An important concept in tumor angiogenesis is that tumor endothelial cells (TECs) are genetically normal and homogeneous. However, we previously reported that TECs differ from normal ECs. Whether the characteristics of TECs derived from different tumors differ remains unknown. To elucidate this, in this study, we isolated two types of TECs from high-metastatic (HM) and low-metastatic (LM) tumors and compared their characteristics. HM tumor–derived TECs (HM-TECs) showed higher proliferative activity and invasive activity than LM tumor–derived TECs (LM-TECs). Moreover, the mRNA expression levels of pro-angiogenic genes, such as vascular endothelial growth factor (VEGF) receptors 1 and 2, VEGF, and hypoxia-inducible factor-1α, were higher in HM-TECs than in LM-TECs. The tumor blood vessels themselves and the surrounding area in HM tumors were exposed to hypoxia. Furthermore, HM-TECs showed higher mRNA expression levels of the stemness-related gene stem cell antigen and the mesenchymal marker CD90 compared with LM-TECs. HM-TECs were spheroid, with a smoother surface and higher circularity in the stem cell spheroid assay. HM-TECs differentiated into osteogenic cells, expressing activated alkaline phosphatase in an osteogenic medium at a higher rate than either LM-TECs or normal ECs. Furthermore, HM-TECs contained more aneuploid cells than LM-TECs. These results indicate that TECs from HM tumors have a more pro-angiogenic phenotype than those from LM tumors. (Am J Pathol 2012, 180:1294–1307; DOI: 10.1016/j.ajpath.2011.11.035)

Tumor angiogenesis is necessary for the progression and metastasis of solid tumors. Tumor blood vessels provide nutrition and oxygen, thereby promoting tumor progression. In addition, tumor blood vessels act as gatekeepers that facilitate the metastasis of tumor cells to distant organs. The progressive growth of tumors and metastasis depends on angiogenesis; therefore, inhibition of tumor angiogenesis is a promising strategy for cancer treatment.

The degree of angiogenesis is determined by the balance between the positive and negative regulating molecules that are released by tumor and host cells in the microenvironment. Recently, tumor blood vessels differed morphologically from their normal counterparts. These morphological abnormalities in tumor blood vessels raise the question of whether tumor endothelial cells (TECs) and normal ECs (NECs) differ at the molecular and functional levels. We previously reported that TECs differ from NECs in characteristics such as cell proliferation, migration, gene profile, and responses to growth factors and several chemotherapeutic drugs. Furthermore, TECs are cytogenetically abnormal. In addition, our previous reports showed that isolated TECs did not lose their specific phenotype for some time after dissociation from the tumor tissue. Some reports showed that tumor blood vessels were heterogeneous. Because TECs play an important role in metastasis, investigation of the possible differences between TECs of tumors differing in their malignancy status should be elucidated.

Supported in part by a grant-in-aid for scientific research from the Ministry of Education, Science, and Culture of Japan (20390506 to K.H.).

Accepted for publication November 21, 2011.

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To analyze the correlation between TEC characteristics and malignancy status of the tumor, we compared the characteristics between high- and low-metastatic tumor-derived endothelial cells (HM-TECs and LM-TECs, respectively) using the following two types of melanoma cells xenografted into nude mice: high-metastatic melanomas (HM tumors) and low-metastatic melanomas (LM tumors).

Materials and Methods

Cell Lines and Culture Conditions

A375 cells (LM tumors) were obtained from American Type Culture Collection (Manassas, VA). A375-SM cells (super-metastatic human malignant melanoma; HM tumors) were a gift from Dr. Isaiah J. Fidler (University of Texas M.D. Anderson Cancer Center, Houston, TX). A375 and A375-SM cells were cultured in minimum essential medium (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS). The cells were cultured at 37°C in a humidified atmosphere containing 5% CO2 and 95% air.

Animals and Treatments

Pathogen-free 6-week-old female nude mice weighing approximately 20 g (Sankyo Labo Service Corporation, Tokyo, Japan) were randomly divided into two groups (n = 5 in each group). LM and HM tumor cells (1 x 10⁶ cells/mouse) were injected s.c. into the right flank of the mice (day 0). No difference in body weight was observed among the groups throughout the experiment. Mice were euthanized under anesthesia on day 42. All procedures for animal experimentation were approved by the institutional guidelines.

Antibodies

Fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD31, CD133, and CD34 antibodies were obtained from eBioscience (San Diego, CA). FITC-conjugated Bandeiraea simplicifolia lectin isolectin B4 (BS1-B4) was obtained from Vector Laboratories (Burlingame, CA). Phosphatidylethanolamine (PE)-conjugated anti-mouse CD31 antibody; purified rat anti-mouse CD31, CD105, and CD144 antibodies; and FITC-conjugated anti-mouse stem cell antigen (Sca-1) antibody were obtained from BD Pharmingen (San Diego). Alexa Fluor 488–conjugated anti-mouse CD90 antibody and allopurinol (APC)-conjugated anti-mouse CD45 were obtained from eBioscience (San Diego, CA). FITC-conjugated rat IgG1, Alexa Fluor 488–conjugated goat anti-rat IgG, and Alexa Fluor 594–conjugated goat anti-rabbit IgG were obtained from Molecular Probes (Eugene, OR). Anti-mouse α-smooth muscle actin (α-SMA) antibody was obtained from Abcam (Cambridge, UK).

IHC Analysis and Determination of MVD

Tumors were dissected from human tumor xenografts in nude mice. The tumor specimens were embedded in cryocryocap (Tissue-Tek; Miles, Elkhart, IN), immediately immersed in liquid nitrogen, and cut into sections (10 µm thick) using a cryotome. The frozen sections were fixed in 100% ice-cold acetone for 10 minutes and blocked with 2% goat serum and 5% sheep serum in PBS for 30 minutes. The sections were incubated with rat anti-mouse CD31 antibody for 16 hours, followed by Alexa Fluor 488–conjugated goat anti-rat IgG for 2 hours. Sections were counterstained with DAPI (Roche, Indianapolis, IN), using an Olympus IX71 microscope (Olympus, Tokyo). To analyze microvessel density (MVD), the vascular area per unit area in sections stained for CD31 was determined using MetaMorph software (Molecular Devices, Tokyo).

Isolation of TECs and NECs

TECs and NECs were isolated as previously described. TECs were isolated from human tumor xenografts of HM and LM tumors (10 nude mice for each tumor type). NECs were isolated from the dermis and used as a control. TECs and NECs were isolated using a magnetic cell sorting system (Miltenyi Biotec, Tokyo) with anti-CD31 antibody. CD31-positive cells were sorted and plated on 1.5% gelatin-coated culture plates and cultured in endothelial growth medium-2 (EGM-2 MV) (Clonetics, Walkersville, MD) and 15% FBS. Diphtheria toxin (DT; 500 ng/mL; Calbiochem, San Diego) was added to TEC subcultures to kill any human tumor cells and NEC subcultures for technical consistency. Human cells express heparin-binding EGF-like growth factor, which is a DT receptor. However, DT does not interact with murine HB-EGF; murine ECs, therefore, survive this treatment. The isolated ECs were purified by a second round of purification with FITC-conjugated BS1-B4.

In Vitro Cell Proliferation Assay

The proliferation of tumor cells (HM and LM tumors) and ECs (HM-TECs, LM-TECs, and NECs) was measured by the MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] assay (CellTiter 96 Non-Radioactive Cell Proliferation Assay; Promega, Madison, WI), which monitors the number of viable cells, according to the manufacturer’s instructions. Cultured cells were seeded at 2 x 10⁴ cells per well in 96-well flat-bottomed plates containing a suitable medium (minimum essential medium for tumor cells and EGM-2 MV for ECs) and allowed to adhere to the plates for 12 hours. The experiment was performed three times, and similar results were observed.

Flow Cytometric Analysis of Cultured Endothelial Cells

After TECs and NECs were dissociated by treatment with 0.5% trypsin-EDTA, cells were incubated with FITC-conjugated BS1-B4 and primary antibodies against CD105 and CD144, Alexa Fluor 488–conjugated anti-CD90, and FITC-conjugated anti-Sca-1 for 20 minutes at 4°C. After washing, cells were incubated...
with Alexa Fluor 488–conjugated goat anti-rat IgG for 20 minutes at 4°C for analysis of the expression of CD105 and CD144. Cells were analyzed using the FACSAria II cell sorter (Becton Dickinson, San Jose, CA). All data were analyzed using FlowJo software version 9.3.1 (Tree Star, Inc, Ashland, OR).

Cell Migration Assay

Migration of cells toward vascular endothelial growth factor (VEGF) was measured in a Boyden chamber, as previously described, with minor modifications.9,11 Real-time RT-PCR was performed with a Total RNA was extracted using the RNeasy Micro Kit RT-PCR and Real-Time RT-PCR previously described, with minor modifications.11,12 VEGFA (VEGF) was measured in a Boyden chamber, as previously described.9,11

Assessment of Tissue Hypoxia

Hypoxic areas in HM and LM tumor cryosections were detected using the Hypoxyprobe-1 TM Kit (HPI, Burlington, MA). In brief, 60 mg/kg pimonidazole was injected i.p. 30 minutes before the animal was sacrificed, and samples were stained with Hypoxyprobe (FITC-conjugated anti-pimonidazole mouse monoclonal antibody). To analyze the hypoxic area in HM and LM tumors, stained samples were visualized under a Fluoview FV1000 confocal microscope (Olympus). The pimonidazole-positive areas were quantified using ImageJ software (NIH, Bethesda, MD).

IHC Analysis

Tumor tissues were dissected from euthanized mice, embedded in cryoprotectant (Tissue-Tek), and immediately immersed in liquid nitrogen. Cryosections of xenografted HM and LM tumors were processed for immunohistochemistry (IHC) using FITC-conjugated anti-mouse Sca-1, Alexa Fluor 488–conjugated anti-mouse CD90, and mouse CD31 antibodies to investigate the colocalization of Sca-1 and CD31 in tumor tissue. After washing, cells were incubated with Alexa Fluor 594–conjugated goat anti-rat IgG to analyze the expression of CD31. Further, cryosections of xenografted HM and LM tumors were processed for IHC using FITC-conjugated anti-mouse CD133, α-SMA, and Alexa Fluor 647–conjugated anti-mouse CD31 antibodies to investigate the colocalization of CD133 and α-SMA in tumor tissue. After washing, slides were incubated with Alexa Fluor 594–conjugated goat anti-rabbit IgG to evaluate the expression of α-SMA.

All IHC samples were counterstained with DAPI and visualized under an Olympus IX71 fluorescence microscope and an Olympus Fluoview FV1000 confocal microscope for each experiment.
**Western Blot Analysis**

Western blot analysis was performed using antibodies specific for phosphorylated Akt, Akt, β-actin, and horseradish peroxidase–conjugated secondary antibody, as previously described. Phosphorylated Akt levels were normalized to total Akt and β-actin levels and analyzed by scanning densitometry using ImageJ software.

**Flow Cytometric Analysis of Stemlike ECs in HM and LM Tumors**

To investigate the populations of stemlike ECs in uncultured cell suspensions of HM and LM tumor tissues, multicolor flow cytometric analysis was performed for CD31, CD45, CD90, and Sca-1. We dissociated tumor tissues and analyzed stained cells by flow cytometry. We classified CD31+CD45+CD133+ cells as endothelial stemlike cells. Cell composition percentages varied among tumor samples, and the range of values is presented. Cell analyses were performed using FACSAria II.

**Measurement of EPCs and Resident Progenitors in HM and LM Tumors**

Tumors were dissected from each of the 10 xenografted mice and digested with collagenase and DNase. Cells were suspended in HBSS containing 0.5% bovine serum albumin and incubated with mouse Fc blocking reagent (Miltenyi Biotec). We classified CD31+CD45−CD133+ cells as endothelial progenitor cells (EPCs) and CD31+CD45+CD133+ as tissue resident progenitors of ECs. Cells were stained with PE-conjugated CD31 (clone 390), APC-conjugated CD45 (clone 30-F11), and FITC-conjugated CD133 (clone 13A4) monoclonal antibodies. Propidium iodide (Sigma-Aldrich) was used to eliminate background noise from dead cells. Antibody batches were titrated to determine the optical concentration to be used. Control tubes included an isotype for each of the fluorochromes (PE-conjugated rat IgG2a, APC-conjugated rat IgG2b, and FITC-conjugated rat IgG1). Subsets of EPCs were measured in HM and LM tumor tissues. In addition, we analyzed CD31+CD34−CD133+ cells in tumor tissues to measure resident progenitors of ECs. Cells were stained with APC-conjugated CD31, FITC-conjugated CD34, and PE-conjugated CD133 monoclonal antibodies. Control tubes included an isotype for each of the fluorochromes (PE-conjugated rat IgG1, APC-conjugated rat IgG2a, and FITC-conjugated rat IgG2a). In HM and LM tumor tissues, EPCs and tissue resident progenitors of ECs were identified as the rare fraction by four-color flow cytometric analysis. Cell analyses were performed using FACSAria II. In a test tube, 1 × 10^6 cells were acquired. Results are expressed as medians and ranges. Groups were compared using a Mann-Whitney test. All data were analyzed using FlowJo software. P < 0.05 was considered statistically significant.

**ALP Assay**

To evaluate bone differentiation, TECs and NECs were seeded at a density of 50,000 cells/well in 12-well plates containing EGM-2 MV. The next day, the medium was removed and replaced with osteogenic differentiation medium (Lonza, Walkersville, MD) supplemented with 5% FBS. After 3 days, cells were washed, fixed in formalin, and stained for alkaline phosphatase (ALP). ALP activity was determined using the TRACP & ALP Double-Stain Kit (Takara Bio, Shiga, Japan).

**Stemness Spheroid Assay**

The EC suspension was inoculated in a microwell chip (STEM Biomedical, Kitakyushu, Japan) and placed in a 35-mm culture dish containing 2 mL of culture medium.

**Karyotype Analysis**

Karyotype analysis of cultured ECs was performed by GTG banding, as previously described. Four or five karyotypes of HM-TECs and LM-TECs were analyzed, and 20 metaphase spreads were counted.

**Fluorescence in Situ Hybridization**

ECs directly isolated from tumor or normal tissues had high purity (95%) and were immunostained for CD31 (green), followed by fluorescence *in situ* hybridization (FISH). After immunostaining with FITC-conjugated CD31 (uncultured ECs) or staining with FITC-conjugated BS1-B4 (cultured ECs), slides were fixed for 45 minutes using Histochoice (AMRESCO, Solon, OH), as previously described. FISH was performed using a Cy3-mouse chromosome 17 locus-specific BAC probe (RP23-146B6; Chromosome Science, Sapporo, Japan). All samples were counterstained with DAPI. Hybridization signals were observed and analyzed using an Olympus IX71 fluorescence microscope. Chromosomes were counted in at least 100 nuclei for each sample. Aneuploid cells were counted three times in each sample. Cells with a single signal for each probe were not included in the analysis because it was difficult to judge whether the single signal was due to monosomy or incomplete hybridization.

**NEC–Tumor Cell Co-Culture Assay**

Transwell chambers (Costar, Cambridge, MA) with membranes with a pore size of 0.4 μm, with some modification, were used.

Changes in the expression of pro-angiogenic genes in NECs co-cultured with HM or LM tumor cells were analyzed by real-time PCR. NECs (6 × 10^4 cells) were suspended in EBM-2 supplemented with 1% FBS and placed in the lower compartment of the Transwell chamber. HM or LM tumor cells (2 × 10^4 cells) were suspended in EBM-2 supplemented with 1% FBS and placed in the upper compartment. After 24 hours of incubation, RNA was isolated from NECs. After the NECs were co-cultured under serum-starved conditions for 72
hours, the number of dead NECs was determined by staining with propidium iodide and counting using the FACS-Aria II cell sorter.

Transwell Migration Assay

NEC migration toward HM or LM tumor cells was examined using the Transwell chamber, with membranes having a pore size of 8 μm, with some modifications.19 HM or LM tumor cells (6 × 10⁴ cells) were suspended in EBM-2, supplemented with 1% FBS, and placed in the lower compartment. NECs (2 × 10⁴ cells) were suspended in EBM-2, supplemented with 1% FBS, and placed in the upper compartment. After 4 hours of incubation, cells that had migrated to the lower surface of the membranes were counted using a microscope at a magnification of ×200.

Statistical Analysis

Differences between experimental groups were evaluated using the Student’s t-test or the Mann-Whitney U-test. P < 0.05 was considered significant, and P < 0.01 was considered highly significant.

Results

HM Tumors Show a More Angiogenic Phenotype than LM Tumors

Before isolating HM-TECs and LM-TECs, we analyzed the biological differences between the two types of melanoma cells [A375 (LM tumors) and A375-SM (HM tumors)] that were to be xenografted into nude mice. Cell proliferation was compared between the two types of tumor cells. HM tumor cells proliferated significantly faster than LM tumor cells (P < 0.05, Figure 1A).

Next, LM and HM tumor cells were xenografted into mice. HM tumors grew more rapidly than LM tumors in nude mice (data not shown). When mice were euthanized on day 42, the number of mice with metastasis for HM tumors was two and zero for LM tumors (Figure 1B). The incidence of metastasized HM tumors was consistent with that in a previous report.20

HM tumors showed a highly active angiogenic phenotype, with a mean MVD of 2.4%, compared with LM tumors, which had a mean MVD of 1.5% (P < 0.05, Figure 1, C and D). Furthermore, mRNA expression levels of the pro-angiogenic genes HIF-1α and VEGFA and the metastasis-associated gene S100A4 in HM tumor cells were significantly

Figure 1. HM melanoma (HM tumor) was more angiogenic than LM melanoma (LM tumor). A: HM tumor cells proliferated significantly faster than LM tumor cells in vitro. *P < 0.05. B: HM and LM tumor cells were xenografted into mice, and animals were sacrificed on day 42. The number of mice with metastasis was two for HM tumors and zero for LM tumors. C: IHC analysis of xenografted LM and HM tumor tissues. Cryosections were stained with FITC-conjugated anti-CD31 antibody and counterstained with DAPI. The CD31-positive vessel area in HM tumors was larger than that in LM tumors. Scale bar = 100 μm. D: Quantitative analysis of MVD. IHC analysis indicated that HM tumors were more angiogenic than LM tumors. *P < 0.05. E: The metastasis-associated gene, S100A4, was up-regulated in HM tumor compared with LM tumor. mRNA expression levels of HIF-1α and VEGFA in HM tumor were higher than those in LM tumors. mRNA expression levels were normalized to GAPDH. *P < 0.05 versus LM tumors.
PCR. TECs and NECs were positive for CD31, VEGFR1, and VEGFR2 by regular previously described, with some modifications.2,7,9 NECs and LM tumor xenografts in nude mice, respectively, as HM-TECs and LM-TECs were isolated from human HM Phenotype than LM-TECs and NECs HM-TECs Show a More Pro-Angiogenic and metastatic potential than LM tumors. These data suggest that HM tumors possess greater angiogenic and higher than those in LM tumor cells (P < 0.05, Figure 1E). These data suggest that HM tumors possess greater angiogenic and metastatic potential than LM tumors.

**HM-TECs Show a More Pro-Angiogenic Phenotype than LM-TECs and NECs**

HM-TECs and LM-TECs were isolated from human HM and LM tumor xenografts in nude mice, respectively, as previously described, with some modifications.2,7,9 NECs were isolated from the dermis (skin ECs) and used as controls. BS1-B4 lectin binding and the expression of CD105 and CD144 indicated high purity of isolated ECs during long-term culture. The expression of CD105 and CD144 and binding of BS1-B4 indicated high purity of isolated TECs and NECs on fluorescence-activated cell sorter (FACS) analysis. Isotype control is on the left. **Figure 2.** HM-TECs showed greater pro-angiogenic than LM-TECs and NECs. A: Characteristics of isolated HM-TECs, LM-TECs, and NECs after long-term culture. The expression of CD105 and CD144 and binding of BS1-B4 indicated high purity of isolated TECs and NECs on fluorescence-activated cell sorter analysis. Isotype control is on the left. B: Cultured TECs and NECs were positive for CD31, VEGFR1, and VEGFR2 by regular PCR. C: Cultured ECs formed tubes on Matrigel-coated plates. D: HM-TECs showed greater proliferative activity than LM-TECs and NECs. A cell proliferation assay demonstrated that HM-TECs proliferated faster than LM-TECs and NECs. *P < 0.05 versus LM-TECs and NECs. E: HM-TECs showed higher motility than LM-TECs and NECs. Cell migration of ECs toward VEGFA was analyzed using a Boyden chamber. EBM-2 containing 0 or 10 ng/mL VEGFA was added in the lower compartment. HM-TECs were significantly more sensitive to VEGFA than LM-TECs and NECs. *P < 0.05 versus LM-TECs and NECs.

Angiogenesis-Related Genes Are Up-Regulated in HM-TECs

TECs have more angiogenic properties than NECs.10,11,21 Because HM-TECs showed a higher proliferation rate and greater migration toward VEGF than LM-TECs and NECs, we compared the expression levels of the angiogenesis-related genes VEGF, VEGFR1, and VEGFR2 in ECs by real-time PCR. Expression levels in HM-TECs were higher than those in LM-TECs and NECs (P < 0.05 versus LM-TECs and NECs, Figure 3A). In tumors, mRNA expression of VEGF was substantially enhanced in the hypoxic area of the tumors, indicating a mechanism by which a hypoxic microenvironment may stimulate tumor angiogenesis.22,23 Hypoxia stimulates HIF, which regulates diverse cellular processes, including angiogenesis.24 Next, the hypoxic area in HM and LM tumors was analyzed using the hypoxia marker pimonidazole. Tumor tissues were double stained with anti-CD31 and anti-pimonidazole antibodies to visualize hypoxic areas. The pimonidazole-positive area in HM tumors was 7.8-fold larger than that in LM tumors (P < 0.05, Figure 3B). The tumor blood vessels and surrounding area were also pimonidazole positive in HM tumors. This suggests that the tumor vessels in HM tumor were exposed to hypoxia. In addition, expression of HIF-1α and transcripts of HIF-1α and its downstream gene CXCL12 in HM-TECs were up-regulated compared with LM-TECs and NECs (P < 0.05 versus LM-TECs and NECs, Figure 3C). These results indicate that intratumoral hypoxia may be due to the pro-angiogenic phenotype of HM-TECs.

**HM-TECs Show Greater Invasive Potential than LM-TECs and NECs**

The key steps in tumor angiogenesis are migration and invasion of ECs through extracellular matrix (ECM) barriers. To compare the invasive potential among ECs, chemoinvasion assays were performed using a Matrigel-coated Boyden chamber. Among ECs examined, HM-TECs showed the greatest invasion of the lower part of the chamber (P < 0.05 versus LM-TECs and NECs, Figure 4A). Because MMP-2 and MMP-9 are involved in cell invasion during angiogenesis, their mRNA expression levels were analyzed by real-time PCR and were up-regulated in HM-TECs compared with those in LM-TECs and NECs (P < 0.05 versus LM-TECs and NECs, Figure
4B). On the other hand, mRNA expression levels of tissue inhibitor of MMP-1 in HM-TECs were lower than those in LM-TECs and NECs (P < 0.05 versus LM-TECs and NECs). Akt promotes invasion of tumor cells by increasing cell motility and MMP-9 production.25 We analyzed Akt phosphorylation of ECs. HM-TECs displayed the highest level of Akt activation under basal conditions (Figure 4C). Phosphorylated Akt levels were normalized for total Akt and -actin. Figure 4D shows the ratios of phosphorylated Akt (Ser 473)/Akt and phosphorylated Akt/ -actin determined by densitometric analysis. The results suggest that the activation of the Akt signaling pathway may be one of the mechanisms underlying the invasive potential of HM-TECs and a cause of its pro-angiogenic phenotype.

**HM-TECs Are Resistant to Anticancer Drugs with Up-Regulation of MDR1–P-gp**

Some studies21,26 have reported that TECs are resistant to chemotherapy-induced apoptosis. Sensitivity to paclitaxel and 5-fluorouracil (5-FU) was analyzed in all EC types. Proliferation of all ECs was inhibited by paclitaxel and 5-FU in a concentration-dependent manner after 72 hours. The half-maximal inhibitory concentration (IC50) of paclitaxel was 0.8 nmol/L for NECs, 1.5 nmol/L for LM-TECs, and 16 nmol/L for HM-TECs. The IC50 of 5-FU was 1.1 μmol/L for NECs, 0.9 μmol/L for LM-TECs, and 3.7 μmol/L for HM-TECs. A significant difference was observed in sensitivity to paclitaxel among HM-TECs, LM-TECs, and NECs in the concentration range of 1 to 20 nmol/L (Figure 5A) and to 5-FU in the concentration range of 0.5 to 1 μmol/L (P < 0.05 versus LM-TECs and NECs, Figure 5B). HM-TECs were more resistant to paclitaxel and 5-FU than other types of ECs.

**MDR1** is a major drug-resistance–related gene and is up-regulated in drug-resistant cells. In the present study, mRNA expression levels of MDR1 in TECs were higher than those in NECs. The mRNA expression levels of MDR1 in HM-TECs were approximately twofold higher than those in LM-TECs and NECs (P < 0.05 versus LM-TECs and NECs, Figure 5C). These results indicate that...
the up-regulation of MDR1 in HM-TECs may be a mechanism of drug resistance.

**HM-TECs Show More Marked Stem Cell Characteristics than LM-TECs**

Previous reports demonstrated that the expression of MDR–P-gp positively correlates with stem cell characteristics in cancer stem cells. Some tumors have undergone vascularization by recruiting vascular progenitor cells. Sca-1 is a marker for vascular progenitor cells and is used extensively as a marker of adult murine hematopoietic stem and progenitor cells. Our previous reports showed that Sca-1 is overexpressed in tumor blood vessels in vivo and in isolated TECs. To elucidate whether stemness characteristics differ among these ECs, expression levels of Sca-1 and CD90 were analyzed in ECs. Real-time PCR revealed that mRNA expression levels of Sca-1 and CD90 in HM-TECs were higher than those in LM-TECs and NECs (P < 0.05 versus LM-TECs and NECs, Figure 6A).

Flow cytometric analysis revealed that expression levels of Sca-1 and CD90 in cultured HM-TECs were significantly higher than those in LM-TECs and NECs (Figure 6B). To investigate the presence of EC-like cells in uncultured cell suspensions prepared from HM and LM tumor tissues, multicolor flow cytometry analysis for CD31, CD45, CD90, and Sca-1 was performed. We classified CD31+CD45−Sca-1+ and CD31+CD45−CD90+ cells as stemlike ECs. Cell composition percentages varied among tumor samples. The percentage of CD31+CD45−Sca-1+ cells in HM tumors was 90.77% ± 0.68% versus 50.9% ± 14.53% in LM tumors. The percentage of CD31+CD45−CD90+ cells in HM tumors was 50.33% ± 4.3% versus 20.8% ± 15.27% in LM tumors (Figure 6C).

IHC analysis revealed that HM tumor blood vessels were partially stained with anti-Sca-1 antibody, whereas LM tumor blood vessels were negative or weakly stained (Figure 6D). Furthermore, HM tumor blood vessels were partially stained with anti-CD90 antibody, whereas LM tumor blood vessels were negative or weakly stained (Figure 6E).

Previous studies reported that TECs had features of mesenchymal stem cells. Next, we investigated stem cell characteristics in HM-TECs and LM-TECs using a stem cell spheroid assay. HM-TECs, but not LM-TECs, showed spheroid morphological features, with a smooth surface and high circularity 72 hours after seeding of the cells into the microchip (Figure 7A). The number of sphere-forming cells in HM-TECs was significantly higher than that in LM-TECs and NECs (P < 0.05 versus LM-TECs and NECs, Figure 7B).

Because previous studies reported that TECs could transdifferentiate into cells forming bone-like tissue, showing features of mesenchymal stem cells, we performed a bone differentiation assay. HM-TECs exhibited ALP activity after 3 days of stimulation with osteogenic differentiation medium, but LM-TECs or NECs did not (Figure 7C). The number of cells with ALP activity in HM-TECs was significantly higher than in LM-TECs and NECs after stimulation with osteogenic medium (P < 0.05 versus LM-TECs and NECs, Figure 7D). These results suggest that HM-TECs may have more marked stem cell characteristics and greater ability to differentiate into bone-like tissue than LM-TECs and NECs.

**HM-TECs Have a Higher Aneuploidy Rate than LM-TECs**

We have previously reported that murine TECs isolated from melanoma and liposarcoma were karyotypically aneuploid, whereas NECs cultured under the same conditions were diploid. Human renal carcinoma–derived TECs have been aneuploid, with down-regulated centromere-associated protein-E (CENP-E). High aneuploidy rates in cancer cells are related to the frequent occurrence of MDR genes. Because HM-TECs were more resistant to paclitaxel than LM-TECs, we hypothe-
sized that the aneuploidy rate may differ between HM-TECs and LM-TECs. Karyotypes of LM-TECs and HM-TECs were analyzed by GTG banding. Representative images are shown in Figure 8A. HM-TECs had more complex abnormal karyotypes than LM-TECs. HM-TECs had several missing chromosomes, markers of unknown origin, and double-minute chromosomes, along with aneuploidy. After ECs were cultured, approximately 85% of HM-TECs, 31% of LM-TECs, and 8% of NECs were aneuploid by FISH (Figure 8B).

Freshly isolated and uncultured TECs were cytospun and immunostained with CD31 (green) and subjected to FISH. FISH, performed using a mouse chromosome 17 probe, revealed that approximately 34% of HM-TECs, 7% of LM-TECs, and 3% of NECs were aneuploid (Figure 8C). These results indicate that HM-TECs had chromosomal abnormalities.

**Lack of Pericyte Coverage and Defects in Vessel Maturity in HM Tumors**

Pericyte coverage is one of the parameters affecting the angiogenic behavior of endothelium in tumor vessels.
Therefore, we analyzed pericyte coverage in both tumor vessels, using antibody against 
\( \alpha \)-H9251-SMA (a marker of mural cells, including pericytes and smooth muscle cells).6 The percentage of intratumoral microvessels covered with H9251-SMA-positive cells was 37.5% in LM tumors and 4.4% in HM tumors (Figure 9A). Pericyte coverage in HM tumor vessels was significantly lower than that in LM tumor vessels (\( P < 0.01 \), Figure 9B). These results suggest that HM tumor blood vessels are more immature than LM tumor blood vessels.

**Involvement of EPCs in HM and LM Tumor Vascularization**

EPCs, a subset of stem cells, can be incorporated into tumor blood vessels.32-33 Up-regulation of the Sca-1 gene in HM-TECs, as shown in Figure 6, suggested that at least some of them were differentiated from progenitor cells. Resident progenitors, such as CD34+CD133+ cells, are also involved in tumor angiogenesis.34,35

IHC analysis revealed that HM tumor blood vessels were partially stained with anti-CD133 antibody, whereas LM tumor blood vessels were negative or weakly stained (Figure 9C).

We classified CD31+CD45−CD133+ cells as EPCs and CD31+CD34−CD133+ cells as resident stem cells.34,36,37 Subsets of EPCs were measured in LM and HM tumor tissues by flow cytometer. The median percentage of EPC cells was 1.87% in HM-TECs and 0.27% in LM-TECs (Figure 9D). There was no difference in the percentage of resident EC progenitors between HM and LM tumor tissues (data not shown).

These results suggest that more EPCs are mobilized during vascularization in HM than in LM tumors and that some of the differences between HM-TECs and LM-TECs may be attributable to a differential contribution of EPCs.

**Co-Culture with HM Tumor Induces a Pro-Angiogenic Phenotype and Resistance to Serum Starvation in NECs**

To investigate the effect of tumor-derived factors on NECs, we performed an NEC–tumor cell co-culture assay *in vitro*. NECs exposed to the soluble factors secreted by HM tumors showed higher mRNA expression levels of VEGF, VEGFR1, and VEGFR2 than those exposed to LM tumor (Figure 10A). The number of dead NECs under...
serum starvation was significantly lower when co-cultured with HM tumor cells than when co-cultured with LM tumor cells (Figure 10B). Furthermore, in the Transwell migration assay, NECs migrated more to HM tumor cells than to LM tumor cells (Figure 10C). These results suggest that an HM tumor–derived factor may have induced resistance to serum starvation and the angiogenic phenotype in NECs. Our findings suggest that ECs are able to acquire a heterogeneous TEC phenotype, depending on type of tumor cells in their microenvironment.

Discussion

Heterogeneous vascular morphological characteristics have been described in various tumor types, at different stages of tumor progression, and even within a single tumor stage.38,39 However, the question arises whether tumors with different malignancy status have heterogeneous characteristics in addition to morphological differ-
ferences in their blood vessels. In this study, we compared the characteristics of TECs isolated from different tumors with different malignancy status. A375 (LM tumor) and A375-SM (HM tumor) cells are suitable for the investigation of TEC heterogeneity with regard to metastatic ability. HM-TECs were more proliferative, motile, sensitive to VEGF, and invasive to ECM than LM-TECs. In addition, HM-TECs showed up-regulation of the angiogenesis-related genes VEGFR1 and VEGFR2-VEGF. Under basal conditions, Akt phosphorylation levels were higher in HM-TECs than in LM-TECs and NECs. VEGF is reported to be involved in angiogenesis and to promote metastasis. In addition, autocrine VEGF is required for the homeostasis of blood vessels, and it mediates cell survival. Our previous study suggested that autocrine VEGF was necessary for cell survival and tube formation in TECs. During angiogenesis, VEGF signaling is mediated through the phosphatidylinositol 3-kinase (PI3K)-Akt pathway.

The PI3K-Akt signaling pathway plays a crucial role in angiogenesis and is involved in VEGF production. Under basal conditions, Akt phosphorylation levels in HM-TECs were higher than those in LM-TECs. This suggests that PI3K-Akt signaling activation by the autocrine loop of VEGF is a reason why the phenotype of HM-TECs is more pro-angiogenic than that of LM-TECs. In addition, our results are consistent with previous reports showing that human renal TECs were more proliferative and overexpressed VEGF on Akt activation under basal conditions.

During tumor neovascularization, ECs can breach their basement membrane, degrade ECM, and migrate to the tumor. These events are necessary for tumor metastasis. The gelatinase–collagenase IV metalloproteases (MMP-2 and MMP-9) are involved in this process and promote angiogenesis. Cell migration, invasion, and matrix remodeling are essential for the morphogenesis of TECs into capillaries. Akt promotes the invasion of HM tumor cells by increasing cell motility and MMP-9 production. The PI3K-Akt signaling pathway functions simultaneously with MMP-2, MMP-9, and VEGF production in ECs. We showed that HM-TECs up-regulated the expression of MMP-2 and MMP-9 and displayed increased Akt phosphorylation under basal conditions compared with LM-TECs. HM-TECs may possess an invasive phenotype, and MMP-2–MMP-9 up-regulation, occurring through the activation of the PI3K-Akt signaling pathway, may be responsible for the invasive potential of HM-TECs.

HM-TECs have relatively marked stem cell characteristics; they transdifferentiate into bonelike cells at a higher rate than LM-TECs or NECs. Recent studies revealed that prostate ECs displayed mesenchymal-like differentiation. In another study, mouse marrow–derived stem cells (Sca-1–CD90–c-kit+) or tissue resident progenitors contributed to tumor vasculature and produced functional ECs. Also, EPCs, a subset of stem cells derived from bone marrow cells, may be incorporated into tumor blood vessels. In particular, EPCs play an important role in early neovascularization. We hypothesized that the phenotypic difference between HM-TECs and LM-TECs may be due to the fact that TECs could be recruited from different sources (ie, EPCs or resident progenitors of ECs), in addition to sprouting ECs. In this study, expression levels of the Sca-1 and CD90 genes in HM-TECs were higher than those in LM-TECs. In addition, the EPC population in HM tumor blood vessels was higher than that in LM tumor blood vessels. These results suggest that EPC incorporation during HM tumor vascularization is more frequent than that during LM tumor vascularization. On the other hand, there was no difference in the tissue resident stem cell population between HM-TECs and LM-TECs. Thus, one mechanism by which HM-TECs acquire stem cell characteristics may be the contribution of EPCs to tumor vascularization.

A recent report suggested that cross talk between tumor cells and stromal cells was essential for tumor progression, acceleration of metastasis, and increased tumor malignancy. Factors secreted by HM tumors may be able to alter the characteristics of NECs to a pro-angiogenic phenotype. We showed that co-culture with HM tumor cells led to an increase in the expression of pro-angiogenic genes and a phenotypic change in NECs. HM-TECs probably acquire their pro-angiogenic characteristics through the soluble factors secreted by HM tumors, which are highly angiogenic. In addition, HM-TECs may be affected in vivo by factors from other types of tumor stromal cells and a hypoxic environment.

Hypoxia may also be one of the mechanisms by which HM-TECs acquire a pro-angiogenic phenotype. Tumor blood vessels were found adjacent to areas of tumor necrosis; an area of tumor necrosis was induced by a rapidly growing tumor, which depleted nutrients and oxygen, ultimately resulting in tumor hypoxia. Tumor vasculature is often leaky because of its immaturity, causing high tissue pressure inside the tumor and shrinkage of tumor vessels, resulting in hypoxia. In this study, pimonidazole staining revealed that HM tumors exhibited more hypoxia than LM tumors. We showed that HM tumor blood vessels were more immature and showed less pericyte coverage than LM tumor blood vessels. This may be one reason why HM tumors are more hypoxic than LM tumors. Furthermore, in HM tumors, some tumor blood vessels and the surrounding area were exposed to hypoxia. Hypoxia in HM tumors may lead to increasingly excessive VEGF production, vascular permeability, and gene instability in HM-TECs. The resulting decrease in blood flow around tumor vessels in HM tumors may also decrease nutrient and oxygen delivery, imposing physiological stress on the tumor. Like cytokines, such as VEGF and CXCL12, hypoxia induces mobilization of EPCs from the bone marrow to the leading edge of necrotic tumors to promote revascularization. Thus, hypoxia and high expression levels of VEGF and CXCL12 in HM tumors may be responsible for the higher EPC incorporation in HM tumor vasculature.

In cancer, high mutation rates of aneuploid cancer cells are related to the frequent acquisition of MDR. Several molecules have participated in the mechanism of drug resistance. P-gp, which is encoded by the MDR1 gene, is a transmembrane glycoprotein that functions as a multidrug transporter. Thus, MDR1–P-gp plays a major role in drug resistance. In this study, we showed that HM-TECs are more resistant to paclitaxel and show
higher mRNA expression levels of MDR1 than LM-TECs. In fact, HM-TECs can acquire drug resistance. Further research on the mechanism of genetic instability in HM-TECs could provide a practical strategy to establish effective anti-angiogenic therapy.

Our results suggest that the heterogeneity of different types of TECs differs according to the malignancy status of the tumor. TECs may be related to their parental tumors by tumor-mediated factors (ie, growth factors, chemokines, and microRNA). Furthermore, TECs may differ depending on the tumor microenvironment, the stage of tumor growth, progression, and metastasis. Some anti-angiogenic drugs gradually lose their effectiveness, possibly through the acquisition of drug resistance. In both preclinical and clinical settings, therapeutic benefits are transitory and are followed by resumption of tumor growth, progression, and metastasis.51,52 Correlations between TEC heterogeneity and its effects on monomorphy anti-angiogenic therapy have not yet been elucidated. From this viewpoint, our results raise several important issues in cancer therapeutics. It is important to understand that even stromal cells can be abnormal in the tumor microenvironment and that TECs are heterogeneous. A novel anti-angiogenic therapy targeting TEC heterogeneity may be developed in the future as a means of improving overall anti-tumor efficacy. Further studies on TEC heterogeneity will facilitate the selection of suitable anti-angiogenic therapies.

Acknowledgments

We thank Dr. Aya Yanagawa, Tomomi Takahashi, Midori Muranaka, and Yuko Suzuki for technical assistance.

References