

Identification of novel targets for antiangiogenic therapy by comparing the gene expressions of tumor and normal endothelial cells

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Since the pioneering work of Judah Folkman, tumor blood vessels are recognized as an important target for cancer therapy.^(1–3) The discovery of bevacizumab, a humanized monoclonal antibody against vascular endothelial growth factor (VEGF), led to the use of antiangiogenic agents as a new approach for the treatment of cancer and hundreds of clinical trials involving antiangiogenic agents are currently underway.^(4–6) However, the benefits of antiangiogenic therapies are often marginal with harmful side-effects, largely because they inhibit normal as well as tumor-induced angiogenesis,^(7–10) and angiogenesis is required not only for tumor progression but also for normal physiological processes.^(11–14) Therefore, identification of novel therapeutic targets based on the difference between tumor and normal angiogenesis is crucial to prevent adverse effects associated with impaired physiological angiogenesis.

Tumor blood vessels differ from their normal counterparts in several ways, such as changes in morphology, altered blood flow and enhanced leakiness.^(15–17) These suggest that tumor endothelial cells (TEC), the main component of tumor vessels, are more relevant tools for developing antiangiogenic cancer

Targeting tumor angiogenesis is an established strategy for cancer therapy. Because angiogenesis is not limited to pathological conditions such as cancer, molecular markers that can distinguish between physiological and pathological angiogenesis are required to develop more effective and safer approaches for cancer treatment. To identify such molecules, we determined the gene expression profiles of murine tumor endothelial cells (mTEC) and murine normal endothelial cells using DNA microarray analysis followed by quantitative reverse transcription–polymerase chain reaction analysis. We identified 131 genes that were differentially upregulated in mTEC. Functional analysis using siRNA-mediated gene silencing revealed five novel tumor endothelial cell markers that were involved in the proliferation or migration of mTEC. The expression of DEF6 and TMEM176B was upregulated in tumor vessels of human renal cell carcinoma specimens, suggesting that they are potential targets for antiangiogenic intervention for renal cell carcinoma. Comparative gene expression analysis revealed molecular differences between tumor endothelial cells and normal endothelial cells and identified novel tumor endothelial cell markers that may be exploited to target tumor angiogenesis for cancer treatment.

therapy than normal endothelial cells (NEC). Some studies based on this concept focused on characterizing the gene expression profiles of TEC to identify molecules (TEC markers) associated with tumor angiogenesis.^(18–24) However, successful application of these TEC markers in the clinic has not been accomplished. This failure may be largely explained by impurities in the TEC during preparations, because isolated TEC were not cultured and their phenotypes were not verified.

Until recently, there were few reports describing the isolation and successful long-term culture of TEC. This is attributed to the technical difficulties caused by the small number of TEC that are enmeshed in a complex tissue that consists of vessel wall components, stromal cells and tumor cells. Moreover, isolated TEC may lose their specific phenotypes during *in vitro* culture. Therefore, most *in vitro* studies on tumor angiogenesis used NEC such as human umbilical vein endothelial cells, human dermal microvascular endothelial cells or bovine aortic endothelial cells.⁽²⁵⁾

To address these issues, we developed a unique method to isolate highly purified murine tumor endothelial cells (mTEC) from

human tumor xenografts or murine normal endothelial cells (mNEC) from dermal tissue of nude mice.^(26,27) Contrary to the stereotype that TEC may lose their specific phenotypes after dissociation from their tumor tissue, the isolated mTEC differed from mNEC in their phenotypic characteristics, including enhanced proliferation, motility, response to growth factors and resistance to chemotherapeutic drugs even after long-term culture.^(28–31) Thus, these mTEC maintain the specific characteristics of TEC *in vivo* and express molecular markers specific for tumor angiogenesis that can distinguish them from mNEC. This unique system for culturing endothelial cells (EC) encouraged us to seek novel molecules specifically associated with tumor angiogenesis.

Using the method described above,^(26,27) we purified and cultured three different types of mTEC and dermis-derived mNEC, compared their gene expression profiles using DNA microarray analysis and quantitative reverse transcription-polymerase chain reaction (qRT-PCR) assays, and identified 131 genes that were differentially upregulated in mTEC. We have already described the suitability of some of these genes including *Bgn*, *Cxcr7* and *Ptgir* as TEC markers.^(32–34) Here, using RNAi techniques, we conducted functional screening of these 131 genes and identified five novel genes associated with the proliferation or migration of mTEC. To validate their applicability to cancer patients, we determined their expression levels in human TEC and tumor vessels isolated from human renal cell carcinoma (RCC) specimens.

Materials and Methods

Cell lines and culture conditions. The human oral squamous cell carcinoma cell line, HSC-3, was supplied by the Japanese Cancer Research Bank (Tokyo, Japan). The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Sigma-Aldrich, St Louis, MO, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS). The human renal clear cell carcinoma cell line, OS-RC-2, was purchased from the RIKEN Cell Bank (Tsukuba, Japan) and cultured in RPMI1640 medium (Sigma-Aldrich) supplemented with 10% FBS. A375SM, a super-metastatic human malignant melanoma cell line, was provided by Dr Isaiah J. Fidler (MD Anderson Cancer Center, Houston, TX, USA).⁽³⁵⁾ The cells were cultured in Minimum Essential Medium (GIBCO, Grand Island, NY, USA) supplemented with 10% FBS. These cells were cultured in a humidified atmosphere containing 5% CO₂ at 37°C.

Antibodies. Antibodies purchased from commercial sources are as follows: mouse anti-human CD31 antibody (BD

Pharmingen, San Diego, CA, USA); Alexa Fluor 647-mouse anti-human CD31 antibody (BioLegend, San Diego, CA, USA); anti-human CD105 antibody (BD Pharmingen); phycoerythrin-conjugated anti-human CD45 antibody (BD Pharmingen); fluorescein isothiocyanate-conjugated anti-human CD45 antibody (BioLegend); rabbit anti-human DEF6 (MBL; Nagoya, Japan); mouse anti-human TMEM176B (Abcam; Cambridge, MA, USA); and Alexa Fluor 594-conjugated anti mouse IgG, Alexa Fluor 488 goat anti-mouse IgG and Alexa Fluor 594 goat anti-rabbit IgG antibody (Invitrogen, Carlsbad, CA, USA).

Isolation of mTEC and mNEC. As described previously,^(27–29) mTEC were isolated from human tumor xenografts (oral carcinoma, renal carcinoma and melanoma) in nude mice and mNEC were isolated from the dermis as controls. The present study was approved by the Animal Care and Use Committee of Hokkaido University (approval ID, 08-0296) and all procedures for animal experiments were performed following the regulation on animal experimentation of Hokkaido University. All purified EC were cultured in EGM-2 MV and used between passages 15–25.

Microarray gene expression analysis. Total RNA was extracted from three types of mTEC (melanoma-derived EC, renal carcinoma-derived EC and oral carcinoma-derived EC) and mNEC using TRIzol (Invitrogen) according to the manufacturer's standard protocol. RNA was quantified using a Ribo-Green RNA Quantitation Kit (Invitrogen) and RNA quality was confirmed using a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Gene expression profiles were obtained from 1.5 µg total RNA per sample using a GeneChip Mouse Genome 430 2.0 Array (Affymetrix, Santa Clara, CA, USA) according to the manufacturer's recommended protocol (GeneChip 3'-IVT Express Kit, P/N 702646 Rev. 7).

RT-PCR and qRT-PCR. Total RNA was extracted using the RNeasy Micro Kit (QIAGEN, Valencia, CA, USA). RT-PCR was performed with modifications. SYBR Green Real-time PCR Master Mix-Plus (Toyobo, Osaka, Japan) was used for qRT-PCR analysis. Cycling conditions followed the manufacturer's instructions according to Opticon Monitor version 3.0 (Bio-Rad, Hercules, CA, USA). Expression levels were normalized to those of genes encoding GAPDH or 18S rRNA. The primers for mouse *Gapdh* (mGAPDH), *Cd31*, *Vegfr1*, *Vegfr2*, *Cd11b*, *Cd45*, human *HBEGF* and human *GAPDH* (hGAPDH) were described previously.⁽²⁸⁾ The primers for mouse *Cd105* (*Eng*) are as follows: 5'-CTTCCAAGGACA GCCAAGAG-3' and 5'-GGGTCATCCAGTGCTGCTAT-3'. The primers used for TEC markers are listed in Table 1.

Table 1. Primer information

Species	Gene	Forward primer	Reverse primer
Mouse	<i>Tmem176b</i>	5'- CTCCAAGTCTACTCTCAAGCTCCA -3'	5'- CCAGAGTCTACAGGAAAGCAGAGA -3'
	<i>Pcdhb22</i>	5'- ATCCGCAACCGAGGTGATG -3'	5'- AATGCGGATTTGCGAGGTG -3'
	<i>Nsg1</i>	5'- GCCTGATGGGTTTGTCTTGA -3'	5'- CACTGGAGTCTTGTCCGTGTAGTA -3'
	<i>Enah</i>	5'- CACATTCAGAGTTGTGGGCAGA -3'	5'- TGCTGCCAAAGTTGAGACCATAC -3'
	<i>Def6</i>	5'- CACCAACGTGAAACTGGAATG -3'	5'- CGGGTCAGGCGCTTTAGAGA -3'
	<i>18SrRNA</i>	5'- GGGAGCCTGAGAAACCGG -3'	5'- GGGTCGGGAGTGGGTAATTT -3'
	<i>Gapdh</i>	5'- TCTGACGTGCCGCTGGAG -3'	5'- TCGCAGGAGACAACCTGGTC -3'
	Human	<i>TMEM176B</i>	5'- CCCTACCACTGGGTACAGATGGA -3'
<i>PCDHB15</i>		5'- GACCAGAGCCGAGTACAACATCAC -3'	5'- GTCCGACACCAGCACGGTTA -3'
<i>NSG1</i>		5'- CCGATGGGTTCTGCTCTCAA -3'	5'- TCTTGTCCGCGTAGTAGCTCTC -3'
<i>ENAH</i>		5'- GTGGCTCAACTGGATTCAGCA -3'	5'- AGGAATGGCAGAGTTTATCACGA -3'
<i>DEF6</i>		5'- CAGGGATACATGCCCTACCTCAAC -3'	5'- CAGCACAGTCTACAAAGTGCTC -3'
<i>GAPDH</i>		5'- ACAGTCAGCCGATCTTCTT -3'	5'- GCCCAATACGACCAAATCC -3'

RNAi experiments. All siRNA (stealth siRNA) were purchased from Invitrogen and transfected at a final concentration of 3 nM using Lipofectamine RNAiMAX reagent (Invitrogen) according to the manufacturer's instructions. Total RNA for qRT-PCR analysis was extracted 48 h after transfection. For proliferation assays, 4×10^3 transfected cells were cultured in 96-well dishes and cell viability was measured 72 h after transfection using Alamar Blue reagent. For migration assays, transfected cells were maintained in six-well dishes for 48 h. After starvation with EBM-2 containing 0.2% bovine serum albumin for 2 h, cells were resuspended in EBM-2 containing 0.2% bovine serum albumin and transferred to the upper chambers of a BD BioCoat Angiogenesis System: Endothelial Cell Migration (BD Biosciences, Franklin Lakes, NJ, USA). Cell migration for 20 h toward the chemoattractant EGM-2MV was measured by labeling the migrated cells with Calcein AM Fluorescent Dye (BD Biosciences) according to the manufacturer's instructions.

Human tissue samples. Surgically resected tissues from three patients diagnosed with RCC (clear cell carcinomas; Table 2) were analyzed. The specimens included tumor tissues and corresponding normal renal tissues 5–10 cm from the tumor. One portion of the sample was immediately snap-frozen in liquid nitrogen and stored at -80°C for immunohistological analysis and another was placed in Hank's Balanced Salt Solution (Life Technologies, Grand Island, NY, USA) on ice until EC isolation. Final diagnosis of RCC was confirmed by pathological examination of formalin-fixed surgical specimens. All protocols were approved by the Institutional (Hokkaido University) Ethics Committee (approval ID, 009-0148) and written informed consent was obtained from each patient before surgery.

Isolation of human renal TEC and NEC. Human TEC (hTEC) and human NEC (hNEC) were isolated from the excised human RCC and normal renal tissues, respectively, followed by flow cytometric analysis as described previously.⁽³³⁾ All purified EC were plated and cultured in EGM-2MV (Lonza, Basel, Switzerland) and 15% FBS.

Immunohistochemistry. Frozen human tissue samples were cut into 8- μm thick sections. Immunofluorescence was performed as previously reported.⁽³³⁾ Primary and secondary antibodies are described above. These samples were counterstained with diamidino-2-phenylindole (DAPI; Roche Diagnostics, Mannheim, Germany) and visualized using an Olympus FluoView FV1000 confocal microscope (Olympus, Tokyo, Japan).

Statistical analysis. All data are expressed as the mean \pm standard deviation and subjected to two-sided Student's *t*-tests. Differences were considered significant for $P < 0.05$.

Results

Isolation and characterization of mTEC and mNEC. We first isolated and cultured three different types of mTEC (Melanoma-EC, Renal carcinoma-EC [Renal Ca-EC] and Oral carci-

noma-EC [Oral Ca-EC]) from human tumor xenografts and mNEC (Skin-EC) from dermal tissues of nude mice as a normal control. These murine endothelial cells (mEC) were positive for the EC markers *Cd31*, *Cd105*, *Vegfr1* and *Vegfr2* and negative for the monocyte marker *Cd11b* and hematopoietic marker *Cd45* using RT-PCR (Fig. 1a). Human *HBEGF*, which is expressed in human tumor cells, was not detected in any of the mTEC (Fig. 1a). These results excluded the possibility that these mEC were contaminated with non-EC such as monocytes, hematopoietic cells and human tumor cells. Furthermore, tube formation was observed when mEC were cultured on matrigel-coated plates (Fig. 1b), indicating that these mEC maintained EC properties after isolation and culture. Thus, our isolation technique yielded highly pure and functional populations of mEC suitable for subsequent analyses.

Expression profiling of isolated mTEC and mNEC. To identify novel markers of tumor endothelium by comparing the gene expression patterns between mTEC and mNEC, total RNA was extracted from eight independent mTEC populations derived from three types of human tumor xenografts (three melanomas, three renal carcinomas and two oral squamous cell carcinomas) and two populations of mNEC derived from two independent samples from the dermis of nude mice. RNA was used to probe an Affymetrix GeneChip Mouse Genome 430 2.0 array to determine transcriptional profiles. We focused on transcripts that were highly expressed in the three types of mTEC compared with mNEC. We detected 180 transcripts expressed in all mTEC with levels five times higher than that in mNEC. We excluded 19 genes with no human orthologs and 30 genes that were expressed less than five times higher by any mTEC compared with mNEC using qRT-PCR (Fig. 2). The DNA microarray and RT-PCR analysis of representative genes including the five novel TEC markers are shown in Table 3, Figure 3 and Supporting Information Table S1. The levels of expression of these genes were higher in the three types of mTEC than that in mNEC. We selected 131 genes as potential TEC markers for functional screening using siRNA-mediated gene silencing (Fig. 2).

Functional validation of TEC markers using RNAi. Excessive angiogenesis occurs through a series of steps including enhanced EC proliferation and migration.⁽³⁶⁾ Therefore, targeting proliferation and/or migration of EC is one of the most attractive and effective strategies for treating angiogenesis-dependent disorders. We reported that mTEC grow faster and migrate better than mNEC.⁽²⁸⁾ These *in vitro* characteristics of mTEC represent enhanced tumor angiogenesis *in vivo* and the genes responsible for increased proliferation or migration of mTEC may serve as ideal targets for antiangiogenic therapy. To identify such molecules, we performed loss-of-function screening of the 131 potential TEC markers in Melanoma-EC, one of the mTEC that showed high activity in the proliferation and migration assays.⁽²⁸⁾ We first cotransfected Melanoma-EC with three different sequences of siRNA per gene. Cell proliferation and/or migration were inhibited by >20% compared

Table 2. Clinical background of renal cell carcinoma specimens

Case no.	Sex	Age (years)	TNM†	Subtype	Grade‡	INF	Vascular invasion
1	F	50	T1b, N0, M0	Clear Cell	G2	INFa	V (-)
2	M	60	T1a, N0, M0	Clear Cell	G2	INFa	V (-)
3	F	48	T2b, N1, M0	Clear Cell	G2	INFa	V (+)

†According to the 7th edition of tumor-node-metastasis (TNM) staging guidelines.³⁷ ‡According to the Fuhrman system. F, female; INF, infiltration pattern; M, male; -, negative; +, positive.

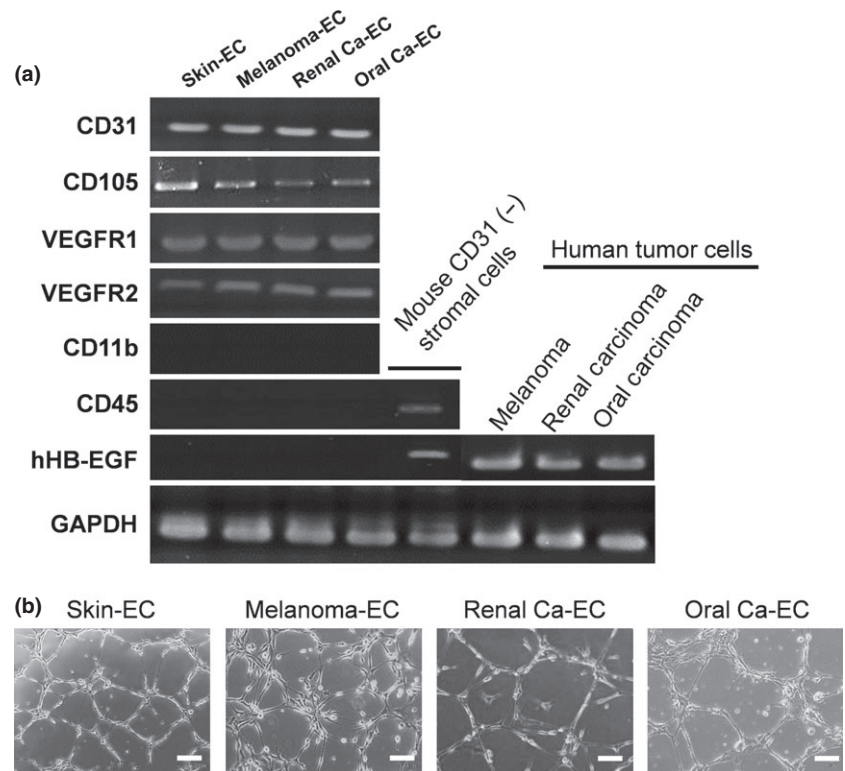


Fig. 1. Characterization of murine tumor endothelial cells (mTEC) and murine normal endothelial cells (mNEC). (a) The expression of *Cd31*, *Cd105*, *Vegfr1*, *Vegfr2*, *Cd11b*, *Cd45* and human *HBEGF* in mTEC (Melanoma-EC, Renal Ca-EC and Oral Ca-EC) and mNEC (Skin-EC) was analyzed using RT-PCR. CD31-negative non-EC fractions and human tumor cells (melanoma, renal carcinoma and oral carcinoma) were also analyzed. (b) Isolated and cultured mEC formed tubes on matrigel-coated plates. Bar, 100 μ m. EC, endothelial cells; Renal Ca-EC, Renal carcinoma-EC; Oral Ca-EC, Oral carcinoma-EC; mEC, murine endothelial cells.

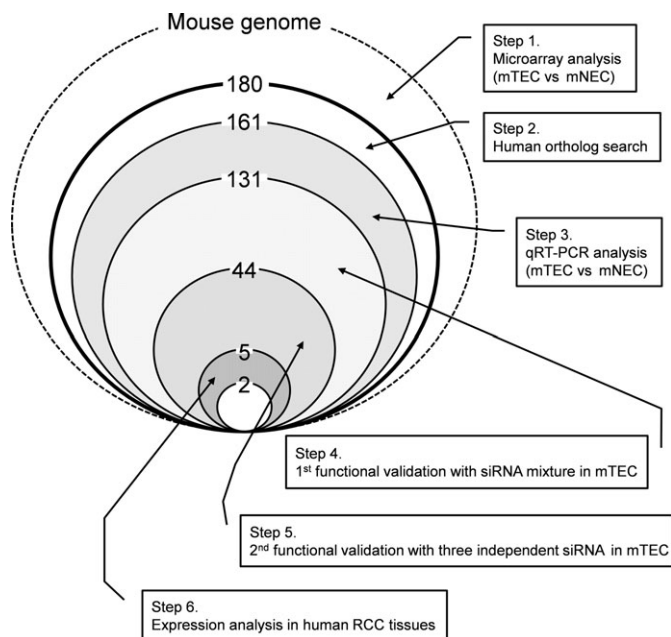


Fig. 2. Schematic of tumor endothelial cell (TEC) marker selection. The strategy and results of TEC marker selection are summarized. We identified five novel TEC markers through analysis of gene expression profiles and functions in murine tumor endothelial cells (mTEC). mNEC, murine normal endothelial cell; RCC, renal cell carcinoma.

with mock-transfected cells using siRNA targeted to 44 genes (Fig. 2). Subsequently, three different siRNA specific for each of the 44 genes were used to independently transfect Melanoma-EC. We performed migration and proliferation assay using siRNA and finally selected five genes (*Def6*, *Nsg1*, *Enah*, *Tmem176b* and *Pcdhb22*; Table 3, Fig. 3) whose

respective siRNA (two or more per gene) inhibited cell proliferation or migration by >30% (Figs 2,4). Cell migration was inhibited by siRNA of *Tmem176b*, *Pcdhb22*, *Nsg1* and *Enah*, but cell proliferation was not inhibited. In contrast, cell proliferation was inhibited by *Def6* siRNA. These were considered potential regulators of proliferation or migration of mTEC. There was no gene whose two or more siRNA inhibited both cell proliferation and migration by >30% (data not shown). Knockdown of each gene was confirmed using qRT-PCR 48 h after transfection (Fig. 4).

Expression of TEC markers in human TEC *in vitro* and *in vivo*. The therapeutic potential of targeting candidate genes largely depends on whether their expression is upregulated in hTEC as well as in mTEC. Therefore, we analyzed the expression of the five putative TEC markers in hTEC isolated from RCC specimens and hNEC from normal renal tissues from the same patients, respectively. Because the EC population represents only a small percentage of the cells present in tumor tissue, sufficient quantities of specimens must be acquired for preparation of hTEC. This technical limitation forced us to choose RCC as the source of hTEC.

The hTEC and hNEC were obtained from three patients. The clinical backgrounds of patients with RCC who donated tissue specimens are shown in Table 2. The binding of ulex europaeus agglutinin 1 (UEA-1 lectin), the expression of CD31 and CD105, and lack of expression of CD45 determined by flow cytometric analysis confirmed the high purity of the isolated human EC (hEC). Representative data are shown in Figure 5(a). The expression levels of *TMEM176B* and *DEF6* revealed by qRT-PCR analysis were significantly higher in hTEC than in hNEC for all paired samples (Fig. 5b). In contrast, we were unable to detect upregulation of *NSG1*, *ENAH* or *PCDHB15* (human ortholog of *Pcdhb22*) in hTEC (Fig. S1). These results indicate that two out of five TEC markers that we identified in mice were upregulated in mTEC (Fig. S2) and hTEC (Fig. 5b). Furthermore, to determine the expression

Table 3. Representative data of DNA microarray analysis

Probe (Mouse430_2)	Gene title	Symbol	Fold increase relative to Skin-EC		
			Oral Ca-EC	Renal Ca-EC	Melanoma-EC
1418004_a_at	Transmembrane protein 176B	<i>Tmem176b</i>	518.29	156.13	61.39
1418941_at	Protocadherin beta 22	<i>Pcdhb22</i>	31.36	20.77	31.96
1423055_at	Neuron-specific gene family member 1	<i>Nsg1</i>	147.51	28.57	53.99
1424800_at	Enabled homolog (Drosophila)	<i>Enah</i>	23.66	14.78	21.43
1452796_at	Differentially expressed in FDCP 6	<i>Def6</i>	5.02	8.36	14.09

EC, endothelial cells; Ca, carcinoma.

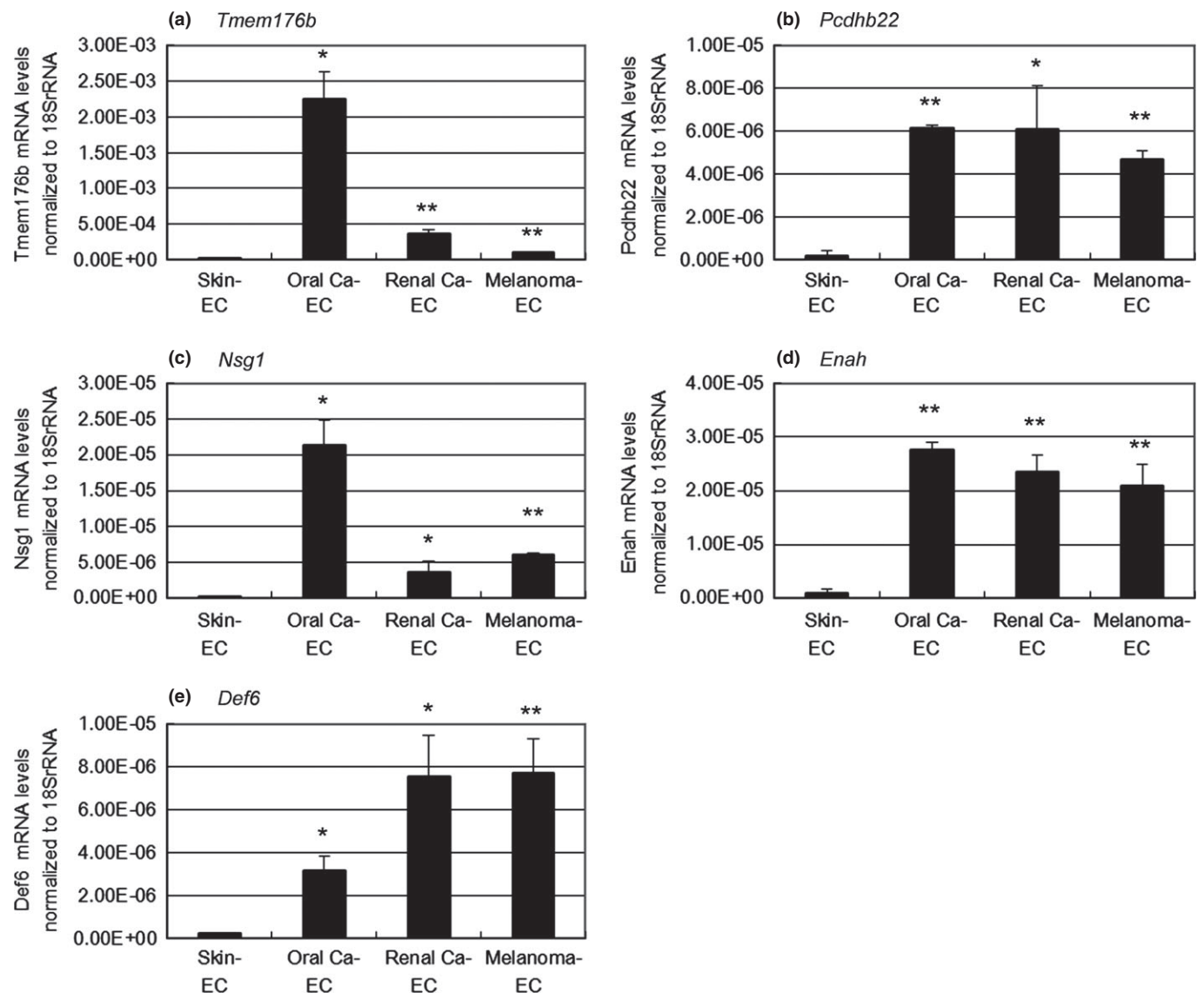


Fig. 3. Analysis of the transcription of tumor endothelial cell (TEC) marker genes. Using qRT-PCR, five novel TEC markers identified here were shown to be selectively upregulated in all types of murine TEC (mTEC) compared with murine normal endothelial cells. The relative expression levels of mRNA were normalized to that of 18S rRNA for each cell type (* $P < 0.05$; ** $P < 0.01$). (a–e) mRNA expression of novel TEC markers (*Tmem176b*, *Pcdhb22*, *Nsg1*, *Enah* and *Def6*). EC, endothelial cells; Ca, carcinoma.

levels of TMEM176B and DEF6 in tumor blood vessels in RCC *in vivo*, we performed immunofluorescence double staining of the frozen sections of human renal tumors and normal kidney tissues (glomerulus) using anti-CD31 with either anti-TMEM176B or anti-DEF6 antibodies. TMEM176B and DEF6

were expressed in tumor blood vessels in renal cancer, but at much lower levels in normal blood vessels (Figs 5c, S3). These results suggest that the transcription of these two genes was upregulated in hTEC *in vivo* and might be involved in tumor angiogenesis in cancer patients.

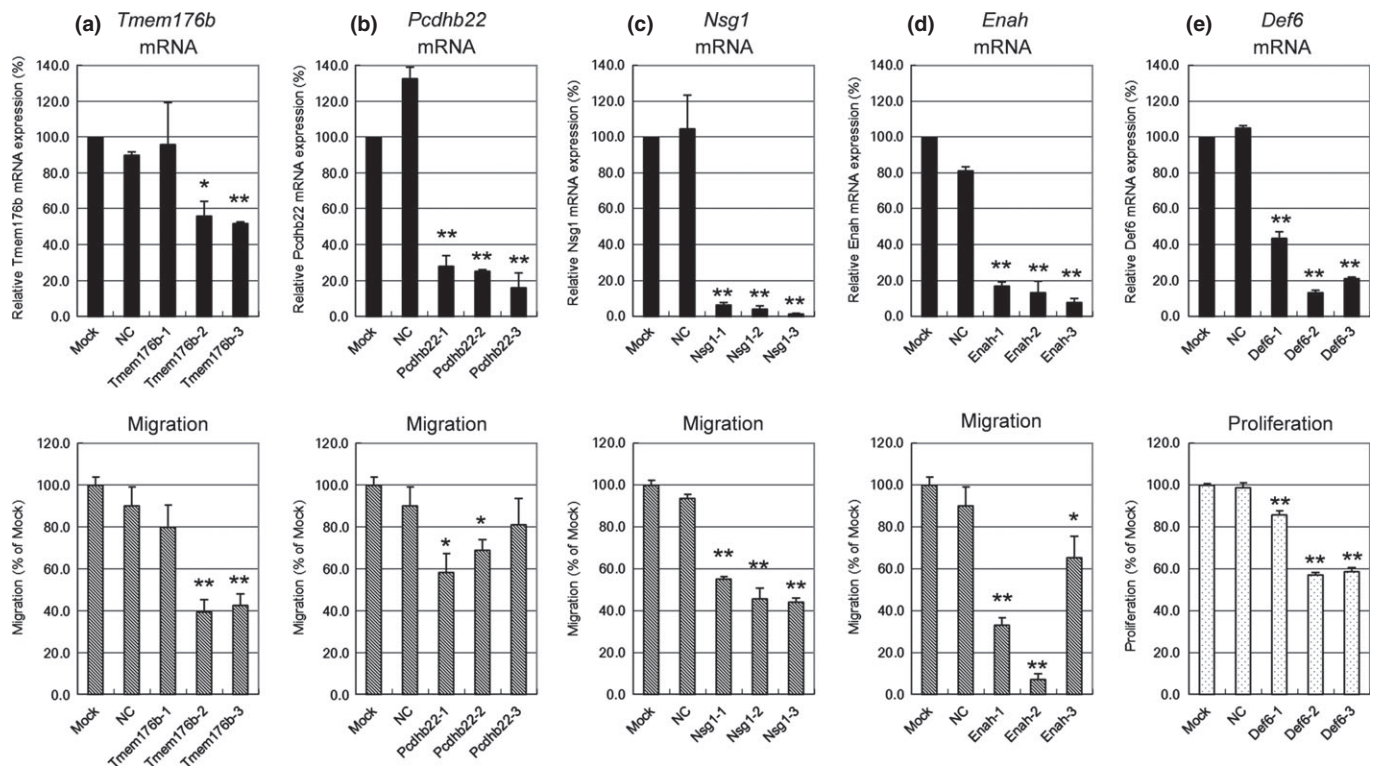


Fig. 4. Functional analysis of tumor endothelial cell (TEC) markers using siRNA-mediated gene silencing. Melanoma-EC were transfected using three different siRNA for each gene. Silencing of each TEC marker 48 h after transfection was evaluated using qRT-PCR (upper panel). Cell proliferation was measured after 72 h using Alamar Blue (lower panel). Cell migration towards the chemoattractant EGM-2MV for 20 h was evaluated using the BD BioCoat Angiogenesis System: Endothelial Cell Migration (BD Biosciences) (lower panel). siRNA-mediated effects of each TEC marker are shown in (a–e) (** $P < 0.01$; * $P < 0.05$). (a) *Tmem176b*; (b) *Pcdhb22*; (c) *Nsg1*; (d) *Enah*; and (e) *Def6*. NC, Negative control siRNA.

Discussion

In the present study, we isolated and cultured mTEC from three different types of human tumor xenografts and mNEC from the dermis of nude mice and compared their gene expression profiles. DNA microarray analysis and qRT-PCR analysis identified 131 genes that were upregulated in mTEC compared with mNEC. Functional analysis of these 131 genes using RNAi revealed that five were involved in the proliferation or migration of mTEC. Two, *DEF6* and *TMEM176B*, were upregulated in hTEC and *in vivo* in tumor vessels of human RCC, suggesting that increased expression of these two proteins contributes to enhanced tumor angiogenesis in cancer patients. To the best of our knowledge, this is the first report that *DEF6* and *TMEM176B* might be involved in tumor angiogenesis and might serve as targets for antiangiogenic therapy of cancer patients.

DEF6, also described as *SLAT* or *IBP*, is highly conserved in vertebrates and acts as a guanine nucleotide exchange factor for Rho-family GTPases, including *RAC1*, *CDC42* and *RHOA*,⁽³⁸⁾ which are involved in cytoskeletal organization, cell cycle progression and extracellular signal transduction, as well as in the proliferation, migration, invasion and metastasis of cancer cells.^(39–42) *DEF6* is overexpressed in cancer cells (as suggested in Fig. 5c) and may have an important function in tumor invasion and metastasis^(43,44); however, its role in tumor angiogenesis is unknown. In the present study, we show for the first time that *DEF6* was upregulated in TEC compared with NEC and might mediate increased proliferation of TEC that enhance tumor angiogenesis. Its role in tumor endothelial function combined with its significance for tumor cell function makes it an appealing candidate as a target for cancer therapy.

TMEM176B, also known as *LR8*, belongs to the *CD20/Fc-RI* and membrane-spanning 4A (*MS4A*) family.^(45,46) It was discovered in human lung fibroblasts and is associated with human small cell lung carcinoma.⁽⁴⁷⁾ Although several recent reports implicate human *TMEM176B* in cancer,^(48–50) no direct evidence is available regarding its function in cancer pathogenesis, including tumor angiogenesis. Here, we report for the first time overexpression of *TMEM176B* in TEC and further show using RNAi that *TMEM176B* mediates TEC migration. Moreover, immunohistochemical analysis revealed expression of *TMEM176B* in tumor vessels and in tumor cells (as suggested in Fig. 5c) as reported previously.⁽⁵¹⁾ Although the contribution of *TMEM176B* in tumor cells to the malignant phenotype is unknown, it might serve as a target for cancer therapy. The physiological function of *TMEM176B* remains to be determined.

Unlike *DEF6* and *TMEM176B*, we were unable to detect upregulation of *NSG1*, *ENAH* or *PCDHB15* (human ortholog of *Pcdhb22*) in hTEC or tumor vessels of human RCC specimens. *ENAH* and *PCDHB15* were expressed in vessels and in mesangial cells of normal tissues and *NSG1* expression was not detected in tumor vessels (data not shown). Because the number of samples was limited and tumors other than RCC remain to be examined, we consider these genes to be worthy of future study.

The present study indicates the power of determining the differential expression of genes between TEC and NEC for identifying potential targets for antiangiogenic therapy. Although TEC markers such as *ANTXR1* (*TEM8*), *CD276* and *JAG1* (*Jagged1*) were previously identified using this

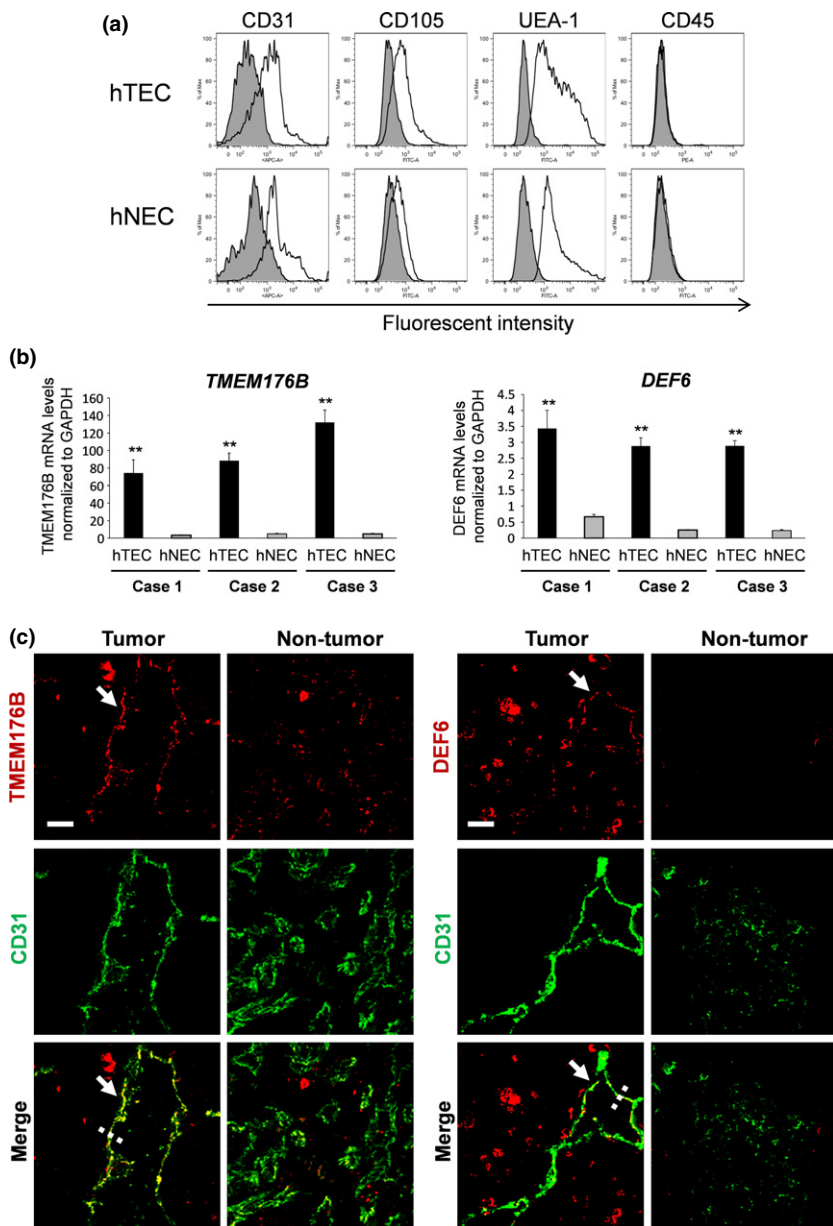


Fig. 5. Analysis of *TMEM176B* and *DEF6* expression *in vitro* and *in vivo*. (a) Verification of endothelial cells (EC) from a human sample. The binding of ulex europaeus agglutinin 1 (UEA-1 lectin), expression of CD31, CD105 and lack of expression of CD45 (white area) indicates high purity of the isolated human tumor endothelial cells (hTEC) and human normal endothelial cells (hNEC). The isotype control is shown in gray. (b) Upregulated expression of *TMEM176B* and *DEF6* in hTEC. qRT-PCR analysis detected high levels of expression of both genes in hTEC compared with the corresponding hNEC in all three cases. Expression levels of the mRNA were normalized to that of *GAPDH* (** $P < 0.01$). (c) Both *TMEM176B* and *DEF6* were strongly stained in tumor vessels using an anti-CD31 antibody in combination with an antibody against either *TMEM176B* or *DEF6*. In contrast, normal vessels (glomerular) of normal renal tissue were weakly stained. All samples were counterstained with DAPI. Profiles of immunofluorescence intensities along the dashed lines are shown in Figure S3(a,b). The signal intensities of *TMEM176B* or *DEF6* in the CD31-positive area of whole sections were analyzed by ImageJ (NIH, Bethesda, MD, USA) quantitatively (Fig. S3c,d). Bar, 20 μm.

technique,^(18–24) the present study is the first, to our knowledge, to demonstrate upregulated expression of the genes encoding *DEF6*, *TMEM176B*, *NSG1*, *ENAH* and *PCDHB15* in TEC. The major difference between the present and previous studies is our unique method for culturing mTEC, which overcomes the loss of the TEC phenotype after dissociation from their tumor tissue. These highly purified mTEC isolated from human tumor xenograft maintain the specific characteristics of TEC *in vivo* during long-term culture^(26–31) and therefore provide a more relevant system for tumor angiogenesis research and the identification of novel TEC markers.

In summary, here we report the identification of novel genes that are relevant to tumor angiogenesis by investigating the differences in gene expression patterns between mTEC and mNEC. Targeting these genes may lead to therapies that do not induce adverse effects associated with altering physiological angiogenesis. Further research will be required, particularly *in vivo* studies, to define the roles of the novel genes

identified here in tumor angiogenesis, invasion and metastatic growth.

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Disclosure Statement

T.O., H.S., T. Kai, Y.M. and H.T. are employees of Dainippon Sumitomo Pharma Co., Ltd. The following genes are patent pending: *Pcdhb22*(PCT/JP2011/056631); *Nsg1*(PCT/JP2010/063124); *Enah*(PCT/JP2011/056639); *Def6*(PCT/JP2010/063669); and *TMEM176B*(JP2010/254205). This does not alter our adherence to all the Cancer Science policies on sharing data and materials.

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Supporting Information

Additional supporting information may be found in the online version of this article:

Fig. S1. Analysis of *NSG1*, *ENAH* and *PCDHB15* expression *in vitro*.

Fig. S2. Analysis of the transcription of *Tmem176b* and *Def6* genes using qRT-PCR.

Fig. S3. Immunofluorescence intensities of TMEM176B, DEF6 and CD31 in human RCC specimens.

Table S1. Representative data of qRT-PCR analysis