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Title	Inhibitory effects of epigallocatechin-3 gallate, a polyphenol in green tea, on tumor-associated endothelial cells and endothelial progenitor cells
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7 **Inhibitory Effects of Epigallocatechin-3 Gallate, a Polyphenol in Green Tea, on**
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10 **Tumor-Associated Endothelial Cells and Endothelial Progenitor Cells**
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31 **Running Titles**
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34 Epigallocatechin-3 Gallate inhibits tumor angiogenesis.
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Summary

The polyphenol epigallocatechin-3 gallate (EGCG) in green tea suppresses tumor growth by direct action on tumor cells and by inhibition of angiogenesis, but it is not known whether it specifically inhibits tumor angiogenesis. We examined the anti-angiogenic effect of EGCG on tumor-associated endothelial cells (TECs), endothelial progenitor cells (EPCs), and normal endothelial cells (NECs). EGCG suppressed the migration of TECs and EPCs but not NECs. EGCG also inhibited the phosphorylation of Akt in TECs but not in NECs. Furthermore, vascular endothelial growth factor-induced mobilization of EPCs into circulation was inhibited by EGCG. Matrix metalloproteinase-9 (MMP-9) in the bone marrow plasma plays key roles in EPC mobilization into circulation. We observed that expression of MMP-9 mRNA was downregulated by EGCG in mouse bone marrow stromal cells. In vivo model, EGCG suppressed growth of melanoma and reduced microvessel density. Our study showed that EGCG has selective anti-angiogenic effects on TECs and EPC. It was suggested that EGCG could be a promising angiogenesis inhibitor for cancer therapy.

Introduction

Tea is one of the most popular beverages consumed worldwide. Drinking tea, especially green tea, inhibits the growth of several tumors in animal models, including cancers of the skin, lung, esophagus, breast, stomach, small intestine, colon, liver, pancreas, and mammary glands,^(1,2) and is associated with a lower incidence of cancer in humans.^(1,3,4)

The components of green tea responsible for these effects are catechins, which are polyphenols with potent antioxidant capacity that have been shown to inhibit mutation, tumor cell growth, tumor initiation, tumor progression, and the activity of urokinase and matrix metalloproteinases (MMPs), that are crucial for cancer growth.⁽⁵⁻⁷⁾ Among these catechins, epigallocatechin-3 gallate (EGCG) has the highest antioxidant capacity and is an effective inhibitor of corneal vascularization *in vivo*.⁽⁸⁾

The tumor microenvironment has recently been regarded as a target of cancer chemoprevention because it plays an important role in tumorigenesis and tumor progression.⁽⁹⁾ Tumor angiogenesis is one of the key processes within the tumor microenvironment, and controlling this process is a very important strategy for preventing invasive cancers.⁽¹⁰⁾ Many molecules regulating tumor angiogenesis have

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7 been identified and characterized recently, including vascular endothelial growth factor
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10 (VEGF).^(11,12)
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13 EGCG has also been shown to inhibit cell proliferation,⁽¹³⁾ binding of VEGF to its
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15 receptors,⁽¹⁴⁾ phosphorylation of VEGF receptor 2 (VEGFR2),⁽¹⁵⁾ MMP activity,⁽¹³⁾ and
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17 interleukin-8 production,⁽¹⁶⁾ in normal endothelial cells such as human vascular
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19 endothelial cells (HUVECs).
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27 An important concept in tumor angiogenesis is that tumor blood vessels contain
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29 endothelial cells that are genetically normal and stable, whereas tumor cells are
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31 typically genetically unstable.⁽¹⁷⁾ However, tumor vessels and tumor-associated
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33 endothelial cells (TECs) differ from their normal counterparts in many respects.⁽¹⁸⁻²⁰⁾
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41 Tumor vessels show structural changes such as fewer pericytes, leakiness.^(19,21)
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45 Moreover, some studies have reported that TECs possess molecular characteristics
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47 distinct from those of normal endothelial cells (NECs). Endothelial cells derived from
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49 human renal cell carcinoma express biological features different from those of NECs.⁽²²⁾
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55 We have reported that endothelial cells in malignant tumors (melanomas and
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57 liposarcomas) are cytogenetically abnormal.^(23,24) In addition, TECs showed enhanced
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7 responsiveness to EGF, with alteration of EGF receptor expression.⁽²⁵⁾ These results
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10 suggest that TECs are different from NECs, contrary to the conventional assumption.
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14 We therefore decided to investigate the differential anti-angiogenic effect of EGCG on
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17 TECs and NECs.
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21 In the tumor microenvironment, endothelial progenitor cells (EPCs) also play a
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24 critical role in tumor angiogenesis. The mobilization of EPCs from the bone marrow
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27 into peripheral blood is regulated by tumor-derived cytokines.^(26,27) In response to these
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30 cytokines, EPCs can contribute to tumor angiogenesis and the growth of certain
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33 tumors.^(26,28,29) EPCs derived from the bone marrow are thus considered to be an
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36 important target for anti-angiogenic therapy. Strategies that block the mobilization of
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39 EPCs into circulation might provide a new approach to inhibiting tumor angiogenesis.
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44⁽²⁶⁾ However, the effects of EGCG on EPC mobilization have not been investigated. It is
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47 of great interest to investigate whether EGCG specifically acts on TECs and EPCs
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50 rather than NECs.
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54 To investigate the effects of EGCG on TECs and EPCs, TECs were isolated and
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57 cultured from human tumor xenografts in nude mice and EPCs were isolated from nude
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7 mouse peripheral blood. NECs were cultured from the skin of nude mice as a control.
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10 We also investigated the effect of EGCG on EPC mobilization in VEGF-treated nude
11 mice. In addition, we isolated bone marrow stromal cells (BMSCs) from oral
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13 carcinoma-bearing mice and investigated the expression of MMP-9 mRNA in the
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15 presence and absence of EGCG in BMSCs to determine whether EGCG affects the
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17 expression of MMP-9 in the bone marrow space, which plays an important role in the
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19 mobilization of EPCs into peripheral circulation.^(26,30) Finally, we analyzed the effects of
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21 EGCG on in vivo tumor growth.
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34 **Materials and Methods**

35 **Chemicals.**

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38 EGCG was purchased from Sigma Chemical Co. (St. Louis, MO) and dissolved in
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40 sterile dimethylsulfoxide (DMSO). PI3K inhibitor LY294002 was purchased from
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48 Calbiochem (San Diego, CA)
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50 **Cell lines and culture conditions.**

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55 Cells of the super-metastatic human malignant melanoma cell line A375SM, kindly
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7 humidified atmosphere of 5% CO₂ and 95% air at 37°C in Minimum Essential Medium
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10 (MEM; Gibco, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine
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13 serum (FBS). The medium was changed every 3 days. The human oral carcinoma cell
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16 line HSC-3 was supplied by the Japanese Cancer Research Bank (Tokyo, Japan). The
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20 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Sigma Chemical
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23 Co.) supplemented with 10% FBS.
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26 27 **Antibodies.**

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30 The antibodies were purchased: rat anti-mouse CD31 and fluorescein isothiocyanate
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33 (FITC)-anti-mouse CD31 (eBioscience, San Diego, CA); FITC-anti-mouse CD133
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36 (eBioscience); FITC-*Bandeirea simplicifolia* lectin 1-B4 (BS1-B4; Vector Laboratories,
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39 Burlingame, CA); FITC-goat anti-rat IgG; phycoerythrin (PE)-goat anti-rat IgG
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43 (Molecular Probes, Invitrogen, OR); normal rat IgG (BD Pharmingen); PE-anti-mouse
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46 VEGFR2 (BD Pharmingen); FITC-Sca-1 (BD Pharmingen).
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50 51 **Isolation of TECs, peripheral blood-derived ECs (EPCs) and NECs.**

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54 All animal procedures were performed in compliance with Hokkaido University
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58 guidelines, and the protocols were approved by the Institutional Animal Care and Use
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7 Committee. ECs were isolated as previously described.⁽²³⁾ In brief, TECs were isolated
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10 from melanoma (A375SM) and oral carcinoma (HSC-3) xenografts in nude mice aged
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13 8–12 weeks (Sankyo Labo, Tokyo, Japan). NECs were isolated from the dermal tissue
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16 as control. ECs were isolated using a magnetic cell sorting system (Miltenyi Biotec,
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19 Tokyo, Japan) according to the manufacturer's instructions using FITC–anti-CD31
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22 antibody. CD31-positive cells were sorted and plated onto 1.5% gelatin-coated culture
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25 plates and grown in EGM-2 MV (Clonetics, Walkersville, MD) and 15% FBS.
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30 Diphtheria toxin (500 ng/mL; Calbiochem, San Diego, CA) was added to TEC
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33 subcultures to kill any remaining human tumor cells⁽³¹⁾ and to NECs to ensure technical
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36 consistency. The isolated ECs were purified by a second round of purification, using
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39 FITC–BS1-B4, and purity was determined by flow cytometry.⁽²³⁾ Peripheral
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44 blood-derived ECs were isolated and grown in previously described with modifications.
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48 ⁽³²⁾ Briefly, mouse peripheral blood was obtained by cardiocentesis. Peripheral blood
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51 mononuclear cells (PBMCs) were isolated using Histopaque 1077 (Sigma, St Louis,
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54 Mo) and centrifuged per manufacturer's instruction. The intermediate phase was
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58 collected and washed with Hanks' Balanced Salt Solution (HBSS) before resuspension
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7 in EGM-2 MV supplemented with 15% FBS . Peripheral blood mononuclear cells ($1 \times$
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10 10^6 per well) were seeded onto 12-well tissue culture plates coated with 0.5% gelatin
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12
13 (Sigma) and 2 $\mu\text{g}/\text{mL}$ of human fibronectin (Chemicon International, Temecula, Calif).
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17 On day 4, non-adherent cells were removed. On day 7, cells were trypsinized and
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passed to 6-well tissue culture dishes coated with 1.5% gelatin. Cells were passaged
at a 1: 3 ratio until they reached 90% confluence. Peripheral blood-derived ECs were
regarded as EPCs since they were Sca-1-positive in culture.

Cell migration assay.

Migration of TECs, NECs, and EPCs was measured by a migration assay using a
Boyden chamber as previously described with modifications.^(33,34) TECs , EPCs and
NECs were treated with and without EGCG (0, 25, 50 μM) in Endothelial Basal
Medium-2 (EBM-2, Clonetics Walkersville, MD)+0.5% FBS for 24 h. ECs and EPCs
(1.5×10^4) were seeded into the upper chambers in EBM-2+0.5% FBS and EGCG was
then added. VEGF (10 ng/mL) was added in the lower chambers as a chemoattractant.
Similarly, after starvation for overnight in EBM-2+0.5% FBS, ECs and EPCs were
pretreated with LY294002 (20 μM) for 2hr and were seeded in EBM-2/0.5% FBS,

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7 including LY294002. After 4 h at 37°C, the cells that migrated through a
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10 fibronectin-coated polycarbonate membrane having 8 µm pores (Corning Costar, Nagog
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13 Park, MA) were fixed in 2% paraformaldehyde and stained with DAPI (4',
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17 6-diamidino-2-phenylindole). The numbers of cells that migrated to the lower side of
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20 the membrane were counted in seven high-power fields. The experiment was performed
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24 three times with similar results.
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27 **Western blotting.**

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30 ECs were harvested in serum-free medium after 16 h and were then treated with EGCG
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33 (100 µM) in EBM-2+0.5% FBS for 24 h. Similarly, after starvation for overnight in
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36 EBM-2+0.5% FBS, ECs were pretreated with LY294002 (20 µM) for 2hr in
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39 EBM-2+0.5%. EGCG was not toxic at these concentrations in these cells. After EGCG
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42 treatment, VEGF (10 ng/mL) was added to the cell cultures for 30 min at room
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45 temperature. ECs were lysed as described previously with some modification.⁽²⁵⁾ Total
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48 protein was measured using a BCA protein assay kit (Pierce, Rockford, IL). Western
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51 blotting was also performed for the detection of the Akt and phosphorylated Akt
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55 (P-Akt), as previously described with modifications.⁽³⁵⁾ After electrotransfer, the
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7 membranes were probed with anti-phospho-Akt (Cell Signaling Technology, Beverly,
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10 MA) in Solution 1 (Can Get Signal Immunoreaction Enhancer Solution; Toyobo, Osaka,
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13 Japan) overnight at 4°C. After washing, the membrane was incubated with anti-rabbit
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16 IgG secondary antibody (Cell Signaling Technology) in Solution 2 (Toyobo) and
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19 chemiluminescence reagents (PerkinElmer, Boston, MA). The membranes were
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22 reported with anti-Akt antibody (Cell Signaling Technology) and β -actin (Sigma). The
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25 level of phosphorylated Akt was normalized for total Akt by scanning densitometry
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28 using Image J software from the NIH (Bethesda, MD).
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34 **Counting the number of CD133⁺/VEGFR2⁺ cells to estimate EPCs number.**

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37 PBS or VEGF (300 ng) was injected intraperitoneally into ten nude mice in each group
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40 to mobilize EPCs from the bone marrow, as previously described.^(36,37) In each group,
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43 EGCG (5 mg/kg) or vehicle was also injected once a day for 2 days. Injection of EGCG
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46 into nude mice had no toxic effects. Forty-eight hours after the first injection, peripheral
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49 blood (700–1000 μ L) was collected under anesthesia in each group ($n = 5$ for each
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52 group) before they were sacrificed. Mononuclear cells were isolated by sucrose gradient
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55 centrifugation from peripheral blood as previously described with modifications⁽³⁸⁾ and
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7 the number of cells was counted. Next, they were incubated with FITC-anti-mouse
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10 CD133 and PE-anti-mouse VEGFR2 in order to count the number of
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13 CD133⁺/VEGFR2⁺ cells by flow cytometry, using a FACS Calibur flow cytometer (BD
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17 Biosciences). A minimum of 10,000 events were counted for each mouse.
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20 **Reverse transcription-PCR (RT-PCR) and quantitative real-time PCR.**

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24 Total RNA was isolated from ECs and BMSCs using the RNeasy Micro Kit (Qiagen,
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27 Santa Clarita, CA) with RNase-Free DNase Set (Qiagen). RNA was quantified by
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30 spectrophotometry. Total RNA (5 µg) was then used for first-strand complementary
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32
33 DNA (cDNA) synthesis in ReverTra-Plus (Toyobo). The cDNA was amplified by
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37 polymerase chain reaction (PCR) with primer pairs specific for mouse stromal derived
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40 factor-1 (SDF-1), fibroblast activation protein (FAP), α -smooth muscle actin (α -SMA),
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42
43 and Sca-1. The annealing temperature was 60°C and the reaction proceeded for 30
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46
47 cycles. PCR products were visualized by ethidium bromide staining and ultraviolet
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51 transillumination. NECs were used as a negative control. The primers were as follows:
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54 GAPDH, forward 5'-TCTGACGTGCCCGCCTGGAG-3', reverse
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58 5'-TCGCAGGAGACAACCTGGTC-3'; SDF-1, forward
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7 5'-CTCGGTGTCCTCTTGCTGTCC-3', reverse
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10 5'-CGGTATCAGGCTGACTGGTTTACCG-3'; FAP, forward
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13 5'-CAAATGTGGCATAGCAGTGG-3', reverse 5'-TTCTGCTCTTGCCATCACAG3';
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17 α -SMA, forward 5'-CTGACAGAGGCACCACTGAA-3', reverse
18
19

20 5'-CATCTCCAGAGTCCAGCACA-3'; Sca-1, forward
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23 5'-GAAGAGGCAGAATTCCAAGG-3', reverse
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27 5'-ATGTGGGAACATTGCAGGAC-3'. In addition, total RNA (5 μ g) was used and
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31 real-time PCR was conducted using SYBR Green Real-time PCR Master Mix Plus
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34 (Toyobo) in BMSCs with or without EGCG (0, 25, 50, 100 μ M). Cycling conditions
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37 were according to the manufacturer's instructions based on the use of Opticon Monitor
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40 version 3.0 (Bio-Rad, Hercules, CA). The expression levels of MMP-9 mRNAs in
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43 BMSCs with or without EGCG treatment were normalized to GAPDH. The primers
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46 were as follows: MMP-9, forward 5'-CTCTACAGAGTCTTTGAGTCCGGCAG-3',
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49 reverse 5'-TCAGGAACTTCCAGTACCAACCGTC-3'.
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52 53 54 **Primary culture and characterization of BMSCs.** 55

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58 BMSCs were isolated from the femoral bone marrow of nude mice. The cells were
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7 triturated with a 20-gauge needle and passed through a 70- μ m nylon mesh cell strainer
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10 (Becton-Dickinson, Franklin Lakes, NJ) to obtain a single-cell suspension in DMEM
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13 (Sigma) supplemented with 20% inactivated FBS, as described previously with
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17 modifications.⁽³⁹⁾ We characterized BMSCs that had been grown under the control
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19
20 condition by RT-PCR to analyze the expression of stromal cell (SDF-1, α -SMA, FAP)
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24 and stem cell marker (Sca-1).
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27 ***In vivo* tumor growth experiments.**

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30 Seven-week-old KSN nude mice were purchased from Sankyo Labo and housed in
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33 pathogen-free conditions. Melanoma was obtained by s.c injection of A375SM (1×10^6
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36 cells) in the flanks of nude mice as described previously⁽²³⁾. Animals were randomized
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39 into two groups. Controls were injected with 0.05% DMSO and one group was injected
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42 with EGCG (20mg/kg) intraperitoneally ($n = 5$, for each group). The infusion was
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45 cooled to room temperature, filtered, and stored at -20°C until use. EGCG was injected
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47
48 every day. Animals were weighed, and tumor growth was monitored for 36 days by
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52 measuring two tumor diameters every 3 days with calipers and calculating the tumor
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55 volumes with the formula: (the shortest diameter)² \times (the longest diameter) \times 0.5. On
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7 day 36, animals were sacrificed and the tumors were removed. Each sample was
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10 snapfrozen. Eight μm cryosections of tumors were stained with anti-CD31 antibody for
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13 histological study. The micro-vessel density (MVD) was measured by computer-aided
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17 image analysis system (Metamorph software).
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20 **Statistical Analysis.**

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24 Differences between groups were evaluated using the student's *t*-test, two ways ANOVA
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27 test.
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34 **Results**

35 36 37 **TECs and peripheral blood-derived endothelial cells (EPC) expressed Sca-1.**

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40 Endothelial cells and peripheral blood-derived endothelial cells (EPC) were
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43 characterized by flow cytometry. All ECs, and peripheral blood-derived endothelial
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46 cells were positive for BS1-B4, which binds specifically to mouse endothelial cells
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49 (Fig.1a: a-d). Peripheral blood-derived endothelial cells expressed the stem cell marker
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52 Sca-1. Furthermore, Sca-1 was overexpressed also in TECs from both melanoma and
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55 oral carcinoma whereas it was expressed at low-level in NECs (Fig.1a: e-h).
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7 Consistently, mRNA expression of Sca-1 was higher in TECs and peripheral
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10 blood-derived endothelial cells, than in NECs by real time RT-PCR (* $P < 0.05$ versus
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13 NECs) (Fig.1b). The cryosections of xenografted-melanoma were processed for
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16 immunohistochemistry using FITC-anti-mouse Stem cell antigen-1 (Sca-1) and PE-
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19 anti-mouse CD31. Representative images demonstrate CD31+ cells (red), Sca-1+ cells
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22 (green), and CD31+/Sca-1+ (yellow). Nuclei were stained with DAPI (blue).
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27 CD31+/Sca-1+ cells (yellow) localized in some tumor vessels. These data suggests that
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EGCG inhibited the migration of TECs and EPCs.

Since the PI3K-Akt signaling pathway has recently been shown to promote survival of
TEC and migration^(22,40) that subsequently lead to angiogenesis, we analyzed the effect
of the PI3K inhibitor LY294002 on VEGF-induced migration in ECs and EPCs.
LY294002 (20 μ M) suppressed the VEGF-induced cell migration of ECs and EPCs.
These results suggest that cell migration of ECs and EPCs is stimulated by VEGF

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7 through activation PI3K signaling pathway. Then, the cell migration toward VEGF was
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10 analyzed *in vitro* with or without EGCG. EGCG significantly inhibited the migration of
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14 TECs and EPCs toward VEGF, as compared with NECs (* $P < 0.05$) (Fig. 2).
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16 17 **EGCG inhibited Akt phosphorylation in TECs.**

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20 During angiogenesis, VEGF signaling is mediated by ligand-dependent signaling
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23 through the PI3K/Akt pathway.⁽⁴¹⁾ The protein kinase Akt plays a central role in mature
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26 endothelial cells. Activation of Akt promotes survival by inhibiting apoptosis^(42,43) and
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29 mediates VEGF-induced migration in endothelial cell.^(44,45) Since it was shown that
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PI3K/Akt pathway is involved in cell migration of ECs and EPC, we analyzed the level
of Akt phosphorylation on these cells, using LY294002 or EGCG. Pretreatment with
LY294002 (20 μ M) decreased VEGF-induced Akt activation in TECs not in NECs.

EGCG reduced the levels of phosphorylated Akt stimulated by VEGF in TECs, as well.
(Fig.3a, b). Specific inhibition of the PI3K/Akt pathway by EGCG in TECs is a possible
explanation for the differential effect of EGCG on cell migration between TECs and
NECs, as shown in Fig. 2.

VEGF receptor 2 (VEGFR2) was upregulated in TECs and EPCs.

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7 VEGFR2 plays a major role in tumor angiogenesis. VEGFR2 is responsible for the most
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10 of the mitogenic and chemotactic effects of VEGF in tumor angiogenesis. Some studies
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13 have provided evidence that EGCG inhibits VEGF-induced phosphoryration of
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17 VEGFR2. ⁽¹⁵⁾ To address the mechanism of differential response to the EGCG between
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20 TECs , EPCs and NECs under VEGF treatment, the expression level of mRNA for
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23 VEGFR2 was analyzed by real-time RT-PCR. TECs and EPCs upregulated VEGFR2
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26 compared to NECs (Fig. 4). It was suggested that the different levels of VEGFR2
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29 expression may be one of reason why EGCG cause inhibitory effects more in TECs, and
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34 EPCs in presence of VEGF, as compared with NECs.

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37 **EGCG suppressed the VEGF-stimulated mobilization of CD133⁺/VEGFR2⁺ cells**
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41 **into peripheral blood.**

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44 To evaluate the effect of EGCG on EPC mobilization, the number of
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47 CD133⁺/VEGFR2⁺ cells in circulation was analyzed in mice with or without EGCG
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51 treatment. VEGF was injected intraperitoneally into mice to mobilize EPCs and then a
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54 low dose of EGCG or vehicle (PBS) was injected once a day for 2 days. Forty-eight
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57 hours after the first injection, PBMCs were isolated and counted. PBMCs were
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7 incubated with anti-CD133 and anti-VEGFR2 antibodies, and the CD133⁺/VEGFR2⁺
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10 cells in circulation were counted by flow cytometry. EGCG alone did not affect the
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13 number of CD133⁺/VEGFR2⁺ cells. The number of CD133⁺/VEGFR2⁺ cells in control
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16 group was 11.86±2.09/μl, whereas the number of CD133⁺/VEGFR2⁺ cells in EGCG-
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19 treated group was 9.71±8.33/μl. However, in VEGF-treated group, the number of
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22 circulating CD133⁺/VEGFR2⁺ cells was lower after treatment with EGCG. The number
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24
25 of CD133⁺/VEGFR2⁺ cells in VEGF- treated group was 19.66±19.12/μl, whereas the
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27
28 number of CD133⁺/VEGFR2⁺ cells in VEGF+EGCG-treated group was 8.8±3.49/μl
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34 (Fig. 5). These results suggest that EGCG inhibits the VEGF-induced mobilization of
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37 EPCs into circulation. There was no significant difference between the VEGF group and
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40 the VEGF + EGCG-treated group.
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44 **EGCG suppressed the expression of MMP-9 in BMSCs from oral**
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46
47 **carcinoma-bearing mice.**
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51 MMP-9 in the bone marrow plays an important role in the mobilization of bone
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54 marrow-derived VEGFR-2⁺ cells into peripheral circulation. ⁽²⁷⁾ In order to test the
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57 effects of EGCG on MMP-9 expression in BMSCs, we isolated BMSCs from oral
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7 carcinoma-bearing mice. Expression of mRNAs for Sca-1, SDF-1, α -SMA and FAP,
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10 was detected in BMSCs but not in skin endothelial cells by RT-PCR (Fig.6a). To
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12
13 investigate whether EGCG affects the expression of MMP-9 mRNA in BMSCs, these
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16 cells were treated with or without EGCG (25, 50, 100 μ M) for 24 h and the expression
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19 levels of MMP-9 mRNAs, normalized to GAPDH, were analyzed by real-time PCR.
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24 MMP-9 mRNA expression in BMSC was down-regulated by EGCG. Relative
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27 expression level of MMP-9 after 25 μ M, 50 μ M and 100 μ M of EGCG treatment was
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31 0.49, 0.46, and 0.37, respectively ($P < 0.05$) (Fig. 6b).
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39 **EGCG inhibited tumor growth in vivo.**

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41 To address the question whether EGCG inhibits tumor growth in vivo, either EGCG or
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44 vehicle as a control was administrated intraperitoneally into the mice which bear
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47 melanoma xenografted subcutaneously. After 36 days, EGCG suppressed melanoma
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50 growth ($689.8 \pm 276.6 \text{ mm}^3$) in vivo, as compared with controls ($354.2 \pm 188.2 \text{ mm}^3$).
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55 There was significant difference between EGCG-treated and control tumors ($P < 0.05$)
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58 (Fig. 7a). Drug treatment was well-tolerated without apparent toxicity or body weigh
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7 changes throughout the study. Cryosections of each tumors were stained with an
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10 anti-CD31 antibody (Fig. 7b). Mice treated with the EGCG showed a significant
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13 reduction in MVD. (control: $3.11 \pm 1.02\%$ vs EGCG: 1.83 ± 0.35 , mean \pm SD. $P < 0.05$)
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17 (Fig. 7c). These results suggest that EGCG inhibits tumor growth with anti-angiogenic
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21 activity.

22 23 24 25 26 27 **Discussion**

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30 We provide new evidences about the mechanism by which EGCG inhibits tumor
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33 angiogenesis. First, EGCG inhibited cell migration toward VEGF in TECs and EPCs,
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37 but not in NECs. Second, EGCG inhibited Akt phosphorylation in TECs. Third, EGCG
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40 suppressed the VEGF-induced mobilization of CD133⁺/VEGFR2⁺ cells into peripheral
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44 circulation *in vivo*. EGCG down-regulated the expression levels of MMP-9 in BMSCs,
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48 which is related to the mobilization of EPCs into peripheral circulation.^(26,27) Finally,
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51 EGCG suppressed *in vivo* tumor growth in nude mice with anti-angiogenic activity.

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54 We demonstrate for the first time that EGCG specifically targets TECs and EPCs.
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58 Although we did not identify the molecules responsible for this specificity, the specific
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7 inhibition of Akt phosphorylation in TECs suggests that the PI3K/Akt pathway mediates
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10 targeted signaling. In our previous studies with isolated TECs, we found that they were
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13 different from NECs in many respects, including sensitivity to growth factors and to
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16 certain drugs such as an EGF receptor kinase inhibitor.^(23,24,46) Activation of Akt
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19 mediates VEGF-induced endothelial cell migration.^(44,45) In our study, PI3K inhibitor
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22 LY294002 significantly reduced cell migration and Akt phosphorylation was inhibited
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25 by LY294002 in TECs and EPCs. These results suggest that PI3K-dependent pathway
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28 modulates cell migration of TECs and EPCs, which we isolated, consistently with other
29
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31 reports on microvascular endothelial cells.⁽⁴⁵⁾ Furthermore, our results show that EGCG
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34 inhibited VEGF-induced Akt phosphorylation in TECs, but not in NECs. These results
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37 suggest that inactivation of Akt signaling is involved in the anti-angiogenic effects of
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40 EGCG on TECs and EPCs causing the inhibition of migration. It was speculated that
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43 selective inhibition by EGCG on TECs and EPCs may be caused from their
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46 upregulation of VEGFR2, which stimulates PI3K/Akt pathway.
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54 EPCs are currently considered to be a novel target for anti-angiogenic therapy, as are
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57 TECs, since they play important roles in tumor metastasis.⁽²⁶⁾ It has been shown that
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7 tumors mobilize bone marrow-derived EPCs, besides recruiting neighboring blood
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10 vessels or ECs, and that EPCs migrate to tumors and become incorporated into their
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13 developing vasculature.⁽⁴⁷⁾ The contribution of EPCs to tumor angiogenesis in mouse
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16 models ranges from 2 to 50%.^(28,48) Several anti-angiogenic drugs have already been
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19 reported to produce inhibitory effects on EPCs.⁽²⁸⁾ EPCs are considered to express
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22 CD133 and VEGFR2.^(36,37,49) Here we showed for the first time, using a mouse model,
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24
25 that EGCG inhibits mobilization of CD133⁺/VEGFR2⁺ cells into peripheral circulation
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28 *in vivo*. It is expected that EGCG will target EPCs besides TECs when used for cancer
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31 therapy.
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37 The expression of MMP-9 mRNA was significantly down-regulated by EGCG
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40 treatment in BMSCs. MMPs play key roles in cancer invasion and metastasis.
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44 Upregulated MMP-9 activity in bone marrow cleaves the membrane-bound stem cell
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47 cytokine mKitL expressed by BMSCs to liberate soluble sKitL. The survival activity of
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50 sKitL then stimulate the mobilization of bone marrow-derived VEGFR2⁺ cells to
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53 peripheral circulation. In MMP-9^{-/-} mice, release of sKitL, hematopoietic cell motility
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56 and the mobilization of bone marrow-derived VEGFR2⁺ cell are impaired.⁽²⁷⁾ EGCG
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7 has been reported to inhibit the activity of MMP-2,3,9 in cancer cells .⁽⁵⁰⁾ In our study,
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10 suppression of MMP-9 expression by EGCG in BMSCs may be one explanation for
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13 reduction of EPCs mobilization. Finally, we demonstrated that EGCG inhibited growth
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17 of tumors in nude mice with impaired angiogenesis.
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20 We conclude that EGCG inhibits tumor angiogenesis by inhibiting PI3K/Akt
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23 signaling specifically in TECs and peripheral blood-derived endothelial cells, but not in
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27 NECs. It might be possible to use EGCG as an anti-angiogenic drug, either as a single
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30 agent or for cancer treatment in adjuvant settings for relatively long periods, since it is
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34 not toxic to normal cells.
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46
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10 (Y. Hida).
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9 human prostate carcinoma LNCaP cells to TRAIL-mediated apoptosis and
10 synergistically inhibits biomarkers associated with angiogenesis and metastasis.
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15 16 **Figure Legends**

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18 **Fig. 1.** Peripheral blood-derived endothelial cells (EPCs) and TECs bind BS1-B4 lectin
19 (mouse endothelial marker) and express Sca-1 (stem cell marker). (a) NECs, TECs, and
20 peripheral blood-derived ECs (EPCs) were characterized by Flow cytometry. (a–d)
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22 NECs, TECs, and peripheral blood-derived ECs (EPCs) were positive for BS1-B4. (e–h)
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24 Peripheral blood-derived endothelial cells (EPCs) expressed the stem cell marker Sca-1.
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26 Sca-1 was overexpressed also in TECs from both melanoma and oral carcinoma
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28 whereas it was expressed at low-level in NECs. (b) The mRNA expression of Sca-1 was
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30 higher in TEC, and peripheral blood-derived ECs (EPCs) compared to NEC in real time
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32 PCR ($*P<0.05$). (c) The cryosections of xenografted melanoma tumors were processed
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34 for immunohistochemistry using FITC-conjugated anti-mouse Stem cell antigen-1
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36 (Sca-1) and PE-conjugated anti-mouse CD31. Representative images are shown
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38 demonstrating CD31 positive cells (red), Sca-1 positive cells (green), and
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40 CD31+/Sca-1+ (yellow). Nuclei were stained with DAPI (blue). CD31+/Sca-1+ positive
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7 cells existed in tumor vessels. Bar; 100µm.
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13 **Fig. 2.** EGCG inhibited TECs and EPCs migration. Endothelial cells (ECs) and EPCs
14 were pretreated with EGCG (25, 50 µM) for 16 h and a PI3 kinase inhibitor LY294002
15 (20 µM) for 2h. Migration of ECs and EPCs toward VEGF was analyzed on
16 fibronectin-coated membranes using a Boyden chamber. Cells that migrated to the
17 lower side of the membrane were counted in seven different high-power fields.
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19 Migration of TECs and EPCs, compared to NECs, toward VEGF was inhibited
20 significantly by EGCG (**P* < 0.05 vs controls). Results represent the average of three
21 experiments.
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41 **Fig. 3.** EGCG inhibited Akt phosphorylation in TECs but not in NECs. (a)
42 Serum-starved ECs were pretreated with EGCG (100 µM) for 16h or PI3 kinase
43 inhibitor LY294002 (20 µM) for 2h before addition of VEGF (10ng/mL). EGCG was
44 not toxic to ECs. ECs were stimulated with VEGF for 30 minutes. Levels of
45 phosphorylated Akt (P-Akt) were determined by Western blotting using an
46 anti-phospho-Akt antibody. After Western blot analysis with antiphospho-Akt (Ser473)
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7 antibody (top), the membrane was stripped and reincubated with anti-Akt antibody
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10 (middle) and β -actin antibody (bottom) to detect of the amount of Akt and β -actin
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17 (b) Levels of phosphorylated Akt were normalized for total Akt by scanning
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20 densitometry using computed image analysis. The graph shows the relative ratio of
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23 phosphorylated Akt (Ser 473) /Akt by densitometric analysis. The experiment was
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26 performed three times with similar results.
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31 **Fig. 4.** VEGF receptor 2 (VEGFR2) is upregulated in TECs, EPCs. TECs and EPCs
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33 showed higher levels of VEGFR2 mRNA compared to NECs by real-time RT-PCR.
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35 Expression levels of VEGFR2 mRNAs were normalized to GAPDH (* $P < 0.05$ versus
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41 NECs).
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45 **Fig. 5.** EGCG suppressed VEGF-stimulated mobilization of CD133⁺/VEGFR2⁺ cells
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48 into circulation. Ten nude mice in each group were injected intraperitoneally with PBS
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51 or a high dose of VEGF (300 ng) to mobilize EPCs from the bone marrow. Next, in
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54 each group, EGCG (5 mg/kg) or vehicle was injected once a day for 2 days. Forty-eight
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58 hours later, peripheral blood was collected from each mouse in each group ($n = 5$ for
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7 each group) before they were killed. PBMCs were collected and incubated with
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10 FITC-anti-mouse CD133 and PE-anti-mouse VEGFR2, and the number of
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13 CD133⁺/VEGFR2⁺ cells was counted by flow cytometry. Although there was no
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17 significant difference between the VEGF group and the VEGF + EGCG group, the low
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20 dose of EGCG (5 mg /kg) moderately decreased the number of VEGF-mobilized
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24 CD133⁺/VEGFR2⁺ cells that migrated into peripheral circulation.
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27 **Fig.6.** EGCG suppressed the mRNA expression of MMP-9 in BMSCs of oral
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30 carcinoma-bearing mice. (a) RT-PCR characterization of BMSCs for expression of stem
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33 cell marker Sca-1, stromal cell markers SDF-1, α -SMA and FAP. Sca-1, SDF-1, α -SMA,
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36 and FAP were expressed in BMSCs but not skin endothelial cells. (b) The possibility
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39 that EGCG affects mRNA expression of MMP-9 in BMSCs was assessed by real-time
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Fig.6. EGCG suppressed the mRNA expression of MMP-9 in BMSCs of oral carcinoma-bearing mice. (a) RT-PCR characterization of BMSCs for expression of stem cell marker Sca-1, stromal cell markers SDF-1, α -SMA and FAP. Sca-1, SDF-1, α -SMA, and FAP were expressed in BMSCs but not skin endothelial cells. (b) The possibility that EGCG affects mRNA expression of MMP-9 in BMSCs was assessed by real-time PCR. BMSCs were pretreated with or without EGCG (25, 50, 100 μ M) for 24 h. Expression levels of MMP-9 mRNAs were normalized to GAPDH. EGCG significantly reduced MMP-9 mRNA levels in BMSCs. The experiment was performed three times with similar results (* P < 0.05 versus 0 μ M).

Fig.7.

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7 EGCG repressed melanoma tumor growth. (a) The melanoma tumor cells were
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10 inoculated subcutaneously into nude mice. Day 0 is defined as the first day of treatment.
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14 Two groups of animals were then treated with the vehicle (0.05% DMSO, n= 5) or
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17 EGCG (20 mg/kg, n = 5) intraperitoneally every day. Tumor growth was significantly
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20 suppressed by EGCG treatment, as compared with controls ($*P < 0.05$) . Means \pm SD are
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23 shown. (b) Immunohistochemical analysis of tumors. Snap-frozen tumor tissue
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27 specimens were processed for immunohistochemical study when mice were sacrificed.
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31 Cryosections were stained with FITC-labeled anti-CD31 antibody (green). The sections
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34 were counterstained with DAPI (blue). The CD31 positive vessel area was decreased in
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37 EGCG-treated tumors compared to controls. Bar; 100 μ m. (c) Microvessel density
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41 (MVD) was analyzed quantitatively. Immunohistochemical analysis of tumors indicated
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45 significant reduction in MVD in tumors after treatment with EGCG ($*P < 0.05$).
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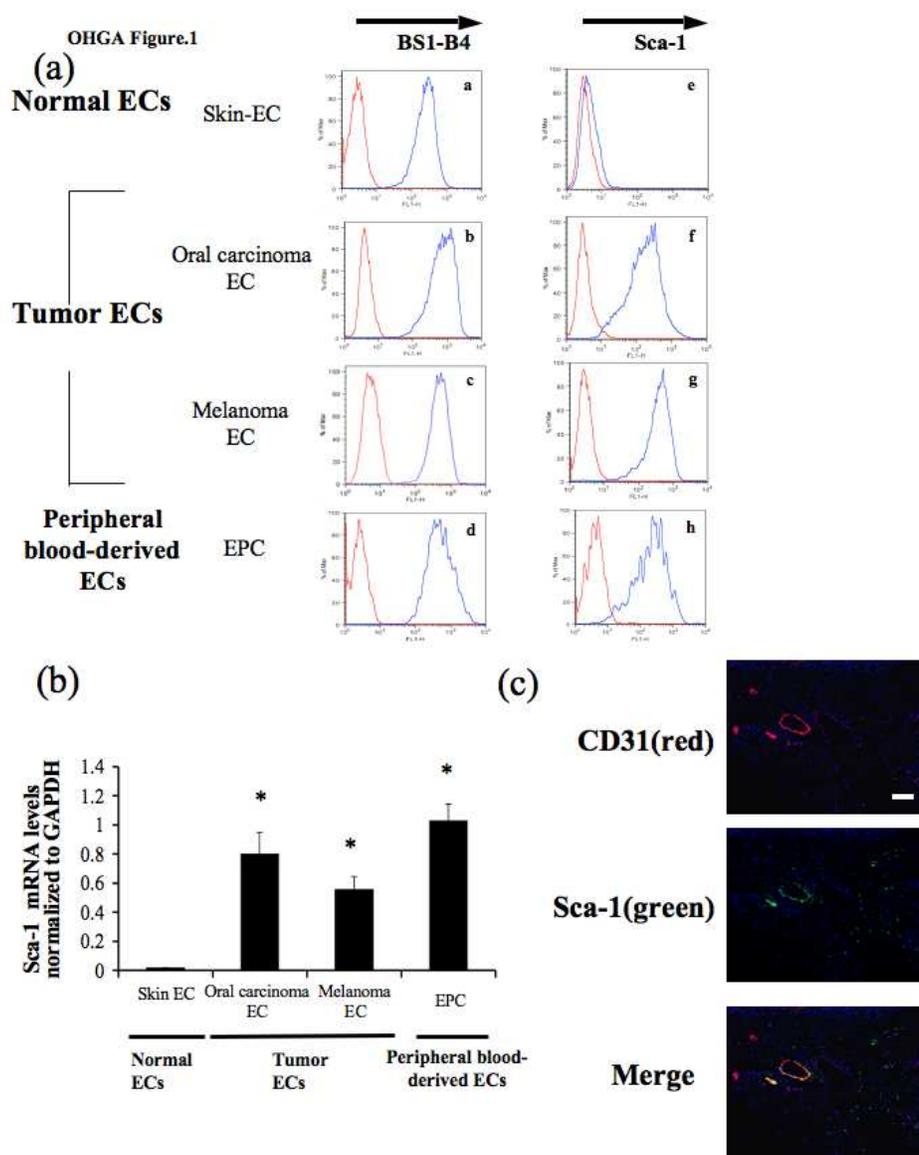


Fig. 1. Peripheral blood-derived endothelial cells (EPCs) and TECs bind BS1-B4 lectin (mouse endothelial marker) and express Sca-1 (stem cell marker). (a) NECs, TECs, and peripheral blood-derived ECs (EPCs) were characterized by Flow cytometry. (a-d) NECs, TECs, and peripheral blood-derived ECs (EPCs) were positive for BS1-B4. (e-h) Peripheral blood-derived endothelial cells (EPCs) expressed the stem cell marker Sca-1. Sca-1 was overexpressed also in TECs from both melanoma and oral carcinoma whereas it was expressed at low-level in NECs. (b) The mRNA expression of Sca-1 was higher in TEC, and peripheral blood-derived ECs (EPCs) compared to NEC in real time PCR (* $P < 0.05$). (c) The cryosections of xenografted melanoma tumors were processed for immunohistochemistry using FITC-conjugated anti-mouse Stem cell antigen-1 (Sca-1) and PE-conjugated anti-mouse CD31. Representative images are shown demonstrating CD31 positive cells (red), Sca-1 positive cells (green), and CD31+/Sca-1+ (yellow). Nuclei were stained with DAPI (blue). CD31+/Sca-1+ positive cells existed in tumor vessels. Bar; 100 μ m.

OHGA Figure.2

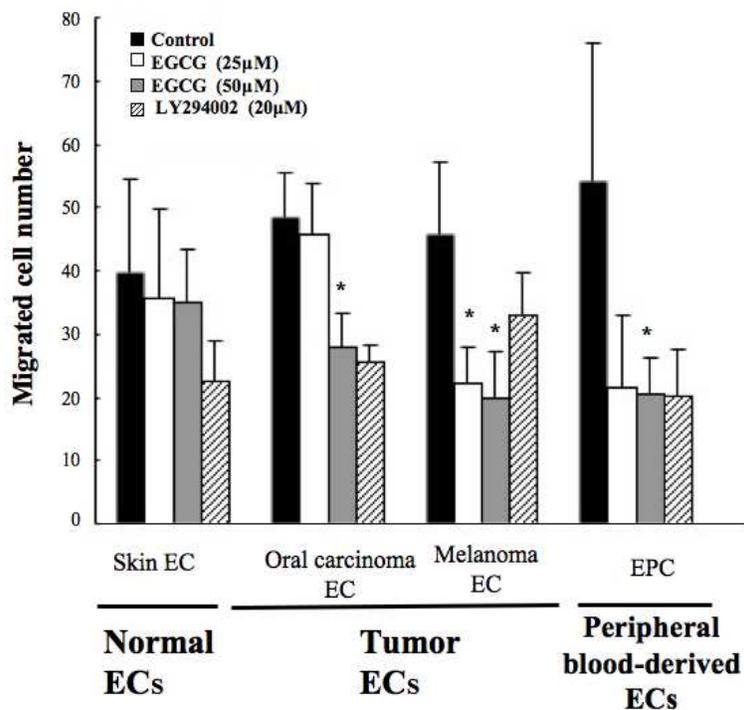


Fig. 2. EGCG inhibited TECs and EPCs migration. Endothelial cells (ECs) and EPCs were pretreated with EGCG (25, 50 µM) for 16 h and a PI3 kinase inhibitor LY294002 (20 µM) for 2h. Migration of ECs and EPCs toward VEGF was analyzed on fibronectin-coated membranes using a Boyden chamber. Cells that migrated to the lower side of the membrane were counted in seven different high-power fields. Migration of TECs and EPCs, compared to NECs, toward VEGF was inhibited significantly by EGCG (* $P < 0.05$ vs controls). Results represent the average of three experiments.

OHGA Figure.3

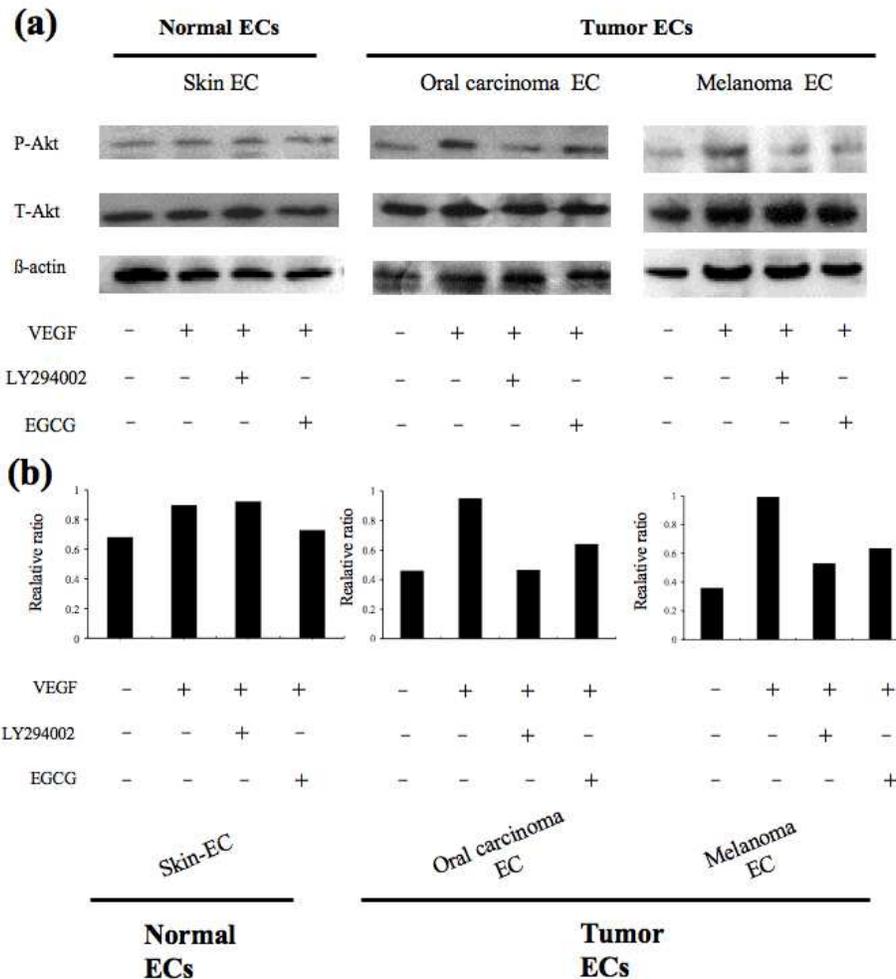


Fig. 3. EGCG inhibited Akt phosphorylation in TECs but not in NECs. (a) Serum-starved ECs were pretreated with EGCG (100 μ M) for 16h or PI3 kinase inhibitor LY294002 (20 μ M) for 2h before addition of VEGF (10ng/mL). EGCG was not toxic to ECs. ECs were stimulated with VEGF for 30 minutes. Levels of phosphorylated Akt (P-Akt) were determined by Western blotting using an anti-phospho-Akt antibody. After Western blot analysis with antiphospho-Akt (Ser473) antibody (top), the membrane was stripped and reincubated with anti-Akt antibody (middle) and β -actin antibody (bottom) to detect of the amount of Akt and β -actin protein.

(b) Levels of phosphorylated Akt were normalized for total Akt by scanning densitometry using computed image analysis. The graph shows the relative ratio of phosphorylated Akt (Ser 473) /Akt by densitometric analysis. The experiment was performed three times with similar results.

OHGA Figure.4

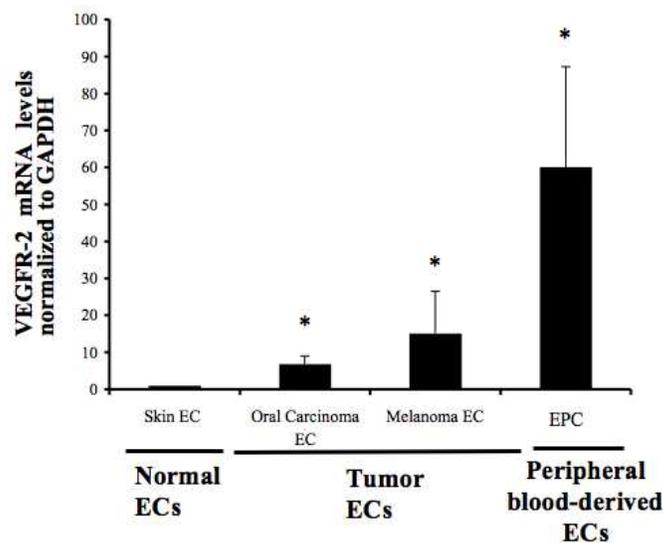


Fig. 4. VEGF receptor 2 (VEGFR2) is upregulated in TECs, EPCs. TECs and EPCs showed higher levels of VEGFR2 mRNA compared to NECs by real-time RT-PCR. Expression levels of VEGFR2 mRNAs were normalized to GAPDH (*P < 0.05 versus NECs) .

OHGA Figure.5

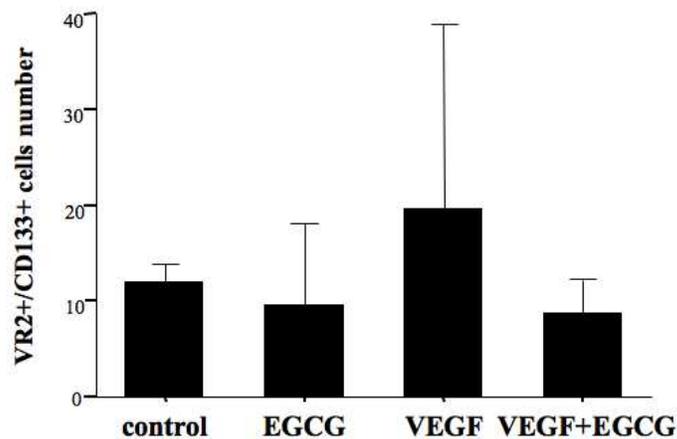
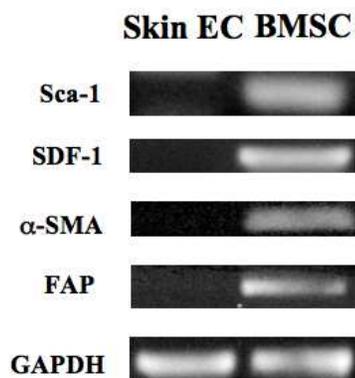


Fig. 5. EGCG suppressed VEGF-stimulated mobilization of CD133+/VEGFR2+ cells into circulation. Ten nude mice in each group were injected intraperitoneally with PBS or a high dose of VEGF (300 ng) to mobilize EPCs from the bone marrow. Next, in each group, EGCG (5 mg/kg) or vehicle was injected once a day for 2 days. Forty-eight hours later, peripheral blood was collected from each mouse in each group (n = 5 for each group) before they were killed. PBMCs were collected and incubated with FITC-anti-mouse CD133 and PE-anti-mouse VEGFR2, and the number of CD133+/VEGFR2+ cells was counted by flow cytometry. Although there was no significant difference between the VEGF group and the VEGF + EGCG group, the low dose of EGCG (5 mg /kg) moderately decreased the number of VEGF-mobilized CD133+/VEGFR2+ cells that migrated into peripheral circulation.

OHGA Figure.6

(a)



(b)

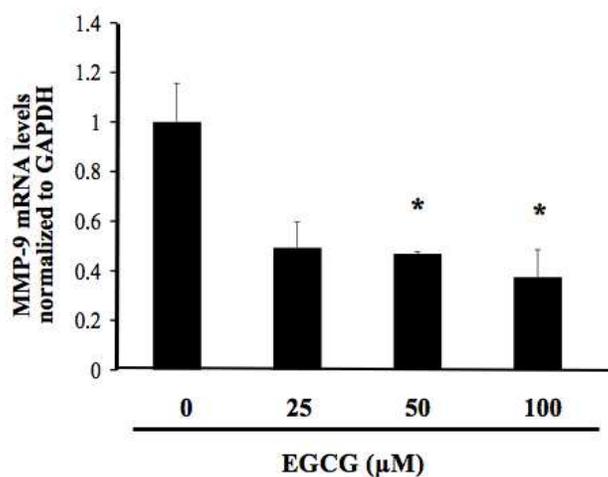


Fig.6. EGCG suppressed the mRNA expression of MMP-9 in BMSCs of oral carcinoma-bearing mice. (a) RT-PCR characterization of BMSCs for expression of stem cell marker Sca-1, stromal cell markers SDF-1, α -SMA and FAP. Sca-1, SDF-1, α -SMA, and FAP were expressed in BMSCs but not skin endothelial cells. (b) The possibility that EGCG affects mRNA expression of MMP-9 in BMSCs was assessed by real-time PCR. BMSCs were pretreated with or without EGCG for 24 h. (25, 50, 100 μ M). Expression levels of MMP-9 mRNAs were normalized to GAPDH. EGCG significantly reduced MMP-9 mRNA levels in BMSCs. The experiment was performed three times with similar results (* $P < 0.05$ versus 0 μ M).

OHGA Figure.7

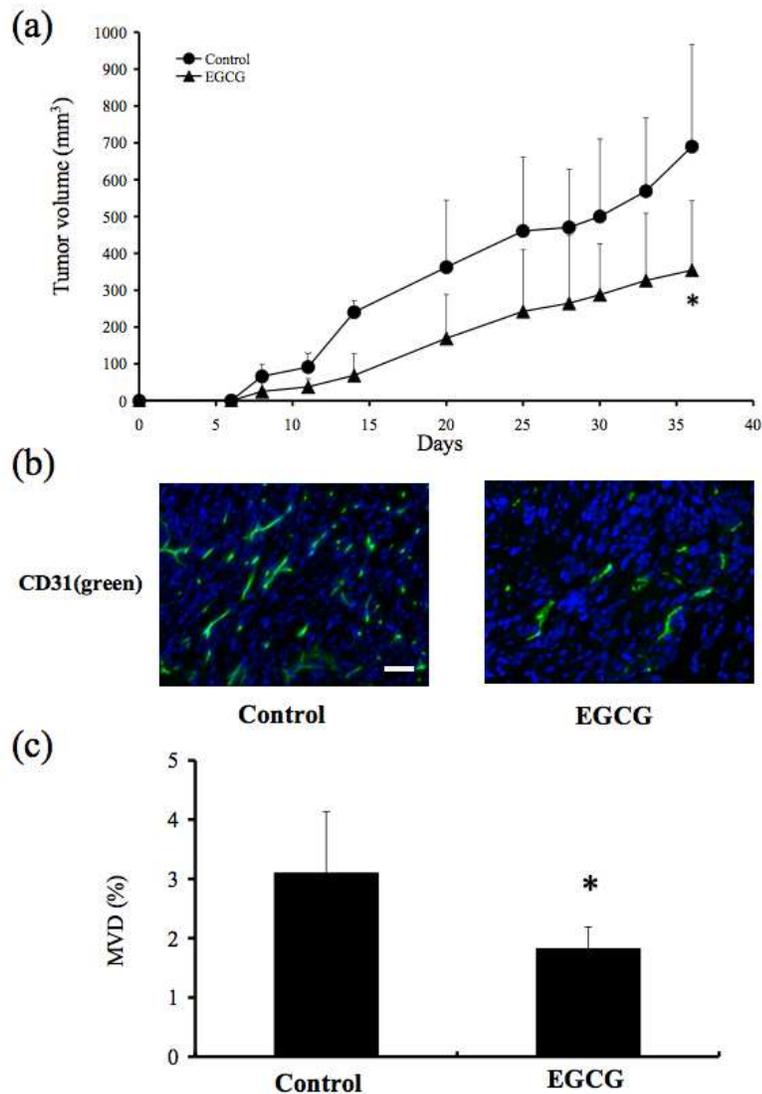


Fig.7.

EGCG repressed melanoma tumor growth. (a) The melanoma tumor cells were inoculated subcutaneously into nude mice. Day 0 is defined as the first day of treatment. Two groups of animals were then treated with the vehicle (0.05% DMSO, n = 5) or EGCG (20 mg/kg, n = 5) intraperitoneally every day. Tumor growth was significantly suppressed by EGCG treatment, as compared with controls (* $P < 0.05$). Means \pm SD are shown. (b) Immunohistochemical analysis of tumors. Snap-frozen tumor tissue specimens were processed for immunohistochemical study when mice were sacrificed. Cryosections were stained with FITC-labeled anti-CD31 antibody (green). The sections were counterstained with DAPI (blue). The CD31 positive vessel area was decreased in EGCG-treated tumors compared to controls. Bar; 100 μ m. (c) Microvessel density (MVD) was analyzed quantitatively. Immunohistochemical analysis of tumors indicated significant reduction in

MVD in tumors after treatment with EGCG (*P < 0.05).

For Review

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