博 士 論 文

Identification of Novel Targets for Antiangiogenic Therapy

（血管新生阻害療法のための新規標的分子の同定）

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Abstract

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

Key Words

tumor endothelial cell, tumor endothelial cell marker, tumor angiogenesis, antiangiogenic therapy

Introduction

Since the pioneering work of Judah Folkman, tumor blood vessels are recognized as an important target for cancer therapy[1-4]. 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[5-8]. However, the benefits of antiangiogenic therapies are often marginal with harmful side effects, largely because they inhibit normal as well as tumor-induced angiogenesis[9-12], and angiogenesis is required for not only tumor progression but also normal physiological processes[13-16]. 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[17-21]. These suggest that tumor endothelial cells (TECs), the main component of tumor vessels, are more relevant tools for developing antiangiogenic cancer therapy than normal endothelial cells (NECs). Some studies based on this concept focused on characterizing the gene expression profiles of TECs to identify molecules associated with tumor angiogenesis. For example, analysis of gene expression profiles of endothelial cells derived from colon carcinomas and normal colonic mucosa by serial analysis of gene expression (SAGE) identified 46 specific genes for TECs that were designated as tumor endothelial markers (TEMs)[22-24]. These studies further identified 13 other novel cell surface proteins as TEMs[25]. Ovarian tumor vascular markers were identified from vascular cells by laser-capture microdissection and certain tumor vascular markers correlated with patients' prognoses[26]. Ovarian TECs were also isolated in a study in which using DNA microarrays, 23 TEMs were identified[27]. Colon carcinoma endothelial cell markers were also identified by SAGE[28]. However, the

successful application of these TEC markers in the clinic has not been accomplished. This failure may be largely explained by the impurities in the TECs during preparations, because the isolated TECs were not cultured, and their phenotypes were not verified.

Currently, there are few reports describing the isolation and successful long-term culture of TECs. This is attributed to the technical difficulties caused by the small number of TECs that are enmeshed in a complex tissue that consists of vessel wall components, stromal cells, and tumor cells. Moreover, isolated TECs may lose their specific phenotypes during *in vitro* culture. Therefore, most *in vitro* studies on tumor angiogenesis employed NECs such as human umbilical vein endothelial cells, human dermal microvascular endothelial cells, or bovine aortic endothelial cells[29].

To address these issues, we developed a unique method to isolate highly purified murine tumor endothelial cells (mTECs) from human tumor xenografts or normal murine endothelial cells (mNECs) from dermal tissue of nude mice[30, 31]. Contrary to the stereotype that TECs may lose their specific phenotypes after dissociation from their tumor tissue, the isolated mTECs differed from mNECs in

their phenotypic characteristics, including enhanced proliferation, motility, response to growth factors, and resistance to chemotherapeutic drugs even after long-term culture[32-35]. Thus, these mTECs maintain the specific characteristics of TECs *in vivo* and express molecular markers specific for tumor angiogenesis that can distinguish them from mNECs. This unique system for culturing ECs encouraged us to seek novel molecules specifically associated with tumor angiogenesis.

By the method described above[30, 31], we purified and cultured three different types of mTECs and dermis-derived mNECs, compared their gene expression profiles by DNA microarray analysis and quantitative reverse transcription-polymerase chain reaction (qRT-PCR) assays, and finally identified 131 genes that were differentially upregulated in mTECs. We have already described the suitability of some of these genes, including *Bgn*, *Cxcr7*, and *Ptgir* as TEC markers[36-38]. Here, by RNAi techniques, we conducted functional screening of 131 genes that were upregulated in mTECs and identified five novel genes associated with the proliferation or migration of mTECs. To validate their applicability to cancer patients, we determined their expression levels in human

TECs and tumor vessels isolated from human renal cell carcinoma (RCC) specimens. The specific markers in tumor endothelium identified in the present study may offer opportunities for developing new therapeutic approaches to specifically inhibit angiogenesis induced by tumor cells.

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) 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 highly metastatic human malignant melanoma cell line, was provided by Dr. Isaiah J. Fidler (MD Anderson Cancer Center, Houston, TX). The cells were cultured in Minimum Essential Medium (GIBCO, Grand Island, NY) supplemented with 10% FBS. These cells were

cultured in a humidified atmosphere containing 5% CO_2 at 37°C.

Antibodies

Antibodies purchased from commercial sources are as follows: mouse anti-human CD31 antibody (BD Pharmingen, San Diego CA), Alexa Fluor® 647-mouse anti-human CD31 antibody (BioLegend, San Diego CA), anti-human CD105 antibody (BD Pharmingen), phycoerythrin-conjugated anti-human CD45 antibody (BD Pharmingen), rabbit anti-human DEF6 (MBL, Nagoya, Japan), mouse anti-human TMEM176B (Abcam, Cambridge, MA), Alexa Fluor® 594-conjugated anti mouse IgG, Alexa Fluor® 488 goat anti-mouse IgG, Alexa Fluor® 594 goat anti-rabbit IgG, and Alexa Fluor® 594 goat anti-rabbit IgG antibody (Invitrogen, Carlsbad, CA).

Isolation of mTECs and mNECs

mTECs and mNECs were isolated as described previously[31-33]. In brief, mTECs were isolated from human tumor xenografts (oral carcinoma, renal carcinoma, and melanoma) in nude mice, and mNECs were isolated from the

dermis as controls. Local animal research authorities approved all procedures for animal experimentation, and animal care was conducted in accordance with institutional guidelines. Excised tissues were minced and digested with collagenase II. After blood cells were removed by single sucrose step-gradient centrifugation using Histopaque 1077, cell suspensions were filtered, and endothelial cells (ECs) were isolated using a magnetic-activated cell sorting system (Miltenyi Biotec, Tokyo, Japan) with the anti-mouse CD31 antibody. CD31-positive cells were sorted and plated onto 1.5% gelatin-coated culture plates and grown in EGM-2 MV (Lonza, Walkersville, MD) and 15% FBS. Diphtheria toxin (DT; 500 ng ml^{-1}; Calbiochem, San Diego, CA) was added to mTECs subcultures for eliminating any human tumor cell remnant and to mNECs subcultures for technical consistency. After subculture for approximately 2 weeks, ECs were further purified using FITC-BS1-B4-Lectin. All purified ECs were cultured in EGM-2 MV and used between passages 15–25.

Microarray gene expression analysis

Total RNA was extracted from three types of mTECs (melanoma-derived

ECs, renal carcinoma-derived ECs, and oral carcinoma-derived ECs) and mNECs using TRIzol (Invitrogen) according to the manufacturer's standard protocol. RNA was quantified using a RiboGreen RNA Quantitation Kit (Invitrogen) and RNA quality was confirmed using a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Gene expression profiles were obtained from 1.5 μg total RNA per sample using a GeneChip Mouse Genome 430 2.0 Array (Affymetrix) 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). 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). 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,*

human *GAPDH* (hGAPDH) were described previously[32]. The primers for mouse *Cd105* (*Eng*) are as follows: 5′-CTTCCAAGGACAGCCAAGAG-3′ and 5′-GGGTCATCCAGTGCTGCTAT-3′. The primers used for TEC markers are listed in Table 1.

RNAi experiments

All siRNAs (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 6-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). Cell migration for 20 h toward the chemoattractant EGM-2MV

was measured by labeling the migrated cells with Calcein AM Fluorescent Dye according to the manufacturer's instructions.

Human tissue samples

Surgically resected tissues from three patients diagnosed with RCC (clear cell carcinomas; Table 3) were analyzed. The specimens included tumor tissues and corresponding normal renal tissues 5–10 cm from the tumor. A part 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 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 Ethics Committee, and written informed consent was obtained from each patient before surgery.

Isolation of human renal TECs and NECs

The excised human RCC and normal kidney tissues described above were processed using magnetic-bead cell sorting with the IMag Cell Separation

System (BD Bioscience) with mouse anti-human CD31 antibody (BD Pharmingen) and anti-mouse IgG1 Magnetic Particles (BD Bioscience) according to the manufacturer's instructions. Isolated human TECs (hTECs) and human NECs (hNECs) were plated and cultured in EGM-2MV (Lonza) and 15% FBS.

Flow cytometry

After dissociation with 0.5% trypsin-EDTA, hTECs and hNECs were incubated with fluorescein-labeled Ulex europaeus agglutinin I (UEA1-Lectin; Vector Laboratories) and primary antibodies against CD31, CD105, and CD45 for 20 min at 4°C. After washing, the cells were incubated with Alexa Fluor® 488 goat anti-mouse IgG for 20 min at 4°C. The cells were analyzed using a FACS Aria II (Becton Dickinson, San Jose, CA, USA). Representative data were analyzed using FlowJo software (Tree Star, Ashland, OR, USA).

Immunohistochemistry

Frozen human tissue samples were cut into 8 μm thick sections. Immunofluorescence was performed after fixing the sections with 100% ice-cold

acetone for 10 min and blocking with 2% goat and 5% sheep sera in phosphate-buffered saline for 30 min. Primary and secondary antibodies are described above. These samples were counterstained with diamidino-2-phenylindole (DAPI; Roche Diagnostics, Germany) and examined using an Olympus FluoView FV1000 confocal microscope (Olympus, Tokyo, Japan).

Results

Isolation and characterization of mTECs and mNECs

We first isolated and cultured three different types of mTECs (Melanoma-ECs, Renal Ca-ECs, and Oral Ca-ECs) from human tumor xenografts and mNECs (Skin-ECs) from dermal tissues of nude mice as a normal control. These mECs were positive for the EC markers *Cd31*, *Cd105*, *Vegfr1*, *Vegfr2* and negative for the monocyte marker *Cd11b* and hematopoietic marker *Cd45* by RT-PCR (Fig. 1A). Human *HBEGF*, which is expressed in human tumor cells, was not detected in any of the mTECs (Fig. 1A). These results excluded the possibility that these mECs were contaminated with non-ECs such as monocytes,

hematopoietic cells, and human tumor cells. Furthermore, tube formation was observed when mECs were cultured on matrigel-coated plates (Fig. 1B), indicating that these mECs maintained EC properties after isolation and culture. Thus, our isolation technique yielded highly pure and functional populations of mECs suitable for subsequent analyses.

Expression profiling of isolated mTECs and mNECs

To identify novel markers of tumor endothelium by comparing the gene expression patterns between mTECs and mNECs, total RNA was extracted from eight independent mTECs populations derived from three types of human tumor xenografts (three melanomas, three renal carcinomas, and two oral squamous cell carcinomas) and two populations of mNECs derived from two independent samples from the dermis of nude mice. RNAs were 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 mTECs compared with mNECs. We detected 180 transcripts expressed in all mTECs with levels five times higher than that in mNECs. We excluded 19 genes with no

human orthologs and 30 genes that were expressed less than 5-times higher by any mTECs compared with mNECs by qRT-PCR. The DNA microarray and RT-PCR analysis of representative genes, including the five novel TEC markers and the four known markers previously identified, are shown in Table 2 and Figure 3. The levels of expression of these nine genes were higher in the three types of mTECs than that in mNECs. We selected 131 genes as potential TEC markers for functional screening by siRNA-mediated gene silencing (Fig. 2).

Functional validation of TEC markers using RNAi

Excessive angiogenesis occurs through a series of steps, including stimulation of endothelial cells (ECs) by autocrine and/or paracrine growth factors, proteolytic degradation of the basement membrane and surrounding extracellular matrix, EC proliferation and migration, and structural reorganization into a three-dimensional tubular structure[39]. Therefore, targeting proliferation and/or migration of ECs is one of the most attractive and effective strategies for treating angiogenesis-dependent disorders. We reported that mTECs grow faster and migrate better than mNECs[32]. These *in vitro* characteristics of mTECs represent

enhanced tumor angiogenesis *in vivo*, and the genes responsible for increased proliferation or migration of mTECs 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-ECs, one of the mTECs that showed high activity in proliferation and migration assays[32]. We first cotransfected Melanoma-ECs with three different sequences of siRNA per gene. Cell proliferation and migration were inhibited by >20% compared with mock-transfected cells using siRNAs targeted to 44 genes (Fig. 2). Subsequently, three different siRNAs specific for each of the 44 genes were used to independently transfect Melanoma-ECs. We finally selected five genes (*Def6*, *Nsg1*, *Enah*, *Tmem176b*, and *Pcdhb22*; Table 2, Fig. 3) whose respective siRNAs (more than two per gene) inhibited cell proliferation or migration by >30%. These were considered potential regulators of proliferation or migration of mTECs (Fig. 2, Fig. 4A–E). Knockdown of each gene was confirmed by qRT-PCR 48 h after transfection (Fig. 4A–E).

Expression of TEC markers in human TECs *in vitro* and *in vivo*

The therapeutic potential of targeting candidate genes largely depends on whether their expression is upregulated in hTECs as well as in mTECs. Therefore, we analyzed the expression of the five putative TEC markers in hTECs isolated from RCC specimens and hNECs 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 hTECs. This technical limitation forced us to choose RCCs as the source of hTECs.

The hTECs and hNECs were obtained from three patients. The clinical backgrounds of patients with RCC who donated tissue specimens are shown in Table 3. The binding of 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 hECs. Representative data are shown in Fig. 5A. The expression levels of *TMEM176B* and *DEF6* revealed by qRT-PCR analysis were significantly higher in hTECs than in hNECs for all paired samples (Fig. 5B), indicating that two out of five TEC markers that we identified in mice were upregulated in mTECs and hTECs. Furthermore, to determine the expression

levels of TMEM176B and DEF6 in tumor blood vessels in RCCs *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 (Fig. 5C). These results suggest that the transcription of these two genes was upregulated in hTECs *in vivo* and may be involved in tumor angiogenesis in cancer patients.

Discussion

In the present study, we isolated and cultured mTECs from three different types of human tumor xenografts and mNECs 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 mTECs compared with mNECs. Functional analysis of these 131 genes using RNAi revealed that five were involved in the proliferation or migration of mTECs. Two, *DEF6* and *TMEM176B*, were upregulated in hTECs and *in vivo* in tumor vessels of human

RCCs, 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 may be involved in tumor angiogenesis and may 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[40-43], 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[44-54]. DEF6 is overexpressed in cancer cells and may have an important function in tumor invasion and metastasis[55]; however, its role in tumor angiogenesis is unknown. In the current study, we show for the first time that DEF6 was upregulated in TECs compared with NECs and may mediate increased proliferation of TECs that enhances 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[56, 57]. It was discovered in human lung fibroblasts and is associated with human small cell lung carcinoma[58]. Although several recent reports implicate human TMEM176B in cancer[59-61], 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 TECs and show further using RNAi that TMEM176B mediates TEC migration. Moreover, immunohistochemical analysis revealed expression of TMEM176B in tumor vessels and in tumor cells as reported previously[62]. Although the contribution of TMEM176B in tumor cells to the malignant phenotype is unknown, it may 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 hTECs or tumor vessels of human RCC specimens (data not shown). ENAH and PCDHB15 were expressed in vessels and in mesangial cells of normal tissues, and NSG1 expression was not detected in tumor vessels. 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. Therefore, we cannot exclude the possibility that NSG1, ENAH, and PCDHB15 may be involved in tumor angiogenesis and still hold promise as targets for therapy.

The present study indicates the power of determining the differential expression of genes between TECs and NECs for identifying potential targets for antiangiogenic therapy. Although TEC markers such as ANTXR1 (TEM8), CD276, and JAG1 (Jagged1) were previously identified by this technique[22-28], the present study is the first, to our knowledge, to demonstrate upregulated expression of the genes encoding DEF6, TMEM176B, NSG1, ENAH, and PCDHB15 in TECs. The major difference between the current and previous studies is our unique method for culturing mTECs, which overcomes the loss of the TEC phenotype after dissociation from their tumor tissue. These highly purified mTECs isolated from human tumor xenograft maintain the specific characteristics of TECs *in vivo* during long-term culture[30-35], and therefore provide a more relevant system for tumor angiogenesis research and the identification of novel TEC markers.

In summary, we report here the identification of novel genes that are relevant to tumor angiogenesis by investigating the differences in gene expression patterns between mTECs and mNECs. Targeting these genes may lead to therapies that do not induce adverse effects associated with altering physiologic 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.

Conclusions

We identified 131 genes that are upregulated in mTECs compared with mNECs. Among these genes, five were involved in the proliferation or migration of mTECs, and two, *DEF6* and *TMEM176B*, were upregulated in hTECs and *in vivo* in tumor vessels of human RCCs. These results suggest that targeting these TEC markers contributes to more effective and safer approaches for cancer treatment.

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Table 1. Primer information

Species	Gene	Forward Primer	Reverse Primer
Mouse	*Tmem176b*	5'- CTCCAAGTCTACTCCTCAAGCTCCA -3'	5'- CCAGAGTCCTACAGGAAAGCAGAGA -3'
	Pcdhb22	5'- ATCCGCAACCGAGGTGATG -3'	5'- AATGCGGATTTGCGAGGTG -3'
	Nsg1	5'- GCCCTGATGGGTTTGTCTTGA -3'	5'- CACTGGAGTCTTGCTCCGTGTAGTA -3'
	Enah	5'- CACATTCAGAGTTGTGGGCAGA -3'	5'- TGCTGCCAAAGTTGAGACCATAC -3'
	Def6	5'- CACCAACGTGAAACACTGGAATG -3'	5'- CGGGTCAGGCGCTTTAGAGA -3'
	Cxcr7	5'- CTACAAACTGCTCAGCACTGAAGG -3'	5'- GCAGTCGCTGCTGTTACATGG -3'
	Col3a1	5'- TGCTCGGAACTGCAGAGACCTA -3'	5'- AGCATCCATCTTGCAGCCTTG -3'
	Dkk3	5'- CAAAGTCGCTTAGCAACAATGGAA -3'	5'- TGGCACCTGAAACCGTCATC -3'
	Antxr1	5'- TGCCCAGCACCAATCTTGAA -3'	5'- GAGCAATCGCCAGGATGGA -3'
	18SrRNA	5'- GGGAGCCTGAGAAACGGC -3'	5'- GGGTCGGGAGTGGGTAATTT -3'
Human	*TMEM176B*	5'- CCCTACCACTGGGTACAGATGGA -3'	5'- CTTCAAGACACAGACAGCCAGGA -3'
	DEF6	5'- CAGGGATACATGCCCTACCTCAAC -3'	5'- CAGCACAGCTCATCAAAGTGCTC -3'
	GAPDH	5'- ACAGTCAGCCGCATCTTCTT -3'	5'- GCCCAATACGACCAAATCC -3'

Table 2. Representative data of DNA microarray analysis

			Fold increase relative to Skin-ECs		
Probe	Gene Title	Symbol	Oral	Renal	Melanoma
(Mouse430_2)			Ca-ECs	Ca-ECs	-ECs
1418004_a_at	transmembrane protein 176B	*Tmem176b*	518.29	156.13	61.39
1418941_at	protocadherin beta 22	*Pcdhb22*	31.36	20.77	31.96
1423055_at	neuron specific gene family member 1	*Nsg1*	147.51	28.57	53.99
1424800_at	enabled homolog (Drosophila)	*Enah*	23.66	14.78	21.43
1452796_at	differentially expressed in FDCP 6	*Def6*	5.02	8.36	14.09
1417625_s_at	chemokine (C-X-C motif) receptor 7	*Cxcr7*	79.69	7.47	34.44
1427884_at	collagen, type III, alpha 1	*Col3a1*	9.61	9.73	14.75
1417312_at	dickkopf homolog 3 (Xenopus laevis)	*Dkk3*	283.71	29.68	18.70
1451446_at	anthrax toxin receptor 1	*Antxr1*	29.03	5.13	6.23

Table 3. Clinical background of the renal cell carcinoma (RCC) specimens

Case No.	M/F	Age (y)	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 staging guidelines.

‡According to the Fuhrman system.

M/F; male/female

INF; infiltration pattern

Fig. 1.

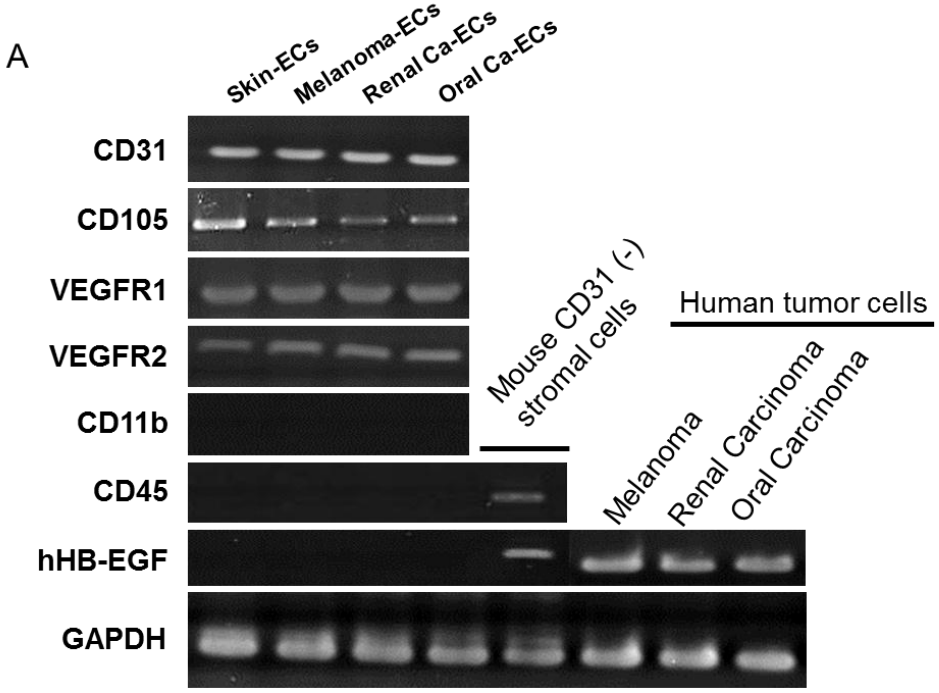
A
Skin-ECs
Melanoma-ECs
Renal Ca-ECs
Oral Ca-ECs
CD31
CD105
VEGFR1
VEGFR2
CD11b
CD45
hHB-EGF
GAPDH
Mouse CD31 (-) stromal cells
Human tumor cells
Melanoma
Renal Carcinoma
Oral Carcinoma
B
Skin-ECs
Melanoma-ECs
Renal Ca-ECs
Oral Ca-ECs

Fig. 2.

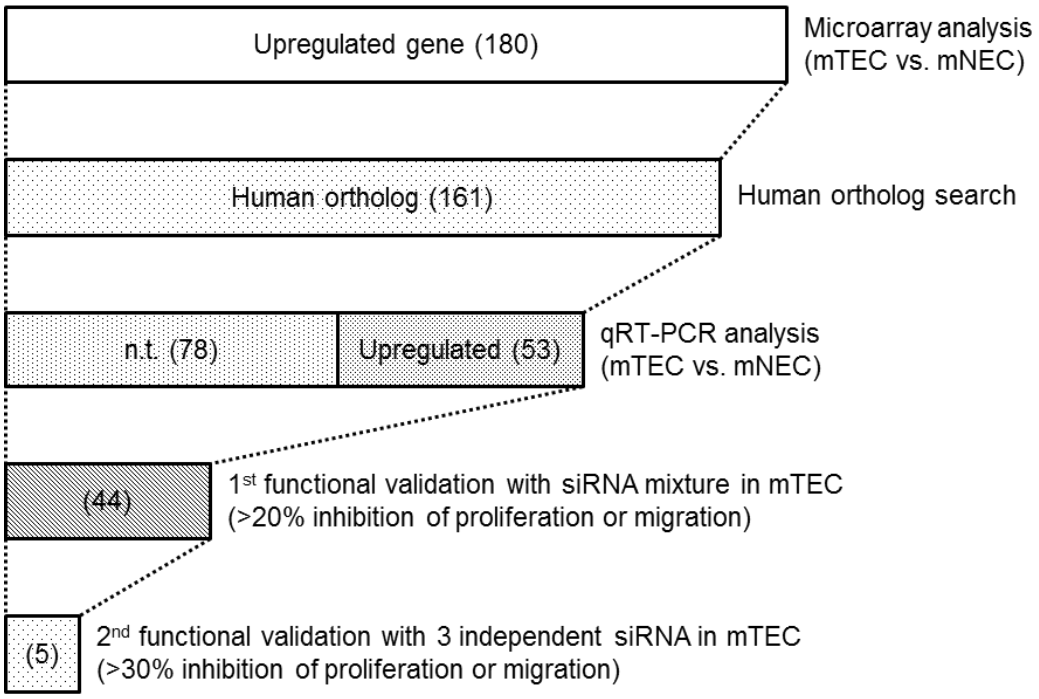
Upregulated gene (180)
Microarray analysis
(mTEC vs. mNEC)
Human ortholog (161)
Human ortholog search
n.t. (78)
Upregulated (53)
qRT-PCR analysis
(mTEC vs. mNEC)
(44)
1st functional validation with siRNA mixture in mTEC
(>20% inhibition of proliferation or migration)
(5)
2nd functional validation with 3 independent siRNA in mTEC
(>30% inhibition of proliferation or migration)

Fig. 3.

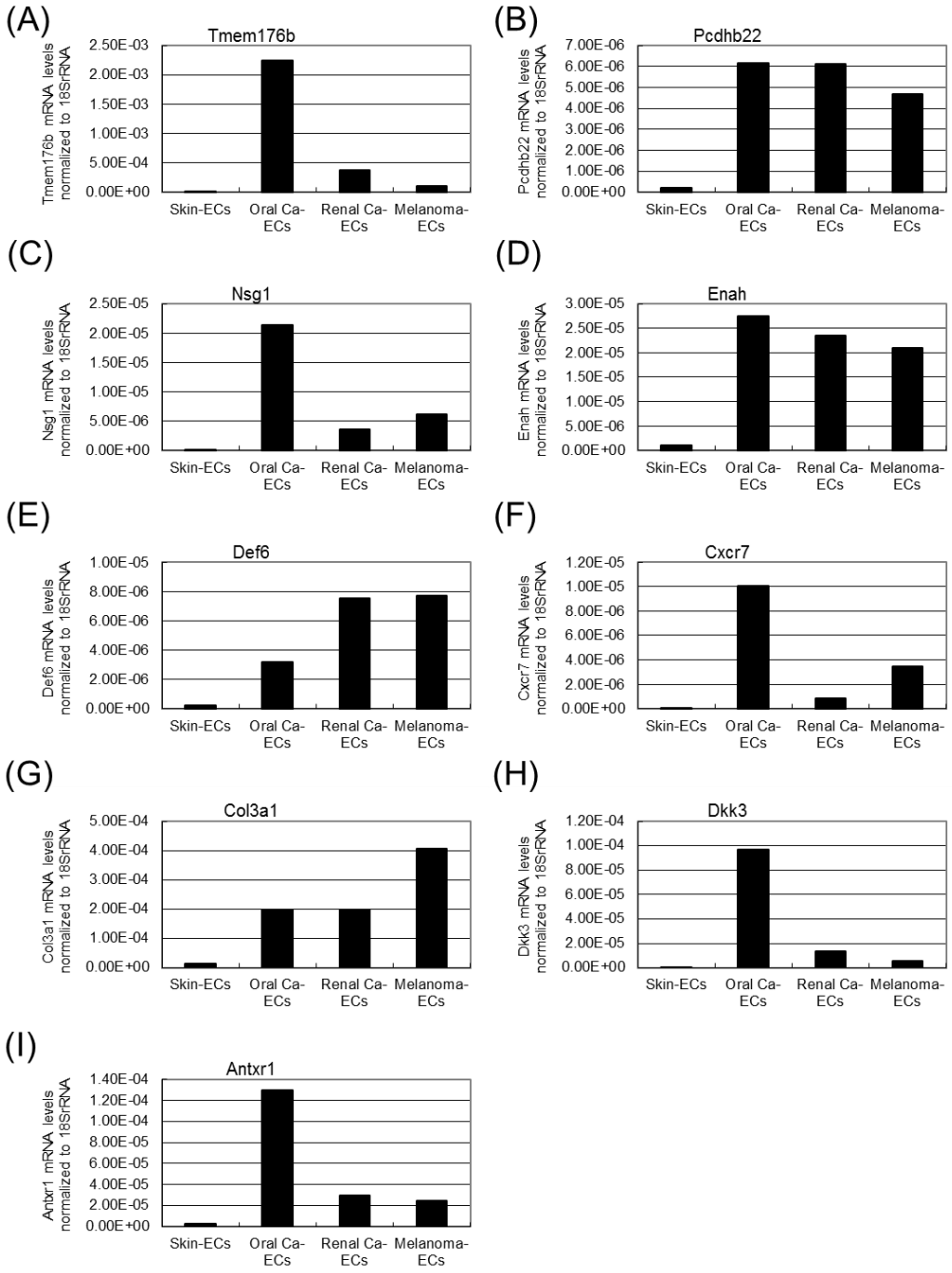
(A)
Tmem176b
Tmem176b mRNA levels normalized to 18SrRNA
2.50E-03
2.00E-03
1.50E-03
1.00E-03
5.00E-04
0.00E+00
Skin-ECs
Oral Ca-ECs
Renal Ca-ECs
Melanoma-ECs
(B)
Pcdhb22
Pcdhb22 mRNA levels normalized to 18SrRNA
7.00E-06
6.00E-06
5.00E-06
4.00E-06
3.00E-06
2.00E-06
1.00E-06
0.00E+00
Skin-ECs
Oral Ca-ECs
Renal Ca-ECs
Melanoma-ECs
(C)
Nsg1
Nsg1 mRNA levels normalized to 18SrRNA
2.50E-05
2.00E-05
1.50E-05
1.00E-05
5.00E-06
0.00E+00
Skin-ECs
Oral Ca-ECs
Renal Ca-ECs
Melanoma-ECs
(D)
Enah
Enah mRNA levels normalized to 18SrRNA
3.00E-05
2.50E-05
2.00E-05
1.50E-05
1.00E-05
5.00E-06
0.00E+00
Skin-ECs
Oral Ca-ECs
Renal Ca-ECs
Melanoma-ECs
(E)
Def6
Def6 mRNA levels normalized to 18SrRNA
1.00E-05
8.00E-06
6.00E-06
4.00E-06
2.00E-06
0.00E+00
Skin-ECs
Oral Ca-ECs
Renal Ca-ECs
Melanoma-ECs
(F)
Cxcr7
Cxcr7 mRNA levels normalized to 18SrRNA
1.20E-05
1.00E-05
8.00E-06
6.00E-06
4.00E-06
2.00E-06
0.00E+00
Skin-ECs
Oral Ca-ECs
Renal Ca-ECs
Melanoma-ECs
(G)
Col3a1
Col3a1 mRNA levels normalized to 18SrRNA
5.00E-04
4.00E-04
3.00E-04
2.00E-04
1.00E-04
0.00E+00
Skin-ECs
Oral Ca-ECs
Renal Ca-ECs
Melanoma-ECs
(H)
Dkk3
Dkk3 mRNA levels normalized to 18SrRNA
1.20E-04
1.00E-04
8.00E-05
6.00E-05
4.00E-05
2.00E-05
0.00E+00
Skin-ECs
Oral Ca-ECs
Renal Ca-ECs
Melanoma-ECs
(I)
Antxr1
Antxr1 mRNA levels normalized to 18SrRNA
1.40E-04
1.20E-04
1.00E-04
8.00E-05
6.00E-05
4.00E-05
2.00E-05
0.00E+00
Skin-ECs
Oral Ca-ECs
Renal Ca-ECs
Melanoma-ECs

Fig. 4.

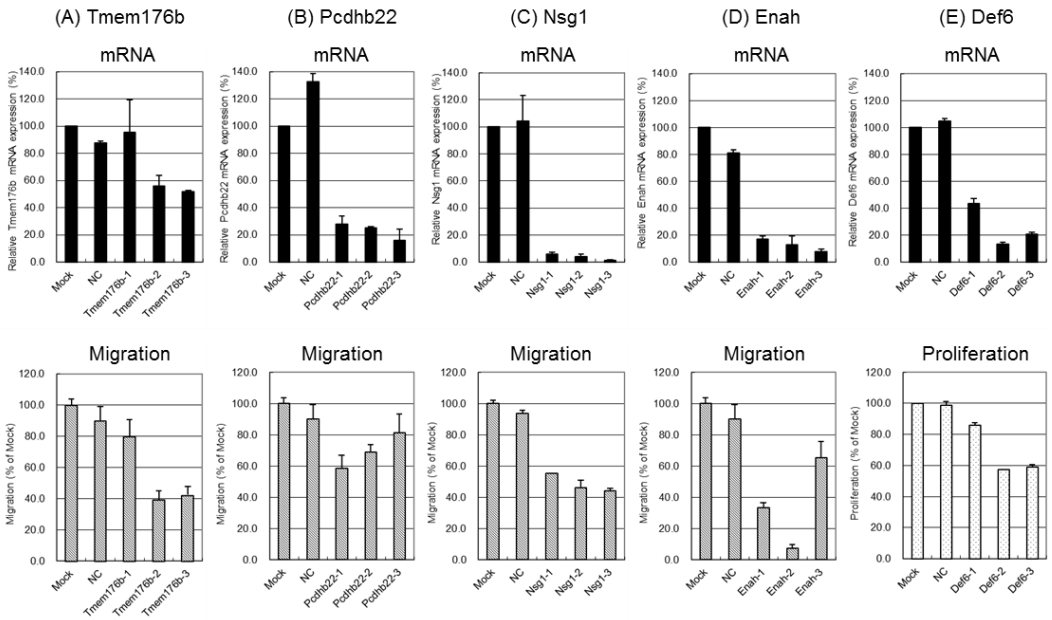
(A) Tmem176b
(B) Pcdhb22
(C) Nsg1
(D) Enah
(E) Def6
mRNA
Migration
Proliferation
Relative Tmem176b mRNA expression (%)
Relative Pcdhb22 mRNA expression (%)
Relative Nsg1 mRNA expression (%)
Relative Enah mRNA expression (%)
Relative Def6 mRNA expression (%)
Migration (% of Mock)
Proliferation (% of Mock)
Mock
NC
Tmem176b-1
Tmem176b-2
Tmem176b-3
Pcdhb22-1
Pcdhb22-2
Pcdhb22-3
Nsg1-1
Nsg1-2
Nsg1-3
Enah-1
Enah-2
Enah-3
Def6-1
Def6-2
Def6-3

Fig. 5.

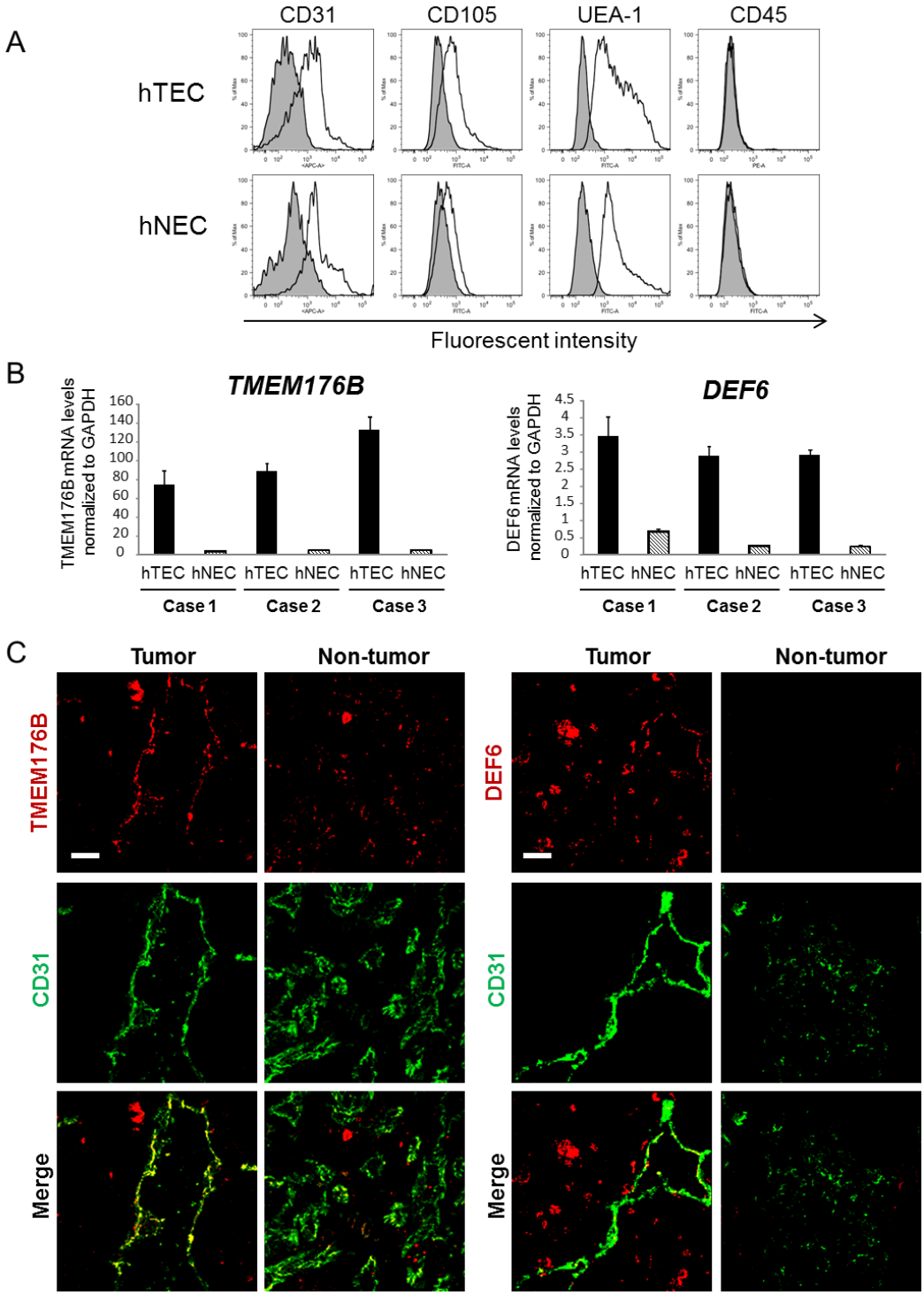
A
CD31
CD105
UEA-1
CD45
hTEC
hNEC
Fluorescent intensity
B
TMEM176B
TMEM176B mRNA levels normalized to GAPDH
hTEC hNEC hTEC hNEC hTEC hNEC
Case 1
Case 2
Case 3
DEF6
DEF6 mRNA levels normalized to GAPDH
C
Tumor
Non-tumor
TMEM176B
DEF6
CD31
Merge

Figure legends

Figure 1. Characterization of mTECs and mNECs. mTECs (Melanoma-ECs, Renal Ca-ECs, and Oral Ca-ECs) were isolated from human tumor xenografts (melanoma, renal carcinoma, and oral squamous cell carcinoma, respectively) and mNECs (Skin-ECs) were from the dermis of nude mice. (A) The expression of *Cd31*, *Cd105*, *Vegfr1*, *Vegfr2*, *Cd11b*, *Cd45*, and human *HBEGF* in mTECs and mNECs was analyzed by RT-PCR. The data indicate high purity of isolated mTECs and mNECs. CD31-negative non-EC fractions and human tumor cells (melanoma, renal carcinoma, and oral carcinoma) were also analyzed. (B) Isolated and cultured mECs formed tubes on matrigel-coated plates. Scale bar; 100 μm.

Figure 2. Schematic of 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 mTECs.

Figure 3. Analysis of the transcription of TEC marker genes. By qRT-PCR,

five novel TEC markers identified here and four known markers were shown to be selectively upregulated in all types of mTECs compared with mNECs. The relative expression levels of mRNAs were normalized to that of 18S rRNA for each cell type. (A)–(E) mRNA expression of novel TEC markers (*Tmem176b*, *Pcdhb22*, *Nsg1*, *Enah*, and *Def6*). (F)–(I) mRNA expression of known TEC markers (*Cxcr7*, *Col3a1*, *Dkk3*, and *Antxr1*).

Figure 4. Functional analysis of TEC markers by siRNA-mediated gene silencing. Melanoma-ECs were transfected using three different siRNAs for each gene. Silencing of each TEC marker 48 h after transfection was evaluated by 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 (lower panel). siRNA-mediated effects of each TEC marker are shown in A–E. (A) *Tmem176b*, (B) *Pcdhb22*, (C) *Nsg1*, (D) *Enah*, and (E) *Def6*.

Figure 5. Analysis of *TMEM176B* and *DEF6* expression *in vitro* and *in vivo*.

hTECs were freshly isolated from RCC tissue and hNECs were isolated from normal renal tissue separated from the tumor in the same specimens. (A) Verification of ECs from a human sample. The binding of UEA-1 lectin, expression of CD31, CD105, and lack of expression of CD45 (white area) indicates high purity of the isolated hTECs and hNECs. The isotype control is shown in gray. (B) Upregulated expression of *TMEM176B* and *DEF6* in hTECs. qRT-PCR analysis detected high levels of expression of both genes in hTECs compared with the corresponding hNECs in all three cases. The expression levels of the mRNAs were normalized to that of *GAPDH*. (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. Scale bar, 20 μm.

Table legends

Table 1. Primer information. List of primers employed in PCR

Table 2. Representative data of DNA microarray analysis. The DNA microarray of representative genes, including the five novel TEC markers and the four known markers previously identified, are shown.

Table 3. Clinical background of the renal cell carcinoma (RCC) specimens. Tumor tissues were surgically resected from three patients diagnosed with RCC (clear cell carcinomas).