, Rockville, MD) and PCI-35 (kindly provided by Dr Hiroshi Ishikura at Hokkaido University). These lines were cultured according to the protocol provided by the suppliers. All cell lines are well characterized in terms of mutational status, as described in a previous report from our group.[21] A normal pancreatic ductal cell line HPDEC-6, a kind gift from Dr MS Tsao, University of Toronto, was propagated as originally described.[22] All cells were routinely monitored for *Mycoplasma* contamination as well as for mouse hepatitis, Sendai, and pneumonia viruses, and the results were consistently negative.

Adenoviral-mediated gene transfer experiments

Suppression of the ETS-1-binding activity was performed using an adenoviral vector encoding only the His-6-tagged EBD (ETS-1-binding domain) but lacking the transactivation domain and most of the automodulation module (adenoviral ETS-1-DN construct, AdETS-1-DN). The adenoviral constructs and transfection conditions have been described previously.[19,23] Briefly, adenovirus vector encoding dominant-negative Ets-1 was constructed by homologous recombination in 293 cells between the transfer cassette bearing the expression unit of dominant-negative Ets-1 and almost the entire adenovirus genome and restriction enzyme-digested adenovirus genome tagged with terminal protein.[19] The adenovirus was applied at a concentration of 1 × 108 plaque-forming units (PFU)/ml, and adenovirus with the genome carrying an enhanced green fluorescent protein (*GFP*) gene (Clontech, Palo Alto, CA) or lacZ were used as controls as described.[19] Infection efficiency was monitored by fluorescence, which showed expression in 80% of cells. Expression of recombinant protein was confirmed by western blot analysis. Conversely, to overexpress ETS-1, we used a similar vector (adenoviral ETS-1, AdETS-1) that encodes a full-length, His6-tagged ETS-1 protein.[19] Gene transfer in tumor cells was carried out using adenoviral vectors at varying multiplicity of infections (MOI) from 10 to 100 for 36 h. The *LacZ* gene transfer (AdLacZ) efficiency was assessed using β-galactosidase staining as described elsewhere.[24] We used the following nomenclature for the infected cells: BxPC3/ETS-1, BxPC3/ETS-1-DN, BxPC3/LacZ, PCI-35/ETS-1, PCI-35/ETS-1-DN, PCI-35/LacZ, Panc-1/ETS-1, Panc-1/ETS-1-DN, and Panc-1/LacZ. His6-tagged ETS-1 was detected by immunocytochemistry using an anti-His6 antibody (Sigma Chemical Co., St Louis, MO), according to the provider's protocol.

RT–PCR

Reverse transcription (RT)–PCR was performed using methods described previously.[25,26] Expression of the *hMSH2* gene was monitored as a positive control as the gene was demonstrated to be expressed in all parental cells.[27]

Samples of 29 primary human pancreatic cancers as well as the corresponding normal pancreatic tissues were also used. These tissues were obtained at Tohoku University Hospital with informed consent and were reviewed by a board-approved pathologist. The protocol was approved by the Ethical Board of Tohoku University Hospital.

Northern blotting
Extracted total RNA (10 μg) was subjected to electrophoresis on a 1% agarose gel containing 5% formalin and then transferred to a Hybond N+ membrane (Amersham, Sweden).[6] We also used human MTN blot membranes containing 16 tissues (Clontech). Probes for ETS-1 and β-actin were obtained from RT–PCR products. Direct sequencing using ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction kit and a 310 DNA sequencer (Applied Biosystems, Foster City, CA) confirmed the sequences of these fragments. Digital autoradiography and quantification of the results were carried out using the BAS 1500 and Image Gauge 3.3 (Fujifilm, Japan) software.

Immunoblotting analysis
Western blotting was performed using 15–20 μg of total lysate as previously described.[28] Conditioned media were obtained, stored, and analyzed as previously described.[29] Primary antibodies used were: polyclonal rabbit anti-human ETS-1, goat anti-human MMP2 and MMP9 (Santa Cruz Laboratories, Santa Cruz, CA), monoclonal mouse anti-human β-actin (Sigma, St Louis, MO) anti-His6, and rabbit anti-human VEGF (Sigma). Secondary antibodies employed were: swine anti-rabbit, swine –anti-goat (Tago Inc., Burlingame, CA) and a sheep anti-mouse peroxidase-conjugated antibody (Amersham, Buckinghamshire, UK). A total lysate of HPDEC-6 was used as a positive control. Immunoreactivity was subsequently demonstrated using the enhanced chemiluminescence ECL western blotting kit (Amersham). The relative intensity of signals was analyzed using the Luminescent Image Analyzer LAS-1000 Plus and Image software Gauge 3.3 (FUJI Photo Film, Co. Ltd, Kanagawa, Japan).

DNA-binding activity, dual luciferase assay and electrophoretic mobility shift assay
DNA-binding assays for ETS-1 proteins were performed with 10 μg of nuclear extract, as described previously.[30] Competition assays were performed with a hundred-fold excess of unlabeled wild-type (WT) or mutant SBE (MT) oligonucleotides. For supershift assays, the nuclear extracts were incubated with 2 μl of ETS-1 antibodies for 30 min at room temperature before the addition of a labeled probe. Reactions were analyzed on a native 4% polyacrylamide gel and exposed to an imaging plate, which was then analyzed with a BAS 2000 image analyzer (Fuji, Tokyo, Japan).

Transfections and luciferase assays
Luciferase assays were conducted as described previously.[31] Briefly, the reporter construct was generated by inserting five head-to-tail ligated copies of the oligo 5′-TCGAGCAGGAAGTTTCG-3′, which contained the PEA3 ETS-binding site from the polyoma virus enhancer, into the pGL3-basic vector, which encoded firefly luciferase (FLS; Promega, Madison, WI). The cells were infected with AdETS-1, AdETS-1-DN or AdLacZ for 36 h at an MOI of 50. Each cell line was then seeded at 0.5×10^5 cells per 10-mm dish and incubated overnight at 37 °C in an incubator containing 5% CO_2. For each transfection, 0.1–0.3 μg of empty vector (pcDNA3; Invitrogen, Carlsbad, CA) and/or pRL-TK (Promega), along with 0.5–1 μg of promoter-luciferase DNA, were mixed together in 50 μl of Opti-MEM (Life Technologies Inc.), and a precipitate formed using LipofectAMINE 2000 (Life Technologies Inc.), according to the manufacturer's recommendations. The empty vector, pcDNA3, was used as a control for normalizing to total plasmid DNA. Cells were washed with Opti-MEM, and complexes were applied to the cells. After 36 h, *FLS* and renilla luciferase (*RLS*) gene activities in extracts from triplicate samples were sequentially measured by using a Dual Luciferase Reporter Assay System (Promega), according to the manufacturer's protocol. Measurements were performed automatically in a Luminescencer (Lumat LB 9507, Berthold, Germany), and results were expressed as fold increase compared to control cells.

In vitro *proliferation assays*
Anchorage-dependent proliferation was monitored by an 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay for 5 days, and a daily proliferation index was calculated for each parental and corresponding hybrid cell line using previously described methods.[32,33] The conversion of MTT to formazan dye was spectrometrically measured for absorbance at 590 nm, using a multi-well plate ImmunoReader System. All experiments were performed in duplicate sets of eight and repeated at least twice. Anchorage-independent proliferation (colony formation assay) was assessed in triplicate by two independent experiments as previously described.[31] The viable colonies were photographed using a Zeiss microscope with ×5 objective. Both colony number and size were measured and averaged using three randomly chosen photographs from each plate, employing NIH 1.62 software. Data were pooled, averaged and then statistically analyzed.

In vivo *proliferation assays*
Tissue sections of 5 μm thickness were prepared from formalin-fixed paraffin-embedded specimens. Immunohistochemical reactions were performed using a mouse anti-proliferating cell nuclear antigen (anti-PCNA, clone PC10, Dako Corporation) and developed with a Zymed Immunomouse kit (San Francisco, CA), as described previously.[34]

Proliferating cells were quantified by counting the PNCA-positive cells as well as the total cells in 10 arbitrarily selected fields (×40 magnification) in a double-blinded manner. The percentage of PCNA-positive cells per 10 fields was calculated according to the following formula: (number of PCNA-positive cells)/(total number of cells) × 100. Negative control slides were prepared by omitting the primary antibody.

Determination of apoptosis
Apoptotic cells were detected by Annexin V/EGFP staining using an ApoAlert Annexin V-EGFP kit

obtained from Clontech. Stained cells were quantified using a Becton Dickinson FACScan, and data were analyzed using CellQuest software (version 3.1, Becton Dickinson).

Invasion assays

Invasion through Matrigel-reconstituted basement membrane was induced by seeding 5000 cells into the upper compartment of the invasion chamber (Becton Dickinson Labware, Franklin Lakes, NJ) using serum-free medium and allowing the cells to invade for 18 h.[29] The invading cells were stained and photographed (× 100 magnification). Cells were counted and averaged, and the data were expressed as the number of migrated cells per high-power field. All experiments were performed in triplicate.

In vivo *gene therapy experiments*

Eight-week-old, male, severe-combined immunodeficient nude mice (Clea Japan Inc., Tokyo, Japan) were maintained under pathogen-free conditions and used in accordance with NIH guidelines. For these experiments, an animal model was employed in which transductions were carried out either *ex vivo* or *in vivo*. In the *ex vivo* transduction model, gene transfer in PCI-35 and Panc-1 cells was achieved using adenoviral vectors encoding for the gene *ETS-1*, *ETS-1/DN*, or *LacZ* for 36 h at an MOI ranging from 50 to 100. Logarithmically growing cells trypsinized from subconfluent monolayers were suspended in medium containing 25% Matrigel Growth Factor Reduced (Becton Dickinson Labware) at a density of 1×10^7 cells per ml. For each inoculation, 3×10^6 cells in 0.3 ml of suspension were injected s.c. into the hind flanks of five nude mice.[33] Data from independent experiments were pooled for statistical analysis. At week 8, when the control tumors reached approximately 2000 mm^3, the mice were killed. For the *in vivo* transduction we used a preestablished xenograft model. Cells were implanted by s.c. injection of 3×10^6 cells in 300 μl of culture medium containing 10% matrigel into the dorsal surface of the 15 mice. Tumors were allowed to develop for 5–7 days until a volume of approximately 100 mm^3 was reached, and then the mice were divided into three experimental groups. Each mouse received intratumoral (i.t.) inoculation with excipient only (phosphate-buffered saline), Ad/LacZ or Ad/ETS-1-DN (10^{10} PFU in 50 μl excipient) using a 32-gauge needle. Every animal received five i.t. injections spaced at 5-day intervals. The tumor diameters were measured before each injection with the use of a caliper. Mice were weighted and observed biweekly for monitoring of detectable side effects. One week after treatment was completed, the mice were killed and the tumors were excised for tissue sections.

Immunohistochemical analysis

Tumor samples were fixed overnight in phospholysine-paraformaldehyde, embedded in optimal cutting temperature compound (Sakura Finetechnical Co., Ltd., Tokyo, Japan) and stored at −80 °C. Tumor samples fixed in 10% formalin and then embedded in paraffin were also used. Tumors grown in nude mice as well as 29 primary human pancreatic tumors were immunostained using rabbit anti-human ETS-1 antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA) as previously described.[17] The degree of ETS-1 expression in the human samples was assessed according to a previously reported method.[17,35] All sections were reviewed independently by pathologists blinded to all clinical and pathologic information (TF and MA). The expression was graded on a scale from 0 to 3. A grade of 0 represented no stain uptake by malignant cells. Neoplastic cells were considered positive when they revealed cytoplasmic or membrane staining of at least moderate intensity and were graded as follows: grade 1, 1–33% positive cells; grade 2, 34–66% positive cells and grade 3, >66% cells positive. Normal ductal structures admixed in some tumor samples did not stain and served as internal negative controls. ETS-1 expression was considered positive when at least one of the pancreatic tissue intensity in >5% of pancreatic cancer cells. Immunohistochemical staining with an anti-PECAM-1 antibody (clone MEC13.3; Pharmingen, San Diego, CA) was performed as previously described.[36] Microvessel density was estimated by counting five non-overlapping areas of tumor infiltration (1 mm^2 area) and the data are presented as means of relative microvessel structures area (in pixels) ± s.d. The immunostaining of gelatinases (MMP-1 and MMP-2) was performed as previously described,[37,38] using goat anti-mouse antibodies (Santa Cruz Biotechnology Inc.).

Statistical analysis

A two-tailed Student's *t*-test was computed by GraphPad Prism 3.0 software (GraphPad Software Inc., San Diego, CA) and used to determine the statistical significance of measured differences. Statistical significance was judged based on *P*-values <0.05.

Results

Expression of ETS-1 in human pancreatic cancer tissues and cell lines

The status of ETS-1 expression was defined using three pancreatic cancer cell lines (BxPC3, Panc-1 and PCI-35), a normal pancreatic ductal cell line (HPDEC-6) and 29 different pancreatic cancer specimens from our tissue library. Endogenous ETS-1 was expressed in all the pancreatic cancer cells, based on mRNA (Figures 1a–c) or protein analysis (Figure 1d). In addition, apparently higher ETS-1 levels were found in SMAD4-null cell lines (Figures 1a–c, lines 1–2), in accordance with our previous findings.[6] The ETS-1 protein levels in human tissue samples appeared to correlate with tumor histology as previously reported.[17] ETS-1 expression was barely detectable in normal pancreatic ductal tissue (Figure 1f). However, whole RNA pancreatic extract (Figure 1e) revealed a relatively abundant expression of ETS-1. This differential expression was confirmed *in vitro* using cultured pancreatic ductal cells (HPDEC-6, Figure 1a, line 4). Remarkably, ETS-1 expression appeared stronger in the islets as compared with either normal or pancreatic

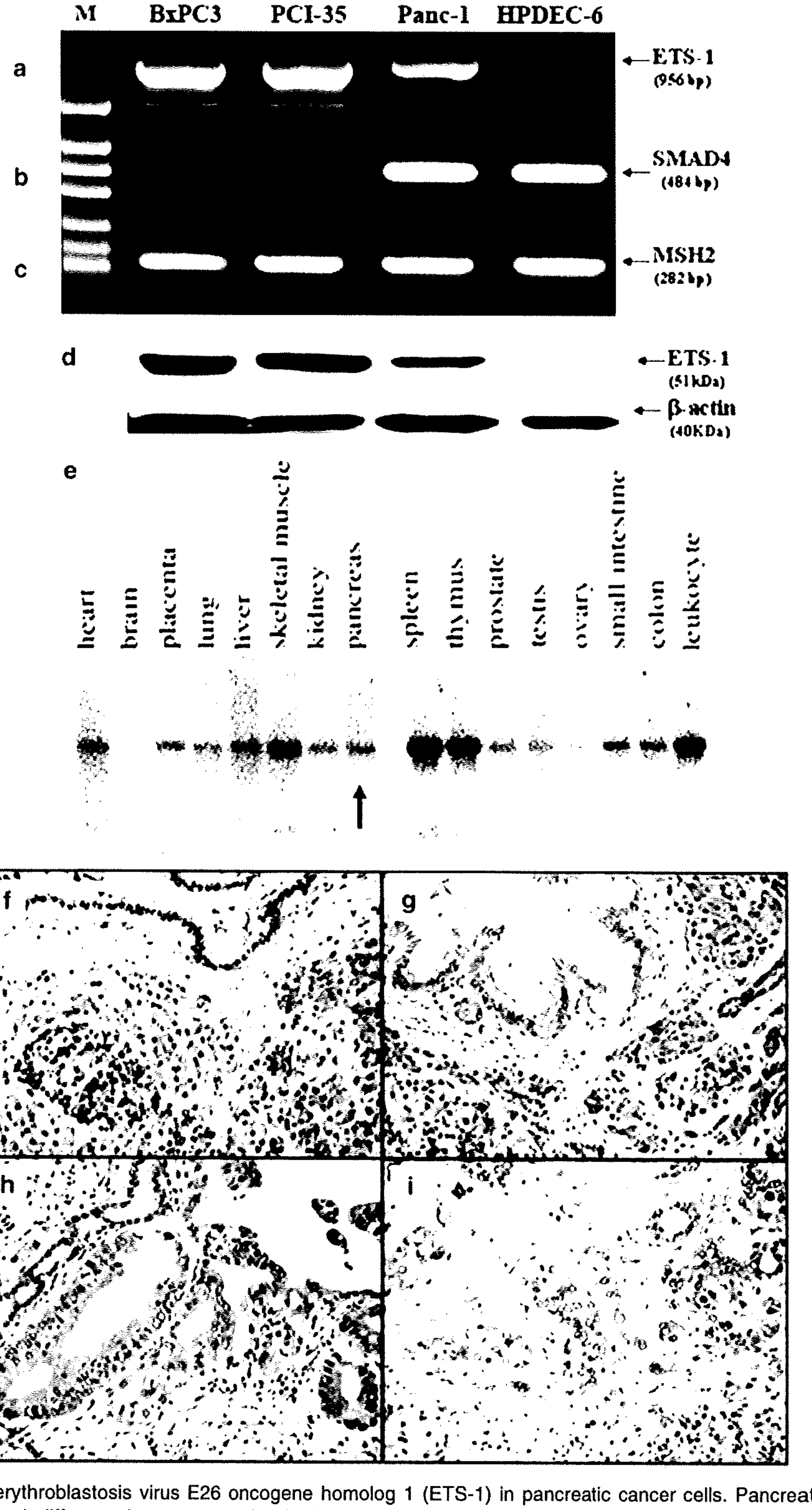

Figure 1 Expression of erythroblastosis virus E26 oncogene homolog 1 (ETS-1) in pancreatic cancer cells. Pancreatic cancer cells (**a–d**), normal pancreas (**e**, **f**), and different tissue types: dysplastic (**g**), moderate (**h**) and poorly (**i**) differentiated pancreatic cancers. PCR amplifications, immunostaining and western blotting were performed using anti-human ETS-1 antibodies as described in Materials and methods section. Original magnifications were × 400. Lane 1, BxPC3; lane 2, PCI 35; lane 3, Panc-1; lane 4, HPDEC-6. ETS-1 RNA expression in normal human tissues (**e**). The MTN RNA membranes containing 16 different normal tissues (Clontech) were probed with ^{32}P-labeled ETS-1 cDNA. Probes for ETS-1 and β-actin were obtained from reverse transcription (RT)–PCR products.

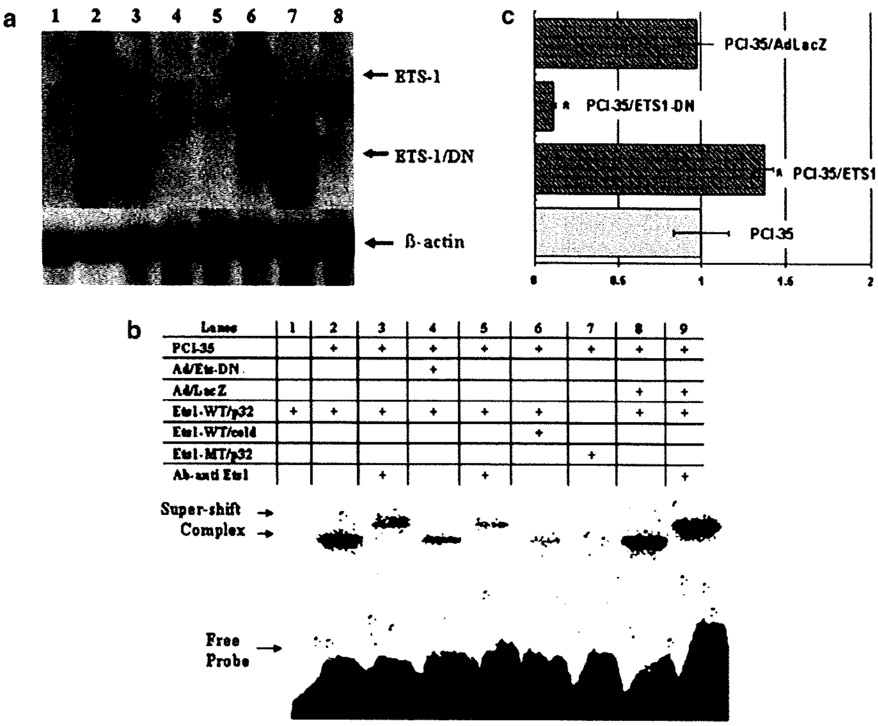

Figure 2 Pancreatic cancer expressing ETS-1 dominant negative (ETS-1-DN). Northern blotting analysis (**a**) was performed using 10 μg of total RNA, showing erythroblastosis virus E26 oncogene homolog 1 (ETS-1) or ETS-1-DN at the indicated levels. Lanes: 1, Panc-1; 2, Panc-1/ETS-1; 3, Panc-1/ETS-1-DN; 4, Panc-1/LacZ; 5, PCI-35; 6, PCI-35/ETS-1; 7, PCI-35/ETS-1-DN; and 8, PCI-35/LacZ. Electrophoretic mobility shift assays (**b**) were performed using an ETS-1-binding probe as described in Materials and methods section. Lanes: 1, unbound probe; 2 and 3, PCI-35/LacZ, 4 and 5, PCI-35/ETS-1-DN, 6 and 7, normal and mutant competitor, respectively. Transactivation of ETS-1 promoter activity (**c**) was accomplished by exogenously adding ETS-1 and ETS-1-DN. The indicated cells were infected with adenoviral ETS-1, adenoviral ETS-1-DN construct or AdLacZ for 36 h at an MOI of 50. Cells were subsequently transfected with pRL-TK (0.25 μg) and 1 μg of ETS-1 expression construct or control pcDNA3 vector, pcDNA3. Luciferase assays were performed after 36 h, and activity is reported as fold induction. Bars, ± s.d. in triplicate assays.

cancer tissues. Among the 29 cases of pancreatic adenocarcinoma, 24 (70.5%) showed positive staining for the ETS-1 protein. Of these, 18 had grade 2 expression and 6 had grade 3 expression. Overall, papillary carcinoma, well-differentiated adenocarcinoma (Figure 1g), and moderately differentiated adenocarcinoma (Figure 1h) expressed gradually higher levels of ETS-1. In contrast, poorly differentiated adenocarcinoma (six cases had grade 0 expression) expressed relatively low levels of ETS-1 (Figure 1i), suggesting that ETS-1 may play a more important role in the early stages of pancreatic tumorigenesis.

Pancreatic cancer cells expressing ETS-1-DN

Suppression of the ETS-1-binding activity was performed using an adenoviral vector encoding the His-6-tagged EBD but lacking the transactivation domain and most of automodulation module (AdETS-1-DN). The expression of exogenous ETS-1-DN RNA was first confirmed by northern blotting, demonstrating expression in transfected cells but not in noninfected or control virus-infected cells. Endogenous ETS-1 RNA was also detected in all parental and infected cells. The RNA levels of ETS-1-DN in PCI-35/ETS-1-DN and Panc-1/ETS-1-DN were significantly higher than those of endogenous ETS-1 (approximately 7- to 8-fold; Figure 2a). To examine the DNA-binding activity of the ectopically expressed ETS-1-DN protein, we performed electrophoretic mobility assays using a ^{32}P-labeled double-stranded ETS-1 probe, as previously described.[30] ETS-1-specific DNA-binding activities were detected to a similar extent in both WT and control cells (Figure 2b, lanes 2 and 8). Furthermore, ETS-1 specific DNA-binding activities appeared markedly reduced in the ETS-1-DN-infected cells (lane 4) when compared with those of parental or control infected cells (lanes 2 and 8). The cold competition experiments showed that the ETS-1/DNA-binding activity was specific (lanes 6), and super-shift assays showed that the DNA-binding consisted of ETS-1 (lanes 3, 5 and 9). Moreover, a mutant ^{32}P-labeled ETS-1 probe failed to exhibit any binding activity (lane 7).

The ETS-1 transactivation activity was further analyzed using a luciferase construct containing an ETS-1-responsive promoter region. As shown in Figure 2c, ETS-1-DN significantly inhibited the transactivation activity of the construct. Taken together, these data show that suppression of functional activity of ETS-1 can be

Table 1 Oncogenic properties of the ETS-1-DN transfectants

Properties	*Cell lines*			
	PCI-35	*PCI-35/ETS-1-DN*	*PCI-35/ETS-1*	*PCI-35/AdLacZ*
(A) Anchorage-independent growth in soft agar, proliferation, invasion and apoptosis index. The averaged results ± s.d. from three independent experiments are represented[a]				
Proliferation index	9.3 ± 1.1	8.9 ± 0.95	9.6 ± 1.31	9.4 ± 1.21
Colony formation (number per 3-cm dish)	227 ± 19.13	231 ± 13.62	238 ± 18.61	225 ± 12.29
Colony size (μm)	384 ± 32.12	378.4 ± 16.21	381 ± 32.93	381 ± 29.66
Matrigel invasion assay[b]	1	0.38 ± 0.11	1.17 ± 0.14	0.97 ± 0.17
Apoptosis index (%)	2.81 ± 0.4	3.11 ± 0.5	2.97 ± 0.3	2.7 ± 0.2
(B) Tumorigenity and proliferating index of parental and hybrid cells in nude mice[c]				
Size of tumors (mm^3 at day 32)	1665 ± 245	356 ± 98	1716 ± 168	1732 ± 39
Latency period (days)	8 ± 2	14 ± 3	9 ± 3	8 ± 3
Microvessel area[d]	2231 ± 121	875 ± 87	2288 ± 106	2175 ± 112
Microvessel density	71 ± 9.6	22 ± 4.9	77 ± 11.6	79 ± 14.1
Proliferating index (PCNA positive cells per × 40 field)	17.7 ± 5.6	16.2 ± 5.1	16.7 ± 6.3	17.1 ± 4.4

Abbreviation: PCNA, proliferating cell nuclear antigen.

[a]The colony number and size were measured and averaged from three randomly chosen photographs of each plate, using NIH 1.62 software. Apoptotic cells were detected by Annexin V/EGFP staining. Data were quantified using a FACScan and analyzed using the CellQuest software.

[b]The invading cells were counted, averaged, and compared to the control cells.

[c]Representative averaged results ± s.d. from the triplicate experiments are shown. The latency period is defined as the period prior to tumors becoming palpable (about 5 mm in diameter).

[d]Microvessel area was quantified by HIH 1.62 software, and the data are presented as means of relative microvessel structures area (in pixels) ± s.d. Microvessel density was estimated by counting five non-overlapping areas of tumor infiltration (1 mm^2 area).

efficiently achieved by adenoviral-mediated gene transfer of a dominant-negative molecule.

ETS-1-DN reduces the vascular density and growth of pancreatic cancer xenografts but has no anti-tumor effect in vitro

Adenoviral-mediated transfer of ETS-1-DN (using AdETS-1-DN) in pancreatic tumor cell lines did not significantly affect their proliferation or degree of apoptosis *in vitro* (Table 1). Nevertheless, AdETS-1-DN-infected cells exhibited a significant decrease in their invasiveness through the matrigel-reconstituted basement membrane, as compared with parental control cells (Table 1). In contrast to the *in vitro* effect, ETS-1 downregulation significantly inhibited the *in vivo* tumor growth in nude mice injected with PCI-35 cells transduced with adenoviral vectors encoding ETS-1-DN, or LacZ (Figure 3a).

In an attempt to test the efficacy of ETS-1-DN as an *in vivo* gene therapy agent, we directly delivered the AdETS-1-DN into preestablished human pancreatic adenocarcinomas grown in nude mice. After the fifth injection, there was a nearly 70% reduction of tumor size in the AdETS-1-DN-treated group, as compared with the saline-injected control group ($P<0.05$), without detectable side effects (Figures 3b–d).

Microvessel density in mouse xenografts demonstrated significantly lower values in tumors with downregulated ETS-1 (Figures 4a and b). A 48% reduction ($P=0.026$) in microvessel density was seen in ETS-1-DN-treated tumors as compared with control cells. The expression of PCNA, a nuclear marker of cell proliferation, showed no significant difference between treated and control tumors (Table 1).

Transcriptional changes in pancreatic cancer cells upon ETS-1 blockade

The autocrine effect of ETS-1-DN in cancer cells was investigated by testing the changes in transcription of a panel of 10 known targets of ETS-1, such as VEGF, basic fibroblast growth factor (bFGF; data not shown), and proteases. Among these, expression of MMP-1 and u-PA were significantly downregulated by transcriptional suppression of ETS-1 (Figures 5a and b). This suggests that transcriptional inhibition of ETS-1 elicits antiangiogenic effects through dysregulation of proteolytic factor gene expression downstream of ETS-1 signaling.

Discussion

ETS transcription factors regulate many genes associated with tumor invasion, angiogenesis, cell adhesion, and organ development. The ETS family is comprised of more than 30 members that share a highly conserved DNA-binding motif termed the ETS domain.[39] In endothelial cells, the ETS-1-DNA-binding site has been reported to be included within the functional promoter site of many genes, including those encoding ECM-degrading enzymes like u-PA, MMP-1, MMP-3, MMP-9 and their inhibitors. Among them, the expression of several MMPs as well as u-PA was correlated with the progression of various cancers.[40,41]

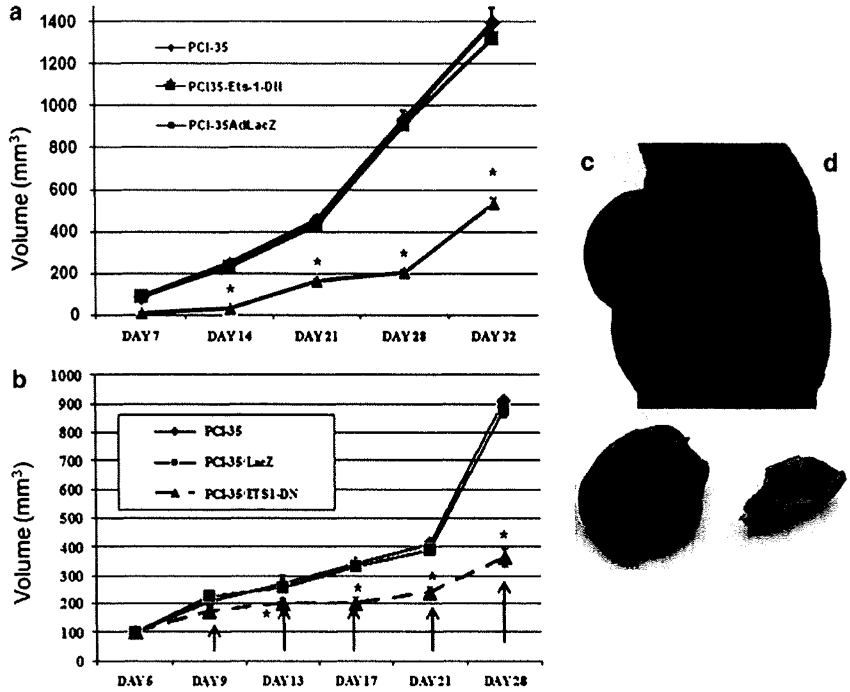

Figure 3 *In vivo* experiments. Logarithmically growing cells were suspended in medium containing 25% Matrigel. For each inoculation, 3×10^6 PCI-35 cells were transduced with adenoviral vectors encoding for erythroblastosis virus E26 oncogene homolog 1 (ETS-1), ETS-1 dominant negative (ETS-1/DN), or LacZ in a 0.3 ml suspension and injected s.c. into the hind flanks of nude mice. At week 8, when the control tumors reached approximately 2000 mm^3, the mice were killed (**a**). For the *in vivo* transduction therapy experiments, tumors were generated by s.c. injection of 3×10^6 cells in 300 µl of culture medium containing 10% Matrigel into the dorsal surface of mice (**b–d**). Tumors were allowed to develop for 5–7 days to about 100 mm^3 volume, and then mice were divided into three experimental groups. Each mouse received intratumoral (i.t.) inoculation with either excipient only (phosphate-buffered saline), Ad/LacZ, or Ad/ETS-1-DN (10^{10} PFU in 50 µl excipient). Each animal received five i.t. injections spaced at 5-day intervals. The tumor diameters were measured using a caliper before each administration. Data from independent experiments were pooled for statistical analysis.

Upregulation of ETS-1 expression has been documented in many types of human tumors. Generally, expression levels of ETS-1 correlate well with the grade of invasiveness and metastasis[42,43] and can therefore be useful for predicting the prognosis of cancer patients. It has been reported that expression of ETS-1 is correlated with the progression of carcinoma cells of the stomach,[15] thyroid,[16] and pancreas.[17] ETS-1 possesses a typical oncogene profile as judged by the correlation of its overexpression with the histological stages of many cancers, including glioma[20] and pancreatic cancer.[17] Although ETS-1 is strongly expressed in the moderately and well-differentiated pancreatic lesions, our data reveal that its levels were lower in poorly differentiated pancreatic cancers, suggesting that ETS-1 is inactivated by an unknown mechanism in these lesions. However, the latter quality was established by examining tumors derived from pancreatic cancer cell lines. Specifically, BxPC-3 produces moderately well-differentiated adenocarcinomas,[44] whereas Panc-1 maintains a poorly differentiated phenotype.[45] Furthermore, in an orthotopic pancreatic cancer model, marked differences with regard to tumor size, metastatic spread, and survival were found, depending on the grade of differentiation. Namely, less differentiated cells (Panc-1) caused higher dissemination scores and mortality than cells displaying greater differentiation. However, it is conceivable that the development of efficacious therapeutic treatments for human cancers relies significantly upon the presence and activity of the primary oncogene target.[46] In our study, the tumoral regression observed with local administration of Ad/ETS-1-DN appears to be significantly higher when the ETS-1 is abundantly expressed (Figure 1). However, IHC revealed that ETS-1 appears to be expressed more strongly in islets than in normal pancreatic ductal tissue or HPDEC-6 cells. To our knowledge, the significance, if any, of ETS-1 expression in the pancreatic islets is not known, and further focused studies may elucidate this phenomenon.

Expression of genes encoding for enzymes involved in degradation of the ECM, such as MMP-1, MMP-3, MMP-7 and MMP-9, is regulated by ETS family proteins such as ETS-1. Hence, it is highly suggestive that ETS-1 contributes to tumor invasion and progression through activation of these enzymes. Indeed, expression of these ECM remodeling enzymes is often detected along with c-ETS-1 mRNA in tumor cells, a phenomenon that correlates with poor survival in human ovarian carcinoma.[47] Moreover, ETS-1 is upregulated in human hepatocellular carcinoma (HCC), where it enhances expression of MMP-7, thus potentially contributing to the progres-

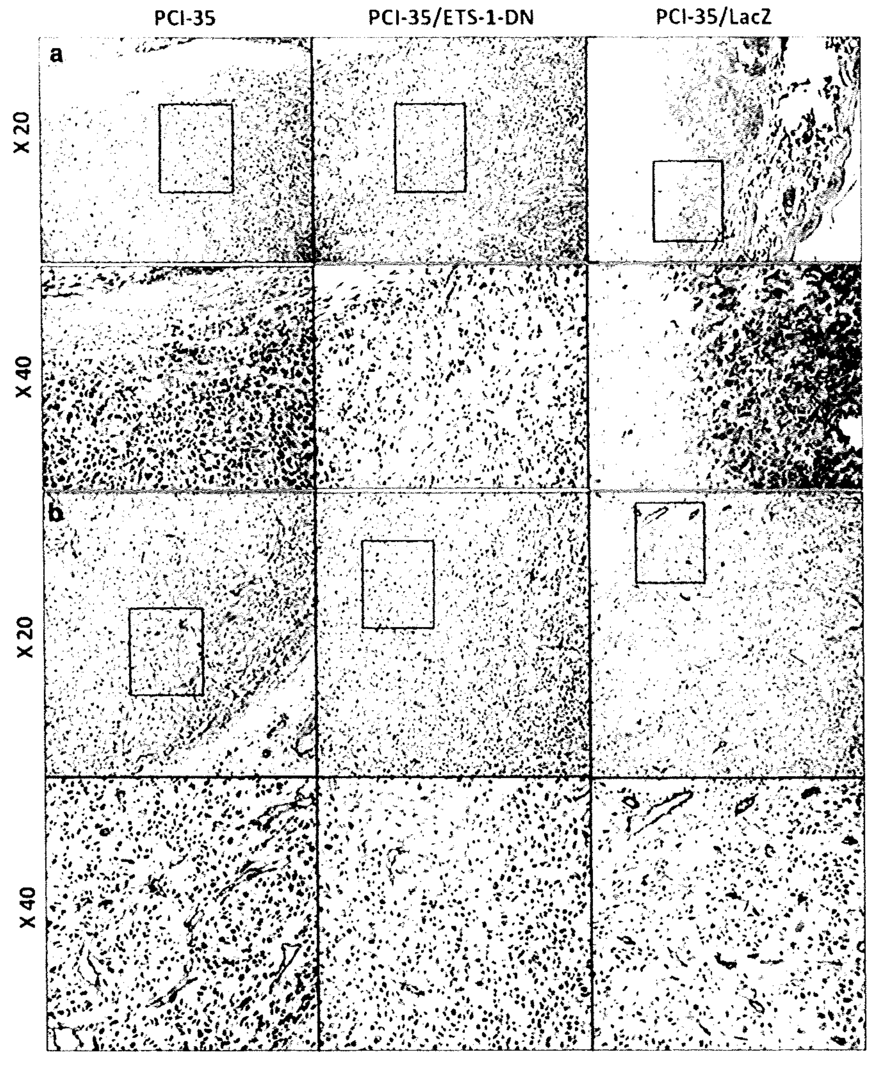

Figure 4 Representative data from immunohistochemical analysis of pancreatic cancer xenografts. Cells were infected as described in Materials and methods section and then inoculated s.c. in nude mice. At day 30, mice were killed and their tumors excised. Sections were stained with polyclonal rabbit anti-erythroblastosis virus E26 oncogene homolog 1 (ETS-1) antibody. A significant decrease of ETS-1 immunoreactivity was observed in ETS-1 dominant negative (ETS-1-DN) as compared with wild-type and ETS-1 tumors (**a**). Microvessel density was estimated using PECAM-1 reactivity. A 48% reduction in microvessel density was seen in ETS-1-DN-treated tumors as compared with control cells (**b**). Original magnifications are indicated on borders and the squares show the subsequently magnified area.

sion of HCC.[48] Although many potential target genes for the ETS family proteins have been identified, most studies have been carried out with the use of *in vitro* assays exclusively. Several studies used a truncated molecule containing EBD to downregulate ETS activity in capillary endothelial or glioma cells.[19,20] Hence, ETS-1 positively regulates angiogenesis, and using a dominant-negative molecule to eliminate ETS-1 activity resulted in angiogenesis inhibition *in vivo*. Consequently, we hypothesized that ETS-1 may be a useful target for gene therapy to combat the progression of pancreatic adenocarcinomas.

In this study, we have shown that ETS-1 is an effective target for gene therapy in pancreatic cancer using an animal model in which transductions were carried out either *ex vivo* or *in vivo*. We have investigated the effect of an ETS-1-DN molecule in a panel of three pancreatic cancer cells using *ex vivo* and *in vivo* gene therapy models. Adenoviral-mediated transfer of ETS-1-DN in pancreatic tumor cell lines did not affect their proliferation rate *in vitro*, but significantly delayed their *in vivo* growth in both nude mouse xenograft models used in this study. Our data clearly indicate that the ETS-1-DN molecule acted in a dominant-negative manner against ETS-1 by competing for its DNA-binding activity. In our system, the inhibition of the endogenous ETS-1 could not be completely abolished, results similar to those reported by Nakano *et al.*[19] using stable ETS-1-DN stable transfectants. This could be because of the use of transient transfections or to an autocrine loop that is able to restore ETS-1-binding activity. ETS-1 is also involved in angiogenesis, which is

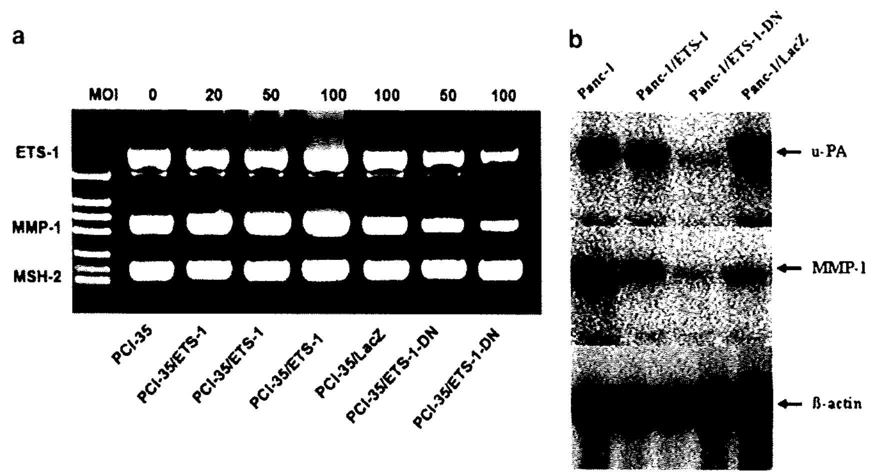

Figure 5 mRNA levels of metalloproteinase-1 (MMP-1) and urokinase-type plasminogen activator (u-PA). The mRNA levels of MMP-1 and u-PA were downregulated following adenoviral-mediated expression of the ETS-1 dominant negative (ETS-1-DN) molecule. PCI-35 and Panc-1 cells were infected with AdLacZ, AdETS-1, or AdETS-1-DN at the indicated MOI for 36 h. Reverse transcription (RT)–PCR showed a MOI-dependent downregulation of MMP-1 following ETS-1 transcriptional suppression (**a**). Panc-1 cells were infected with the above vectors at an MOI of 50 for 36 h, and then 10 μg of total RNA were subjected to northern blotting analysis (**b**). The blot was probed sequentially with u-PA, MMP-1 and β-actin probes.

essential for tumor progression. In fact, induction of ETS-1 expression appears to be a common phenomenon in endothelial cells following stimulation by angiogenic growth factors.[30] Angiogenesis is a necessary and required step for the transition from a small harmless cluster of cells to a large tumor, as well as for independent metastases.[49] We achieved a significant suppression of the tumor-induced angiogenesis by curtailing ETS-1-binding activity. It has been shown that VEGF and bFGF induce ETS-1 in endothelial cells, and ETS-1 then confers an angiogenic phenotype to endothelial cells through induction of u-PA and MMPs. In support of previous studies, our findings clearly indicate that the ETS-1-DN antiangiogenic effect is partly mediated through downregulation of MMP-1 and u-PA, both known downstream targets of ETS-1. Intriguingly, the expression levels of angiogenic factors such as VEGF, bFGF, MMP-2 and MMP-9, all well-known ETS-1 targets, were found to be unchanged by the experimental suppression of ETS-1 activity (data not shown). Nonetheless, such discrepancies appear to be based on differences in cellular make-up, which may include interactions between positive and negative regulators specific to different cell types.[50] For example, ETS-1 induces Fli-1 in endothelial cells but not in fibroblasts.[51]

Although demonstrating the efficiency of ETS-1-DN as an antiangiogenic agent, this study raises important questions regarding the deregulated targets downstream of ETS-1. Here, we demonstrated that downregulation of MMP-1 and u-PA occurs after transcriptional repression of ETS-1. It is conceivable that other known or unknown targets could be affected by the ETS-1 signaling cascade, and further mechanistic studies are warranted. However, the relevance of these future studies will depend upon the recognition of all the facets of the physiological roles of these transcription factors. Moreover, because the binding of ETS-1 to various ETS targets in certain tissues depends on the particular cellular context, future studies should identify the *in vivo* targets, including known or novel genes, in specific cell lineages. To this end, we are employing microarrays designed to reveal new targets downstream of ETS-1 signaling in pancreatic cancer.

Taken together, these results suggest that eliminating the ETS-1-binding activity efficiently suppresses the angiogenic and invasive abilities of pancreatic cancer cells. Moreover, this study indicates that ETS-1 should be considered a potential anticancer target for *in vivo* gene therapy in pancreatic cancer.

Abbreviations

AdETS-1-DN, adenoviral ETS-1-DN construct; EBD, ETS-1 DNA-binding domain; ETS-1, erythroblastosis virus E26 oncogene homolog 1; ETS-1-DN, ETS-1 dominant negative.

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RESEARCH LETTER

Vitreous levels of vasohibin-1 and vascular endothelial growth factor in patients with proliferative diabetic retinopathy

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Abbreviations

PDR proliferative diabetic retinopathy
PEDF pigment epithelium-derived factor
VEGF vascular endothelial growth factor

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To the Editor: Intraocular neovascularisation develops in many ischaemic retinal diseases, e.g. diabetic retinopathy, ischaemic retinal vein occlusion and retinopathy of prematurity. The new vessels are fragile and often rupture, leading to vitreous haemorrhage, tractional retinal detachment, neovascular glaucoma and subsequent vision decrease. The formation of new vessels is dependent on a local balance of stimulators and inhibitors of angiogenesis [1]. Among the stimulators, vascular endothelial growth factor (VEGF) has been shown to play a major role in mediating active neovascularisation in patients with diabetic retinopathy [2]. In addition, several studies have shown that the concentration of VEGF in the intraocular fluids was significantly elevated in eyes with proliferative diabetic retinopathy (PDR) [3–5]. On the other hand, pigment epithelium-derived factor (PEDF) is a potent inhibitor of angiogenesis, and lower levels of PEDF have been found in the vitreous of eyes with active diabetic retinopathy [4]. It has also been shown that the vitreous level of endostatin, another inhibitor of angiogenesis, is correlated with the level of VEGF and that endostatin is produced in the fibrovascular membrane of eyes with PDR [5].

Vasohibin-1, a novel angiogenesis inhibitor, is mainly produced in endothelial cells and is induced by stimulation with VEGF or fibroblast growth factor 2. Vasohibin-1 selectively affects endothelial cells and inhibits angiogenesis [6]. We therefore hypothesised that vasohibin-1 is present in the vitreous of eyes with PDR and is associated with the vitreous level of VEGF. To examine this hypothesis, we measured the vitreous levels of vasohibin-1 and VEGF in 49 samples from 46 patients. This study was conducted in accordance with the tenets of the Declaration of Helsinki as revised in 2000 and was carried out with the approval of the Institutional Review Board of Tohoku University. Informed consent was obtained from all patients after an explanation of the purpose and procedures of the study.

The level of VEGF, measured by ELISA, was significantly higher in the vitreous samples from patients with PDR↑ (584.0±641.5 ↑pg/ml ↑[mean±SD]; n=37) than in samples from control patients with idiopathic macular hole or idiopathic epiretinal membrane (19.61±19.80 pg/ml; n=12; p=0.004; Student's t test). Western blot analysis using anti-vasohibin-1 mouse monoclonal antibody showed a 42 kDa band and/or a 36 kDa band of different intensity levels in 34 (92%) of the 37 vitreous samples of patients with PDR (Fig. 1a). No signal was detected in any control samples. To investigate the association between the vitreous levels of vasohibin-1 and VEGF, we determined the band intensity of the samples relative to that of given recombinant vasohibin-1 (100 fmol) using the publicly available ImageJ software (National Institutes of Health, Bethesda, MD, USA; http://rsb.info.nih.gov/ij/). We observed a statistically significant correlation between the vitreous concentration of vasohibin-1 and VEGF in the 37 samples with PDR (r=0.469, p=0.005; Spearman correlation coefficient by rank test) (Fig. 1b).

What is the source of the 42 kDa and 36 kDa forms of vasohibin-1 seen in the vitreous samples and detected by western blotting? An earlier study reported that the 36 kDa form was generated by processing the 42 kDa form and that both forms had anti-angiogenesis activity [7]. The forced production of vasohibin-1 in HUV-SV8 cells demonstrated that, secreted vasohibin-1 is mostly the larger 42 kDa form and that the other major form (36 kDa) of the protein accumulates within the cells or pericellular milieu [7]. In our study, immunohistochemical analysis showed that vasohibin-1 was produced in the fibrovascular membranes of eyes with PDR (Fig. 1c,d). We suggest that one of the sources of vasohibin-1 is the endothelial cells in the fibrovascular membranes. As the blood–retina barrier in eyes with PDR is damaged, we cannot completely exclude the possibility that the vasohibin-1 in the vitreous cavity was from the blood. However, the concentration of vasohibin-1 in the plasma for the samples available from patients with PDR was very low (1.8±2.6 fmol/ml [mean±SD]; n=7) and was not statistically correlated with that in the vitreous (r=0.259, p=0.54; Spearman correlation coefficient by rank test), suggesting that the vasohibin-1 in the vitreous is mostly derived from the intraocular tissues. It remains to be determined whether the production of vasohibin-1 in the retinas of eyes with PDR is upregulated and whether vasohibin-1 is secreted into the vitreous cavity, because vasohibin-1 is produced in retinal cells as well as endothelial cells in normal mouse retinas [8].

Hypoxia induces VEGF, VEGF induces the production of vasohibin-1 in endothelial cells and vasohibin-1 inhibits angiogenesis as a negative feedback regulator [6]. On the other hand, hypoxia, as well as inflammatory cytokines (e.g. TNF-α and IL-1β, levels of which are increased in the vitreous of eyes with PDR), may reduce the VEGF-stimulated induction of vasohibin-1 in endothelial cells [6]. Many growth factors play an important role in the complex pathogenesis of diabetic retinopathy, in which hypoxia triggers angiogenesis. The results of this study show that the vitreous level of vasohibin-1 was positively correlated with that of VEGF, which suggests that vaso-

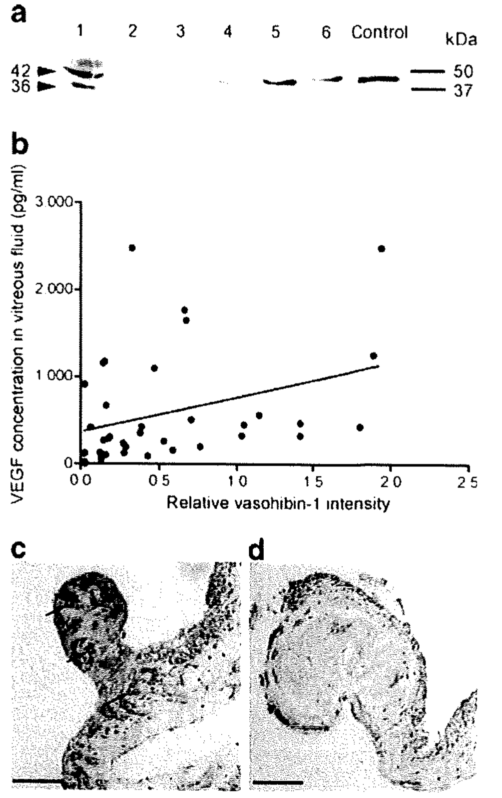

Fig. 1 **a** Detection of vasohibin-1 in vitreous fluid. Western blot shows 42 kDa and/or 36 kDa vasohibin-1 bands in each 7.5 µl of undiluted vitreous samples. Six vitreous samples (lanes 1–6) and 100 fmol recombinant vasohibin-1 (control) were loaded in this representative western blot. **b** Correlation between the vitreous concentration of vasohibin-1 and VEGF (r=0.469, p=0.005; Spearman correlation coefficient by rank test). The band intensities in the western blots were determined by ImageJ software. Relative vasohibin-1 intensity was calculated as the ratio of sample band intensity to control band intensity. Sample band intensity represents the sum of the 42 and 36 kDa vasohibin-1 bands. Exposure time of western blot was fixed to 30 s before the bands were saturated. Western blot analyses were performed in duplicate for each sample. VEGF concentration in the vitreous fluid was measured by ELISA using a human VEGF immunoassay (R&D Systems, Minneapolis, MN, USA). **c** Expression of vasohibin-1 in the surgically removed fibrovascular membranes of eyes with PDR. Fibrovascular membrane contains many new vessels. Immunohistochemistry using anti-vasohibin-1 mouse monoclonal antibody showed signals in the endothelial cells (arrows). **d** Absorbed antibody was used for the controls; no significant signal was seen. Scale bars, 100 µm

hibin-1 regulates retinal angiogenesis as an inhibitor. An intraocular injection of recombinant vasohibin-1 strongly suppressed retinal neovascularisation in mice with ischaemic retinopathy [9], suggesting that vasohibin-1 could be a good candidate for development as a therapeutic agent for diabetic retinopathy, especially in terms of mechanisms different from those in anti-VEGF therapy.

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Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

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A possible role of vimentin on the cell surface for the activation of latent transforming growth factor-β

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ABSTRACT

Latent TGF-β (LTGF-β) has to be converted to active TGF-β for its activities. Previously, we reported that certain fragments of latency associated peptide (LAP) augmented LTGF-β activation via increase in binding of LTGF-β to the endothelial cell (EC) surface followed by cell-associated proteolysis. By searching for EC membrane proteins crosslinked with the LAP fragment, we identified the molecule bound to LAP fragment as vimentin. Moreover, the LAP fragment-induced LTGF-β activation was attenuated by anti-vimentin antibody. These results indicate that binding of the LAP fragment to vimentin on the cell surface is indispensable for LTGF-β activation by the LAP fragment.

Structured summary:
MINT-6806227: *vimentin* (uniprotkb:P48616) *binds* (MI:0407) to *LAP* (uniprotkb:P18341) by *competition binding* (MI:0405)
MINT-6806183: *LAP* (uniprotkb:P18341) *binds* (MI:0407) to *vimentin* (uniprotkb:P48616) by *cross-linking studies* (MI:0030).

1. Introduction

Transforming growth factor-β (TGF-β) is a multi-functional cytokine involved in a wide variety of process. TGF-β is synthesized and secreted as a latent complex composed of mature TGF-β, latency associated peptide (LAP) and latent TGF-β (LTGF-β) binding protein (LTBP) [1]. In order to execute its activities, TGF-β has to be converted from a latent form to an active one, namely mature TGF-β [1]. To date, various mechanisms for the activation of LTGF-β have been proposed including shear forces [2]. However, those can be mainly classified into two categories [1]. One is the structural change of LTGF-β through interaction with other molecules, such as thrombospondin-1 (TSP-1) [3] or integrin αvβ6 [4]. The other is the partial enzymatic cleavage or degradation of LTGF-β [5]. Among several enzymes that activate LTGF-β, the best-characterized one is plasmin, a serine protease, which removes LAP from LTGF-β [5,6]. We have focused on the activation mechanism involving in proteolysis on endothelial cell (EC) [6–9].

Targeting of LTGF-β to cell surface of ECs, where protease activities exist, is one of the important steps for LTGF-β activation. We developed a monoclonal antibody (mAb) against LAP (KM704). KM704 inhibited the activation of LTGF-β by blocking its binding to cells [7]. We assumed that the epitope of KM704 would be the binding site of LTGF-β to cell surface. Therefore, we synthesized several peptides containing the sequence of putative epitopes, and examined if the peptides inhibit the binding of LTGF-β. Surprisingly, some augmented its binding and enhanced LTGF-β activation, although none of them inhibited [9]. Next, we synthesized the relevant peptides supposed to be cleaved by plasmin from LAP, since plasmin activity on the cell surface seems to be necessary in the unique phenomenon [9]. One of the peptides, Peptide-25 (Leu132 to Arg152 in the LAP molecule) bound to EC surface, and increased binding of LTGF-β to there and the following LTGF-β activation [9].

The purpose of this study was to identify the cell surface binding molecule of Peptide-25. We identified the molecule bound to Peptide-25 as vimentin and confirmed that Peptide-25 specifically binds to vimentin protein on endothelial cell surface. Finally, we demonstrated that vimentin is involved in the LTGF-β activation.

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