In Vitro Cellular & Developmental Biology - Animal (2022) 58:758–770
https://doi.org/10.1007/s11626-022-00717-3

VEGF-A promotes the motility of human melanoma cells through the VEGFR1–PI3K/Akt signaling pathway

Koichi Koizumi[1] · Tomoaki Shintani[2] · Yasutaka Hayashido[3] · Atsuko Hamada[3] · Mirai Higaki[3] · Yukio Yoshioka[1] · Akihiko Sakamoto[4] · Souichi Yanamoto[1,3] · Tetsuji Okamoto[1,3,5]

Received: 13 July 2022 / Accepted: 12 August 2022 / Published online: 23 August 2022 / Editor: J. Denry Sato

Abstract

Vascular endothelial growth factor A (VEGF-A) and its receptors (VEGFR1 and R2) play important roles in the progression of malignant melanoma through tumor angiogenesis. However, it is not clear whether the VEGF-A/VEGFR1 signaling pathway is involved in the proliferation and migration of melanoma cells. Thus, the effect of VEGF-A on cell migration was investigated in human melanoma cell lines. Of several splicing variants of VEGF-A, $VEGF_{165}$ is the most abundant and responsible for VEGF-A biological potency. $VEGF_{165}$ facilitated the migration of melanoma cells in both a chemotactic and chemokinetic manner, but cell proliferation was not affected by $VEGF_{165}$. $VEGF_{165}$ also induced the phosphorylation of Akt. In addition, $VEGF_{165}$-induced cell migration was inhibited significantly by VEGFR1/2 or a VEGFR1-neutralizing antibody. Furthermore, the downregulation of VEGFR1 via the transfection of VEGFR1-targeting antisense oligonucleotides suppressed $VEGF_{165}$-induced cell migration. Moreover, wortmannin, an inhibitor of phosphatidylinositol-3 kinase (PI3K) in the PI3K/Akt pathway, suppressed $VEGF_{165}$-induced Akt phosphorylation and $VEGF_{165}$-induced cell migration. These findings suggest that the motility of melanoma cells is regulated by signals mediated through the PI3K/Akt kinase pathway with the activation of VEGFR1 tyrosine kinase by $VEGF_{165}$. Thus, the downregulation of signaling via VEGF-A/VEGFR1 might be an effective therapeutic approach that could prevent the progression of malignant melanoma.

Keywords Melanoma · Cell motility · VEGF-A · VEGFR1 · PI3K/Akt signaling pathway

Introduction

One characteristic property of malignant tumors is their ability to invade the surrounding tissues and form metastatic foci in distant organs. Metastasis involves a series of steps, including the detachment of cancer cells from the primary lesion, migration into connective tissues, intravasation into the circulation, and implantation into distant organs (Bravo-Cordero *et al*. 2012; Clark and Vignjevic 2015).

Tumor cells are known to produce growth factors and cytokines, such as vascular endothelial growth factor (VEGF), transforming growth factors, and basic fibroblast growth factors, which have various biological activities in tumor cells and stroma cells, including endothelial cells and fibroblasts (Hayashido *et al*. 1998; Guo *et al*. 2021; Motwani and Eccles 2021). VEGF is a potent angiogenic factor that binds to two tyrosine kinase-type receptors, VEGF receptor-1 (VEGFR1)/fms-like tyrosine kinase (Flt-1) and VEGFR2/kinase insert domain receptor (KDR)/fetal liver kinase 1, which are specifically and highly expressed in

Koichi Koizumi and Tomoaki Shintani contributed equally to this work.

✉ Yasutaka Hayashido
hayashiy@hiroshima-u.ac.jp

1 Department of Oral Oncology, Graduate School of Biomedical and Health Sciences, Hiroshima University, Hiroshima 734-8553, Japan

2 Center of Oral Clinical Examination, Hiroshima University Hospital, Hiroshima 734-8551, Japan

3 Oral Maxillofacial Surgery, Hiroshima University Hospital, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8551, Japan

4 Oral Maxillofacial Surgery, Hiroshima University Hospital, Hiroshima 734-8551, Japan

5 School of Medical Sciences, University of East Asia, Shimonoseki 751-8503, Japan

vascular endothelial cells. The interaction of VEGF and VEGFRs has a stimulatory effect on the proliferation and migration of vascular endothelial cells (Vaisman *et al*. 1990; Myoken *et al*. 1991). Importantly, VEGF is known to be upregulated in several tumors and to contribute to tumor angiogenesis.

The VEGF family consists of VEGF-A–E and placental growth factor (PlGF). VEGF-A plays a central role in tumor angiogenesis in relation to blood vessel sprouting, repair, and regeneration (Dvorak 2021). VEGF-A consists of several splice variants with different numbers of amino acids, such as $VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$, and $VEGF_{189}$. $VEGF_{165}$ is the most abundant and responsible for VEGF-A biological potency (Dvorak 2021). Although VEGF-A binds to both VEGFR1 and VEGFR2, VEGF-B and PlGF bind only to VEGFR1. The affinity of VEGF-A to VEGFR1 is about tenfold higher than its affinity to VEGFR2, whereas the tyrosine kinase activity of VEGFR1 is about tenfold lower than that of VEGFR2 (Shibuya 2006, 2011; Apte *et al*. 2019). VEGFR1 contributes to pathological angiogenesis in tumors, rheumatoid arthritis, and cerebral ischemia, and VEGFR2 is the regulator of both physiological and pathological angiogenesis (Dvorak 2021). Although PlGF is not involved in physiological angiogenesis, it participates in pathological angiogenesis in cancer tissues via VEGFR1 (Dewerchin and Carmeliet 2012).

In general, tumor cells have the ability to produce VEGFs, whereas their expression of VEGFRs is strongly suppressed. Previous studies have shown that VEGFRs are expressed in many types of cancers, including melanoma, pancreatic, lung, and ovarian cancers, suggesting that VEGFs might regulate tumor progression through not only paracrine mechanisms but also autocrine mechanisms (Gitay-Goren *et al*. 1993; Frank *et al*. 2011; Shibuya 2011; Borsotti *et al*. 2015).

Melanoma is a malignant tumor derived from melanocytes in the skin and mucous membrane (Iversen and Robins 1980; Yde *et al*. 2018; Ahmed *et al*. 2020). Melanoma frequently metastasizes due to its ability to migrate effectively and form a vascular network in tumor tissues (Streit and Detmar 2003; Pasquali *et al*. 2018). Moreover, melanoma is known to express high levels of PlGF and VEGF-A. In vivo studies have shown that when melanoma cells are inoculated into transgenic mice that overexpress PlGF, tumor growth is increased significantly and metastatic potential is relatively higher than that in control mice inoculated with melanoma cells (Lacal *et al*. 2000; Graziani *et al*. 2016; Lacal and Graziani 2018). Furthermore, VEGFR1-expressing melanoma cells have been shown to be more invasive compared with melanoma cells that do not express VEGFR1, and the blockade of VEGFR1 using a specific monoclonal antibody reduces VEGF-A- and PlGF-inducible extracellular matrix invasion (Hennequin *et al*. 1999). These results suggest that a signal mediated via VEGFR1 might regulate the invasion of melanoma cells. However, the mechanism underlying the tumor-produced VEGF-regulated invasion and metastasis of melanoma remains unclear. Thus, in the present study, we examined the expression of VEGF-A and VEGFR1 in human melanoma cells and investigated the effects of $VEGF_{165}$/VEGFR on the migration and proliferation of human melanoma cells as well as the $VEGF_{165}$/VEGFR-related signaling pathway.

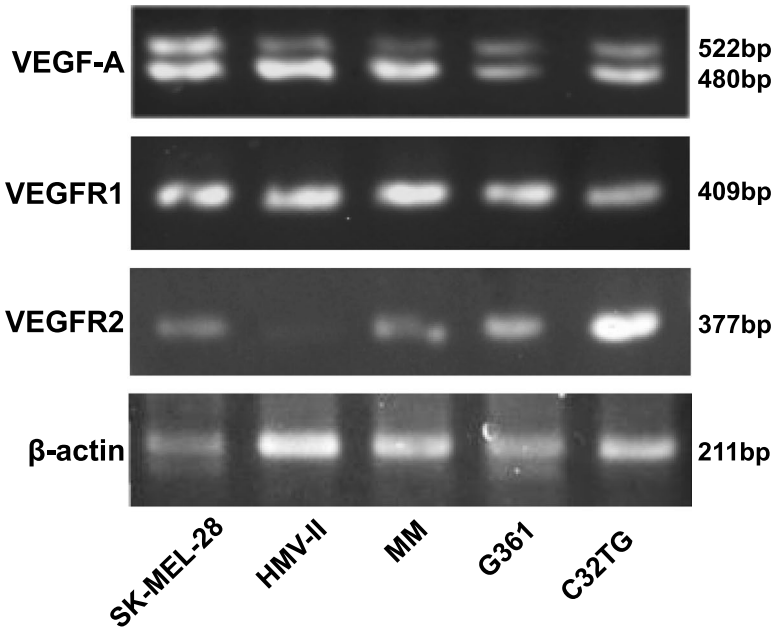
Materials and methods

Chemicals and antibodies Insulin, transferrin, 2-aminoethanol, sodium selenite, 2-mercaptoethanol, oleic acid conjugated with fatty acid–free bovine serum albumin (BSA), and PlGF were purchased from Sigma-Aldrich (St. Louis, MO). Recombinant Human $VEGF_{165}$ and Human VEGF Quantikine ELISA Kits were obtained from R&D Systems Inc. (Minneapolis, MN). Type I collagen solution (Native Collagen Acidic Solution, IAC-50) was purchased from Koken (Tokyo, Japan). The VEGFR1/2 tyrosine kinase activity inhibitor [CB676475, (4-[(4′-chloro-2′-fluoro) phenylamino]-6,7-dimethoxyquinazoline)] was purchased from Calbiochem (San Diego, CA), and the VEGFR2 kinase inhibitor II [(Z)-5-bromo-3-[(4,5,6,7-tetrahydro-1H-indol-2-yl) methylene]-1,3-dihydroindol-2-one] was purchased from Merck Biosciences (Nottingham, UK). Wortmannin, a kinase inhibitor of phosphatidylinositol-3 kinase (PI3K), was obtained from Sigma-Aldrich.

Rabbit polyclonal anti-phospho-VEGFR1 antibody (Y1059; CSB-PA000747) and rabbit polyclonal anti-phospho-VEGFR2 antibody (Y1048; CSB-PA009634) were purchased from Cusabio Technology (Houston, TX). Rabbit polyclonal anti-VEGFR1 antibody (A1277) and rabbit polyclonal anti-VEGFR2 antibody (A5609) were purchased from ABclonal (Boston, MA). Rabbit monoclonal anti-phosphorylated Akt antibody (Ser473; #4060), rabbit monoclonal anti-phosphorylated extracellular signal-regulated kinase-1/2 (Erk1/2) (Thr202/Tyr204; #4370), rabbit monoclonal anti-Akt antibody (#4685), anti-Erk1/2 antibody (#4695), rabbit monoclonal anti-β-Actin (#4970), and horseradish peroxidase (HRP)–conjugated anti-rabbit IgG antibody (#7074) were purchased from Cell Signaling Technology (Danvers, MA). Anti-VEGFR1 blocking monoclonal antibody (KM1750) was kindly provided by Dr. Shibuya (Jobu University, Isesaki, Japan) and Dr. Shitara (Kyowa Hakko Kirin Co., Ltd, Tokyo, Japan).

Cells and culture The human melanoma cell lines SK-MEL-28 (RRID:CVCL_0526) (Shiku *et al*. 1976), HMV-II (RRID:CVCL_1282) (Kasuga *et al*. 1976), G361 (RRID:CVCL_1220) (Peebles *et al*. 1978), and C32TG (RRID:CVCL_2324) (Jia *et al*. 1997) were provided by

(A)



(B)

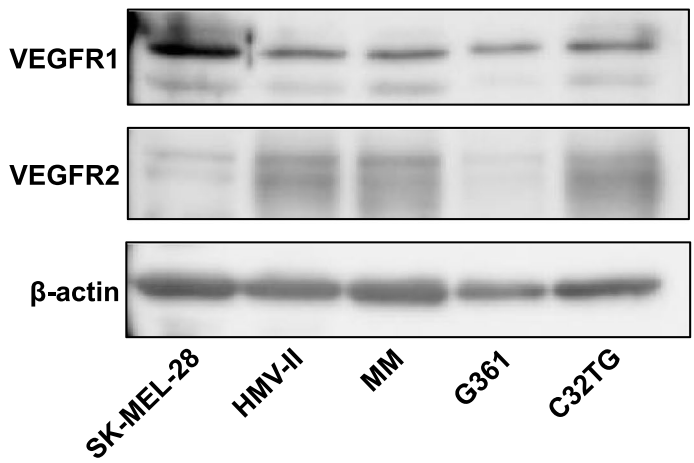
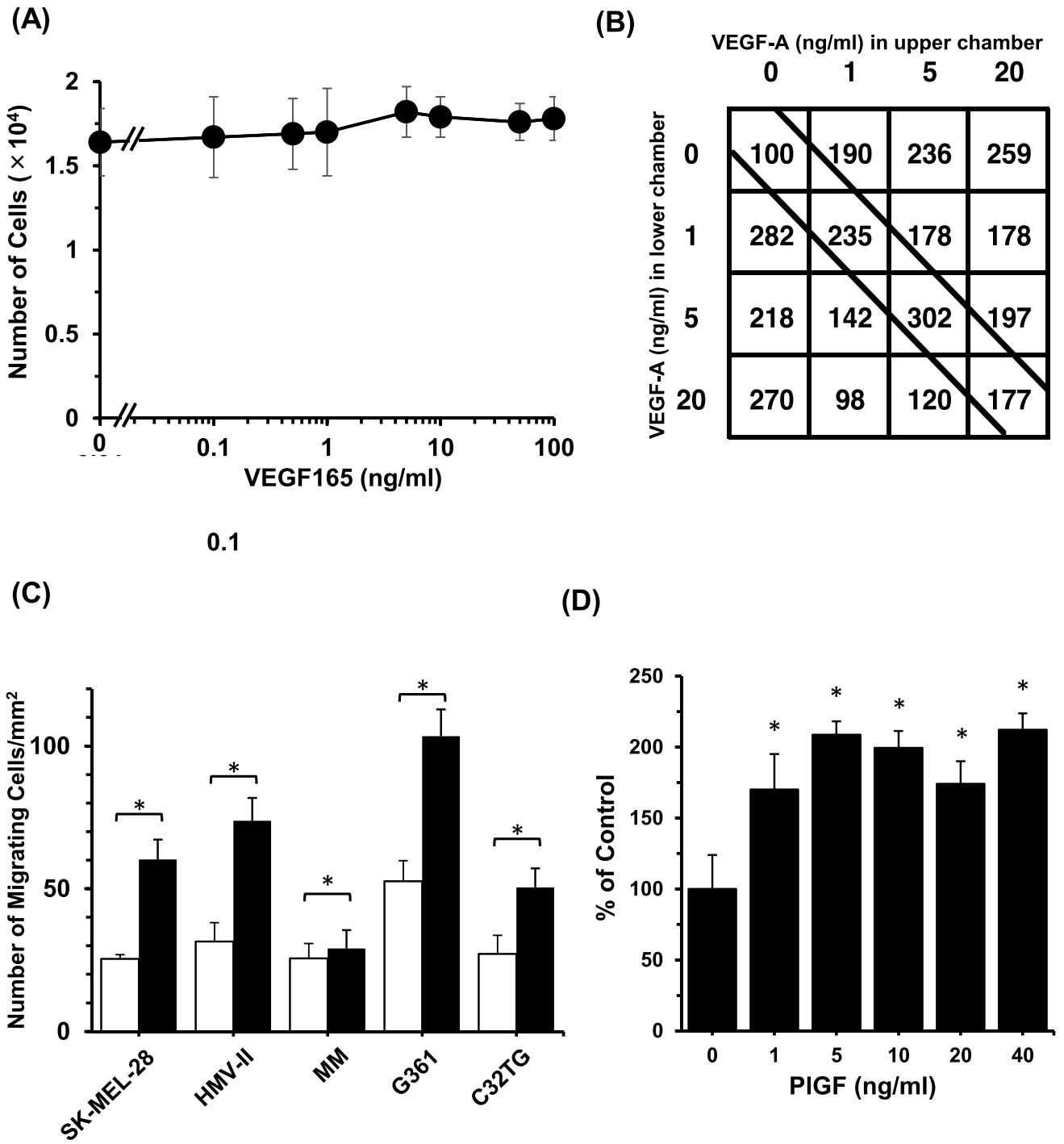


Figure 1. Expression of vascular endothelial growth factor (VEGF)-A, VEGFR1, and VEGFR2 in human melanoma cells. Total RNA was extracted from melanoma cells, and the expression of VEGF-A, VEGFR1, and VEGFR2 mRNAs was analyzed using RT-PCR. All cells expressed the mRNAs of $VEGF_{165}$ and $VEGF_{189}$ (*A*), and the expression of VEGFR1 and VEGFR2 mRNAs (*B*) was also observed. Melanoma cells (80% confluent) were cultured in serum-free medium for 24 h, and $VEGF_{165}$ protein in the culture supernatants was quantified using ELISA (*C*).

(C)

Cells	$VEGF_{165}$ (pg/ml/10^5)
SK-MEL-28	77.4 ± 10.2
HMV-II	45.0 ± 17.6
MM	176.0 ± 8.4
G361	3.4 ± 1.0
C32TG	58.0 ± 10.4

RIKEN BRC (Tsukuba, Japan) and used in this study. Malignant melanoma (MM) cells established in our laboratory from a patient with malignant melanoma of the gingiva were also used (Okamoto *et al*. 1996). These cell lines are free from mycoplasma contamination using e-Myco™ plus Mycoplasma PCR Detection Kit (iNtRON, Seongnam-Si,



South Korea) and have been authenticated using short tandem repeat (STR) profiling (BEX Co., Ltd., Tokyo, Japan) within the last 3 mo (Supplementary Figure S1 and Table S1). The STR profiles of these cell lines, except for MM cell line, matched with the publicly available reference profiles (ICLAC Databases. 2021). As MM cell line has not yet deposited to the cell bank, the STR profile of MM cell line did not match with any other STR data in the databases.

All cells were grown in DF medium (1:1 mixture (by volume) of Dulbecco's modified Eagle medium (DMEM) and Ham F-12 medium) supplemented with 5% fetal bovine serum in a humidified 95% air/5% CO_2 atmosphere at 37 °C in a CO_2 incubator (Thermo Fisher Scientific, Waltham, MA). Cell proliferation was estimated as follows. The wells of 24-well tissue culture plates were coated with 100 µg/mL of type I collagen, and cells (5×10^3) suspended in DF 6F serum–free medium supplemented with 10 µg/mL of insulin,

◂**Figure 2.** Effect of $VEGF_{165}$ on the proliferation and motility of melanoma cells. SK-MEL-28 cells (5×10^3) suspended in DF 6F serum–free medium containing 10 μg/mL of insulin, 5 μg/mL of transferrin, 10 μM of 2-aminoethanol, 10 nM of sodium selenite, 10 μM of 2-mercaptoethanol, and 9.4 μg/mL of oleic acid conjugated with fatty acid–free bovine serum albumin (BSA) were seeded in each well of a 24-well tissue culture plate coated with type-I collagen. After 24 h, the indicated concentrations of $VEGF_{165}$ were added. The number of cells was measured after cultivation for 5 d (*A*). The effect of $VEGF_{165}$ on the migration of SK-MEL-28 cells was investigated using a modified Boyden chamber method. SK-MEL-28 cells (1×10^5) suspended in DF medium containing 0.1% BSA and the indicated concentrations of $VEGF_{165}$ were added to the upper and lower chambers. After incubation for 24 h at 37 °C, the number of cells that had migrated to the lower surface of the filter was counted a percentage of the untreated control. Cell motility was estimated using checkerboard analysis (*B*). Melanoma cells (1×10^5) were added to the upper chambers and cultured with (+) or without (−) 5 ng/mL of $VEGF_{165}$ in both the upper and lower chambers. After cultivation for 24 h, the number of cells that had migrated was counted (*C*). SK-MEL-28 cells (1×10^5) were added to the upper chamber, and the indicated concentrations of PlGF were added to both the upper and lower chambers. After cultivation for 24 h, the number of cells that had migrated was counted (*D*). All experiments were performed in triplicate, and data are means ± SD. In *B* and *D*, the data represent percentages of the untreated control. $^*p<0.05$.

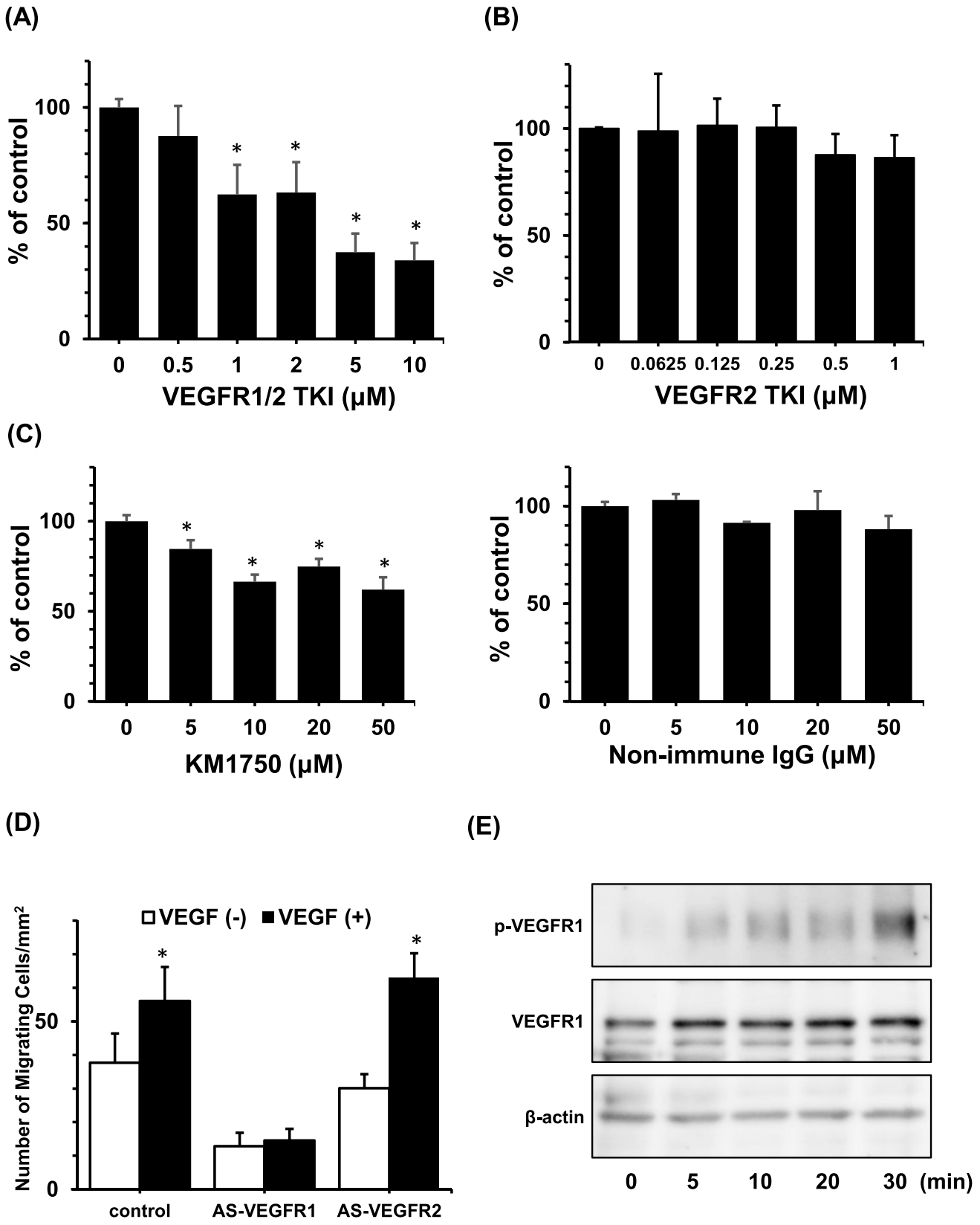
5 μg/mL of transferrin, 10 μM of 2-aminoethanol, 10 nM of sodium selenite, 10 μM of 2-mercaptoethanol, and 9.4 μg/mL of oleic acid conjugated with fatty acid–free BSA were seeded in each well of the culture plates (Sato *et al*. 1987). After 24 h, various concentrations of $VEGF_{165}$ were added, and the cells were cultured in 5% CO_2 for 5 d at 37 °C. Subsequently, the number of cells was counted using a Coulter counter (Beckman Coulter, Tokyo, Japan), and the measurements were collected in triplicate. All reagents used in the cell culture were free from mycoplasma and viral pathogens.

RNA extraction and RT-PCR for VEGF-A and VEGFR mRNAs Total RNA was isolated from the cells using TRIzol reagent (Thermo Fisher Scientific) following the manufacturer's protocol, and RNA quality was determined according to the following criteria: RNA concentration > 0.5 μg/μL; OD 260/280 = 1.8–2.0. Reverse transcription was performed using the Super Script First-strand Synthesis System (Life Technologies, Carlsbad, CA). PCR was performed for VEGF-A and VEGFRs with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) used as the internal control. Following an initial incubation at 94 °C, each PCR cycle consisted of incubation for 30 s at 94 °C, 30 s at 55 °C, and 1 min at 72 °C. After the final cycle, the samples were incubated for a further 7 min at 72 °C and then kept at 4 °C before analysis via agarose gel electrophoresis. The following primers were used: VEGF-A forward primer, 5′-CTTGCCTTGCTGCTCTACC-3′; VEGF-A reverse primer, 5′-CACACAGGATGGCTTGAAG-3′; VEGFR1 forward primer, 5′-CATGAGGATGAGAGCTCCTGAG-3′; VEGFR1 reverse primer, 5′-AGGCCAACAGAGTGCTGCTGTC-3′; VEGFR2 forward primer, 5′-CCTGTCCACTTACCTGAGGAG-3′; VEGFR2 reverse primer, 5′-CTGGCTACTGGTGATGCTGTC-3′; GAPDH forward primer, 5′-GCTCTCTGCTCCTCCTGTTC-3′; and GAPDH reverse primer, 5′-ACGACCAAATCCGTTGACTC-3′.

Immunoblot analysis Cells were lysed using cell lysis buffer (50-mM Tris HCl (pH 7.4), 150-mM NaCl, 1-mM EDTA, 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS), and 0.5% sodium deoxycholate) supplemented with 1% protease inhibitor cocktail (Sigma-Aldrich). The lysates were centrifuged at 15,000 × *g* and 4 °C for 15 min, and the supernatants were collected. Samples containing 20 μg of total protein were electrophoresed on 10% SDS–polyacrylamide gel under reducing conditions and transferred to polyvinylidene difluoride (PVDF) membrane filters (Bio-Rad Laboratories, Hercules, CA). The filters were blocked using TBS-T (20-mM Tris HCl (pH 7.5), 137-mM NaCl, and 0.1% Tween 20) containing 5% skim milk for 1 h at room temperature, after which they were incubated with primary antibodies and then with HRP-conjugated secondary antibody. Rabbit monoclonal anti-β-actin was used as a loading control antibody. Protein bands were visualized using enhanced chemiluminescence detection (Clarity ECL Substrate; Bio-Rad Laboratories).

ELISA for soluble $VEGF_{165}$ To obtain conditioned media, 80% confluent melanoma cells in 6-well plates were washed twice with DF and incubated with 2 mL of DF for 24 h. The conditioned media were then centrifuged at 10,000 × *g* and 4 °C for 30 min to remove cells and debris. The amount of soluble $VEGF_{165}$ in the conditioned media was measured using a Human VEGF Quantikine ELISA Kit according to the manufacturer's instructions. The levels of $VEGF_{165}$ detected were corrected according to the number of cells.

Cell motility assay Cell motility was analyzed using a modified Boyden chamber assay with Transwell inserts (6.5 mm in diameter) containing 8-μm pores (Corning Costar, Cambridge, MA) as described previously (Chen 2005; Hayashido *et al*. 2007). The filters were coated with 100 μg/mL of type-1 collagen to enhance cell attachment. Melanoma cells (1×10^5) resuspended in DF medium containing 0.1% BSA were added to the upper compartment of each Transwell insert, and $VEGF_{165}$ or PlGF (Sigma-Aldrich) was added to the upper or lower compartment. After incubation for 24 h at 37 °C, the Transwell inserts were fixed with methanol and stained with Diff-Quik (Dade Behring AG, Dudingen, Switzerland). The cells on the upper surface of the filter were wiped with a cotton swab, and the number of cells on the lower surface of the filter was counted under a low-power field (×50) using light microscopy. Five fields were counted


(A)
% of control
100
50
0
0 0.5 1 2 5 10
VEGFR1/2 TKI (μM)
(B)
% of control
100
50
0
0 0.0625 0.125 0.25 0.5 1
VEGFR2 TKI (μM)
(C)
% of control
100
50
0
0 5 10 20 50
KM1750 (μM)
% of control
100
50
0
0 5 10 20 50
Non-immune IgG (μM)
(D)
Number of Migrating Cells/mm²
VEGF (-) VEGF (+)
50
0
control
AS-VEGFR1
AS-VEGFR2
(E)
p-VEGFR1
VEGFR1
β-actin
0 5 10 20 30 (min)

◂**Figure 3.** Effects of VEGFR tyrosine kinase inhibitors (TKIs), VEGFR1-neutralizing antibody, and VEGFR antisense oligonucleotides (ASOs) on the $VEGF_{165}$-mediated motility of melanoma cells. SK-MEL-28 cells treated with 4-[(4′-chloro-2′-fluoro) phenylamino]-6,7-dimethoxyquiazorine, a VEGFR1/2 TKI, for 1 h were suspended in DF medium containing 0.1% BSA and then added to the upper compartments of Transwell chambers. After incubation with 5 ng/mL of $VEGF_{165}$ in both the upper and lower compartment for 24 h at 37 °C, the number of cells that migrated to the lower surface of the filter was counted. The VEGFR1/2 TKI suppressed $VEGF_{165}$-induced cell migration in a dose-dependent manner (*A*). After the treatment of SK-MEL-28 cells with (Z)-5-bromo-3-[(4,5,6,7-tetrahydro-1H-indol-2-yl) methylene]-1,3-dihydroindol-2-one, a VEGFR2 TKI, for 1 h, the number of cells that migrated when incubated with 5 ng/mL of $VEGF_{165}$ in both the upper and lower chambers for 24 h was determined. The VEGFR2 TKI did not affect $VEGF_{165}$-induced cell migration (*B*). After treatment of SK-MEL-28 cells with KM1750, a VEGFR1-neutralizing antibody, or nonimmune IgG for 1 h, the number of cells that migrated in the presence of 5 ng/mL of $VEGF_{165}$ was investigated using a modified Boyden chamber assay. KM1750 suppressed $VEGF_{165}$-induced cell migration in a dose-dependent manner (*C*). The motility of SK-MEL-28 cells transfected with VEGFR1 or VEGFR2 ASOs was investigated using a modified Boyden chamber assay. The motility of SK-MEL-28 cells transfected with control oligonucleotide or VEGFR2 ASO was enhanced in terms of migration induced by 5 ng/mL of $VEGF_{165}$. The transfection of VEGFR2 ASO significantly reduced the migration of SK-MEL-28 cells, and the addition of $VEGF_{165}$ did not enhance cell motility (*D*). SK-MEL-28 cells were cultured in the presence of $VEGF_{165}$ for the indicated periods, and the expression of phosphorylated VEGFR1 (p-VEGFR1) was analyzed using immunoblotting. VEGFR1 phosphorylation was observed 5 min after treatment with $VEGF_{165}$ (*E*). In *A*–*D*, data are represented as percentages of the untreated control and are the means ± SD of three replicates. $*p < 0.05$.

in each of the three different experiments, and the results were expressed as the mean number of migrating cells/mm^2 ± the standard deviation (SD). To assess the chemotactic or chemokinetic response of $VEGF_{165}$, checkerboard analysis was performed by adding various concentrations of $VEGF_{165}$ to both the lower and upper Transwell chambers.

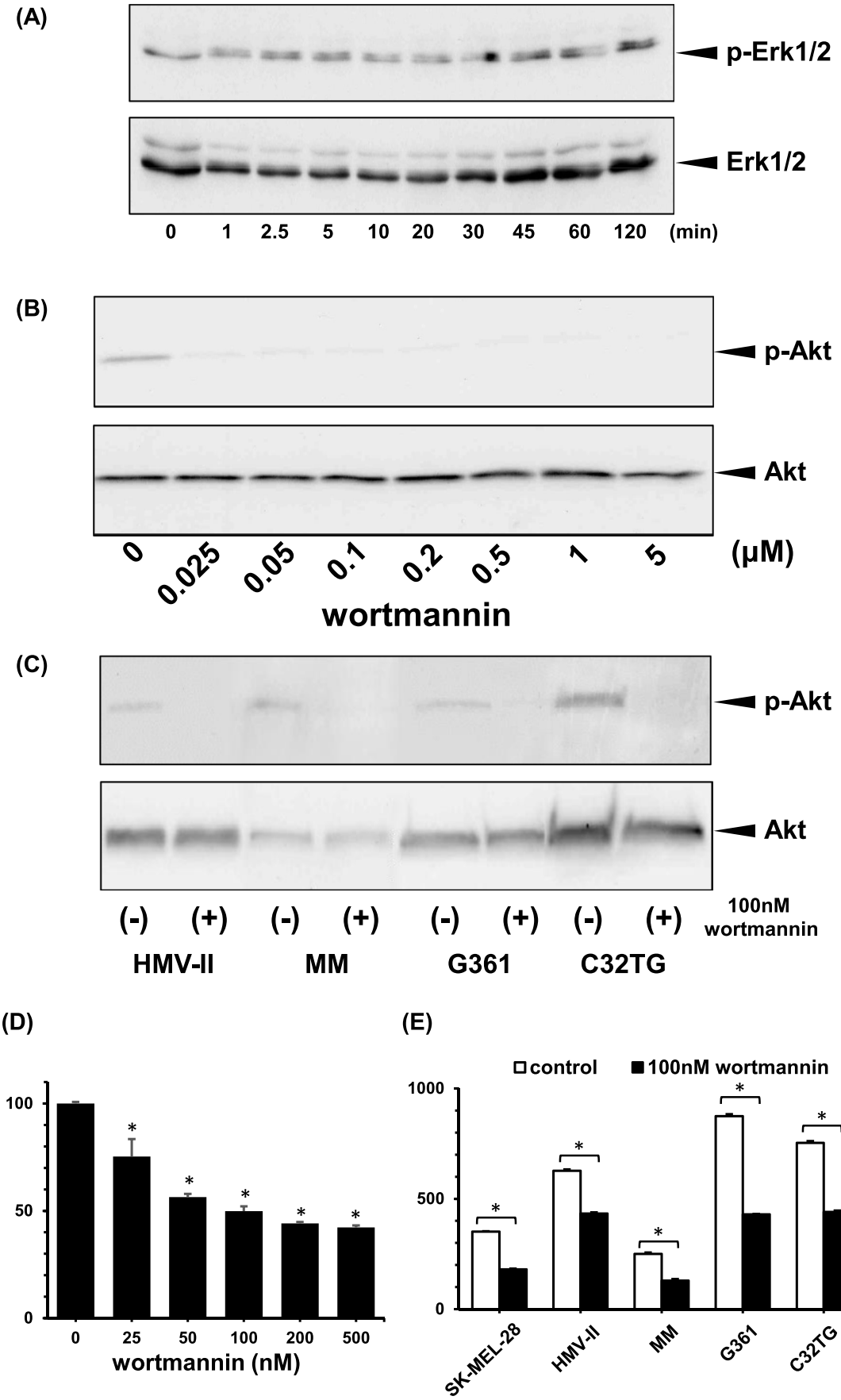
Phosphorylation assay Melanoma cells were cultured on 6-well plates until near confluence and starved with serum-free DF overnight. The cells were then incubated with 5 ng/mL of recombinant human $VEGF_{165}$ for the indicated periods, washed with ice-cold phosphate-buffered saline containing 1 mM of sodium vanadate, and lysed with cell lysis buffer supplemented with protease inhibitor cocktail and 1 mM of sodium vanadate. The samples were separated on 10% SDS–polyacrylamide gels under reducing conditions and transferred onto PVDF membrane filters. The phosphorylation of VEGFR1 and VEGFR2 was examined using immunoblotting with rabbit polyclonal anti-phospho-VEGFR1 antibody and rabbit polyclonal anti-phospho-VEGFR2 antibody, respectively. The phosphorylation of ERK1/2 and Akt was assessed using rabbit anti-phospho-ERK1/2 monoclonal antibody and rabbit anti-phospho-Akt monoclonal antibody, respectively. Total VEGFR1, VEGFR2, Akt, and MEK1/2 were detected using rabbit anti-VEGFR1 antibody, rabbit polyclonal anti-VEGFR2 antibody, rabbit anti-ERK1/2 monoclonal antibody, and rabbit anti-Akt monoclonal antibody, respectively. After incubation with the primary antibodies, the membranes were incubated with HRP-conjugated secondary antibody, and protein bands were detected using an enhanced chemiluminescence reagent.

Antisense oligonucleotides (ASOs) and transfections To downregulate VEGFR1 or VEGFR2, morpholino antisense ASOs specific for VEGFR1 or VEGFR2 (GeneTools, Philomath, OR) were used. The sequences of the ASOs were as follows: VEGFR1, 5′-AAGCCAGGGCCGAGCCGCACATAAT-3′; VEGFR2, 5′-GCAGCACCTTGCTCTGCATCCTGCA-3′. A standard control morpholino oligonucleotide (5′-CCTCTTACCTCAGTTACAATTTATA-3′) was used as a negative control. Delivery of the oligonucleotides into the cells was performed according to the GeneTools protocol. Briefly, 80–100% confluent SK-MEL-28 cells were treated with 10 μM of the morpholino ASOs or the standard control oligonucleotide and 6 μM of Endo-Porter reagent (GeneTools). After 24 h, the cells were used in the subsequent experiments.

Statistical analysis Statistical analysis was performed using BellCurve for Excel (Social Survey Research Information Co., Ltd., Tokyo, Japan). All data are presented as the means ± SD of at least three independent experiments. Student's *t*-test was used to compare the differences between groups, which were considered significant at $p < 0.05$.

Results

Expression of VEGF-A, VEGFR1, and VEGFR2 in melanoma cells The mRNA expression of VEGF-A, VEFGR1, and VEGFR2 in melanoma cells was examined using RT-PCR. The PCR products of $VEGF_{121}$, $VEGF_{165}$, and $VEGF_{189}$, which are splicing variants of VEGF-A, were detected in all cell lines (Fig. 1*A*), as was the expression of VEGFR1/Flt-1 and VEGFR2/KDR mRNAs (Fig. 1*B*). VEFGR1 and VEGFR2 protein expression was examined using immunoblotting. VEGFR1 protein was expressed in all melanoma cells, whereas VEGFR2 protein was not detected via immunoblotting. To investigate $VEGF_{165}$ secretion by melanoma cells, the amount of $VEGF_{165}$ in the conditioned media was assayed using an ELISA. The concentrations of $VEGF_{165}$ in the conditioned media were as follows: 77.4 ± 10.2 pg/mL/10^5 cells in SK-MEL-28 cells; 45.0 ± 17.6 pg/mL/10^5 cells in HMV-II cells; 176.0 ± 8.4 pg/mL/10^5 cells in MM cells; 3.4 ± 1.0 pg/mL/10^5 cells in G361 cells; and 58.0 ± 10.4 pg/mL/10^5 cells in C32TG cells (Fig. 1*C*).


(A)
p-Erk1/2
Erk1/2
0 1 2.5 5 10 20 30 45 60 120 (min)
(B)
p-Akt
Akt
0 0.025 0.05 0.1 0.2 0.5 1 5 (μM)
wortmannin
(C)
p-Akt
Akt
(-) (+) (-) (+) (-) (+) (-) (+) 100nM wortmannin
HMV-II MM G361 C32TG
(D)
100
50
0
0 25 50 100 200 500
wortmannin (nM)
(E)
control
100nM wortmannin
1000
500
0
SK-MEL-28
HMV-II
MM
G361
C32TG

◂**Figure 4.** Effects of a PI3K inhibitor on the $VEGF_{165}$-mediated motility of melanoma cells. SK-MEL-28 cells were cultured in the presence of $VEGF_{165}$ for the indicated periods, and the expression of phosphorylated Erk (p-Erk) and phosphorylated Akt (p-Akt) was analyzed using immunoblotting. SK-MEL-28 cells expressed p-Erk constitutively, and $VEGF_{165}$ did not alter Erk phosphorylation (*A*). After the treatment of SK-MEL-28 cells with the indicated concentration of wortmannin, a PI3K inhibitor, for 1 h, the cells were cultured with 5 ng/mL of $VEGF_{165}$ for 1 h, and the phosphorylation of Akt was then examined using immunoblotting. Wortmannin suppressed the $VEGF_{165}$-induced phosphorylation of Akt in a dose-dependent manner (*B*). After treatment with 100 nM of wortmannin for 1 h, HMV-II, MM, G361, and C32TG cells were cultured with 5 ng/mL of $VEGF_{165}$ for 1 h, and the phosphorylation of Akt was then examined. $VEGF_{165}$-induced phosphorylation of Akt was suppressed in all cells (*C*). After the treatment of SK-MEL-28 cells with the indicated concentrations of wortmannin for 1 h, the motility of SK-MEL-28 cells cultured with 5 ng/mL of $VEGF_{165}$ for 24 h was analyzed using a modified Boyden chamber assay. Wortmannin suppressed the $VEGF_{165}$-induced migration of SK-MEL-28 cells in a dose-dependent manner (*D*). After the treatment of melanoma cells with 100 nM of wortmannin for 1 h, the number of cells that migrated following incubation with 5 ng/mL of $VEGF_{165}$ for 24 h was analyzed using a modified Boyden chamber assay. Wortmannin suppressed $VEGF_{165}$-induced cell migration significantly (*E*). In *D* and *E*, data are represented as percentages of the untreated control. All data are the means ± SD of three replicates. $*p < 0.05$.

Effect of $VEGF_{165}$ on the proliferation of melanoma cells $VEGF_{165}$ at concentrations of 0.1–100 ng/mL did not affect the proliferation of SK-MEL-28 cells (Fig. 2*A*) or the proliferation of the other melanoma cells (data not shown). By contrast, purified $VEGF_{165}$ half-maximally stimulated human umbilical vein endothelial cell (HUVEC) proliferation at 41 pM (1.8 ng/mL) and maximally stimulated HUVEC growth at 200 pM (8.8 ng/mL) (Myoken *et al.* 1991).

Effect of $VEGF_{165}$ on the migration of melanoma cells The effect of $VEGF_{165}$ on the migration of melanoma cells was analyzed using a modified Boyden chamber method. Checkerboard analysis indicated that $VEGF_{165}$ induced chemotactic and chemokinetic migration in SK-MEL-28 cells, and 5 ng/mL of $VEGF_{165}$ added to both the upper and lower chambers led to the highest enhancement of migration (Fig. 2*B*). In all melanoma cells, 5 ng/mL of $VEGF_{165}$ added to the upper and lower chambers enhanced migration significantly ($p < 0.05$) compared with that in the controls (Fig. 2*C*).

Effect of PlGF on the proliferation and migration of melanoma cells To clarify whether the $VEGF_{165}$-induced migration of melanoma cells is regulated via VEGFR1 or VEGFR2, the effect of PlGF on the migration of melanoma cells was investigated. PlGF increased cell migration significantly ($p < 0.05$) in a dose-dependent manner (Fig. 2*D*) but did not affect cell proliferation (data not shown). These results suggested that $VEGF_{165}$-induced migration of melanoma cells was mediated only by VEGFR1.

Participation of VEGFRs in the migration of melanoma cells SK-MEL-28 cells treated with CB676475 VEGFR1/2 tyrosine kinase inhibitor (TKI), VEGFR2 kinase inhibitor II, or anti-VEGFR1 blocking monoclonal antibody for 1 h were then suspended in DMEM containing 0.1% BSA and added to an upper Transwell compartment. After incubation in the presence of 5 ng/mL of $VEGF_{165}$ for 24 h, the number of cells that migrated to the lower surface of the filter was counted. Treatment with VEGFR1/2 TKI led to the suppression of $VEGF_{165}$-induced cell motility in a dose-dependent manner (Fig. 3*A*). In contrast, VEGFR2 TKI did not alter $VEGF_{165}$-induced cell motility (Fig. 3*B*). The treatment of SK-MEL-28 cells with KM1750, a neutralizing antibody for VEGFR1, also suppressed $VEGF_{165}$-induced cell migration in a dose-dependent manner (Fig. 3*C*).

The suppression of VEGFR1 by a morpholino ASO targeting VEGFR1 led to a marked decrease in the migration of SK-MEL-28 cells, and the migration of SK-MEL-28 cells transfected with a VEGFR1-targeting morpholino ASO was not stimulated by $VEGF_{165}$ (Fig. 3*D*). Conversely, the migration of SK-MEL-28 cells transfected with a VEGFR2-targeting morpholino ASO was enhanced by $VEGF_{165}$. These experiments with inhibitors of VEGF receptor activities confirm the conclusion from the experiments with PlGF-treated melanomas that $VEGF_{165}$ stimulated melanoma migration through VEGFR1. To assess VEGFR signaling in melanoma cells, the phosphorylation of VEGFRs was analyzed using western blot analysis following the addition of $VEGF_{165}$. Treatment with $VEGF_{165}$ led to phosphorylation of VEGFR-1 (Fig. 3*E*).

Participation of $VEGF_{165}$ in the ERK signaling pathway To examine the effect of $VEGF_{165}$ on Erk phosphorylation in melanoma cells, the expression of phosphorylated Erk in SK-MEL-28 cells cultivated with $VEGF_{165}$ was analyzed using immunoblotting. Phosphorylated Erk was expressed constitutively in SK-MEL-28 cells, and $VEGF_{165}$ did not alter the expression of phosphorylated Erk (Fig. 4*A*).

Participation of the PI3K/AKT signaling pathway in $VEGF_{165}$-induced cell migration The participation of PI3K in Akt phosphorylation was investigated using wortmannin, a PI3K inhibitor, which suppressed $VEGF_{165}$-induced Akt phosphorylation in SK-MEL-28 cells in a dose-dependent manner (Fig. 4*B*). Similarly, wortmannin suppressed $VEGF_{165}$-induced Akt phosphorylation in the other melanoma cells (Fig. 4*C*). Wortmannin also suppressed $VEGF_{165}$-induced SK-MEL-28 cell migration in a dose-dependent manner (Fig. 4*D*), and it suppressed

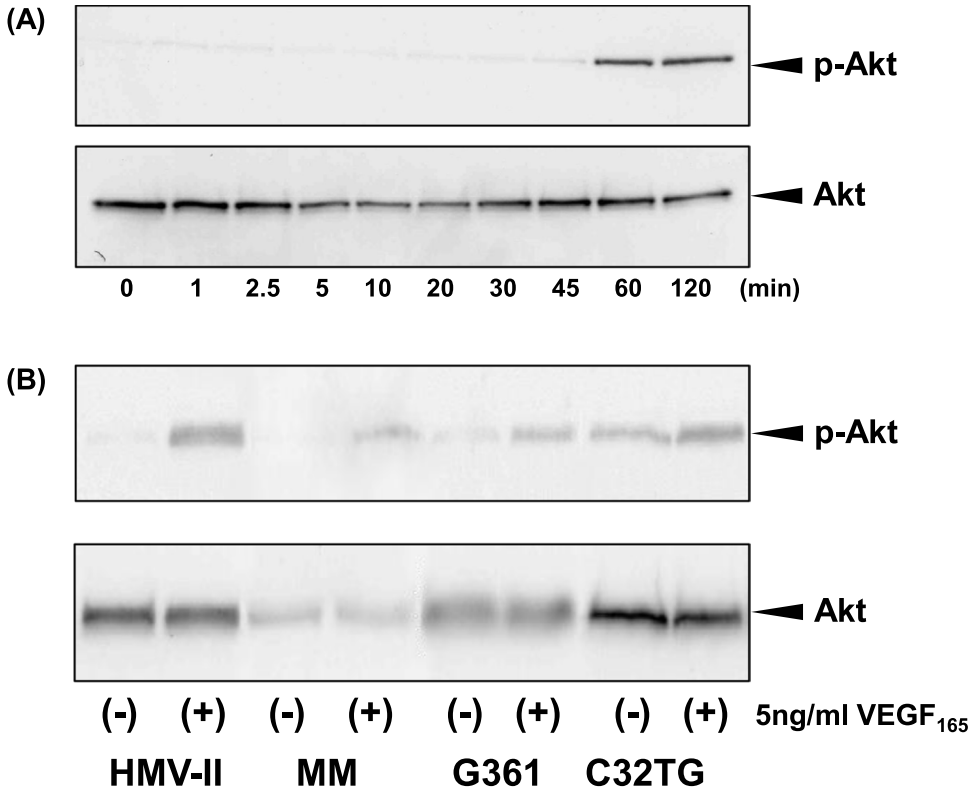


Figure 5. Effects of $VEGF_{165}$ on mitogen-activated protein kinase and phosphatidylinositol-3 kinase (PI3K)/Akt activation in melanoma cells. SK-MEL-28 cells were cultured in the presence of $VEGF_{165}$ for the indicated periods, and the expression of phosphorylated Akt (p-Akt) was analyzed using immunoblotting. Akt phosphorylation was observed 1 h after treatment with $VEGF_{165}$ (*A*). Other melanoma cells were cultured for 1 h with or without 5 ng/mL of $VEGF_{165}$, and Akt phosphorylation was then analyzed using immunoblotting. p-Akt was observed in all cells treated with $VEGF_{165}$ (*B*).

$VEGF_{165}$-induced cell migration significantly ($p < 0.05$) in the other melanoma cells (Fig. 4*E*).

Activation of the PI3K/AKT signaling pathway by VEGFR1 The effect of $VEGF_{165}$ on the phosphorylation of Akt in SK-MEL-28 cells was analyzed using immunoblotting.

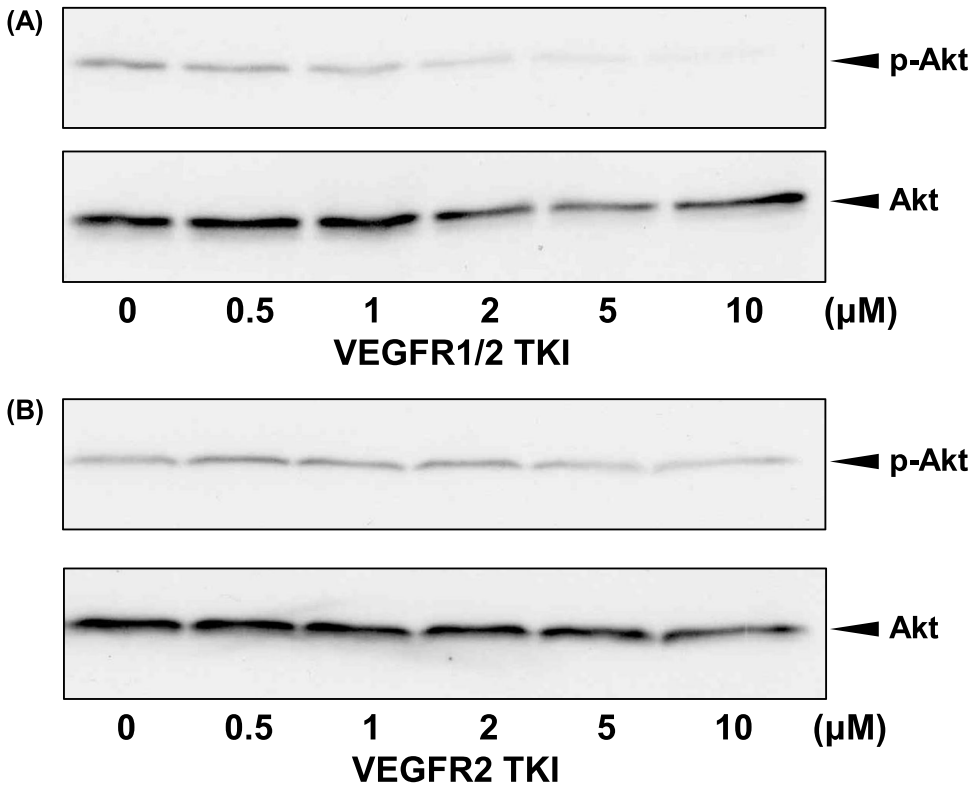


Figure 6. Effects of VEGFR TKIs on $VEGF_{165}$-induced Akt phosphorylation. After the treatment of SK-MEL-28 cells with VEGFR1/2 TKI or VEGFR2 TKI for 1 h, the cells were cultured in the presence of 5 ng/mL of $VEGF_{165}$ for 1 h, and the expression of p-Akt was then examined using immunoblotting. VEGFR1/2 TKI suppressed Akt phosphorylation by $VEGF_{165}$ in a dose-dependent manner (*A*). VEGFR2 TKI did not alter Akt phosphorylation (*B*).

Phosphorylated Akt was observed 1 h after the $VEGF_{165}$ treatment was applied (Fig. 5*A*). Furthermore, the $VEGF_{165}$ treatment led to phosphorylation of Akt in other melanoma cells (Fig. 5*B*). To determine whether $VEGF_{165}$-induced Akt phosphorylation is regulated via VEGFR1 or VEGFR2, the effects of VEGFR1- or VEGFR2-targeting TKIs on $VEGF_{165}$-induced Akt phosphorylation were investigated. VEGFR1/2 TKI suppressed the $VEGF_{165}$-induced phosphorylation of Akt (Fig. 6*A*), whereas VEGFR2 TKI did not alter the expression of phosphorylated Akt (Fig. 6*B*). These results suggest that the PI3-kinase pathway in melanomas is activated by VEGFR1 but not VEGFR2.

Discussion

The expression of VEGF-A in malignant tumors is closely related to tumor progression and prognosis (Aoyagi *et al*. 2010; Martins *et al*. 2013). The biological functions of VEGF-A are exerted through its binding to two tyrosine kinase receptors, VEGFR1 and VEGFR2, expressed in vascular endothelial cells. VEGF-A plays an important role in tumor angiogenesis by enhancing the proliferation and motility of endothelial cells (Motwani and Eccles 2021). Several studies have shown that various cancer cells produce VEGF-A and express its receptors, VEGFR1 and/or VEGFR2 (von Marschall *et al*. 2000; Carrillo de Santa Pau *et al*. 2009; Hlobilkova *et al*. 2009; Sopo *et al*. 2019). Thus, VEGF-A could promote tumor development and progression by regulating the proliferation and motility of tumor cells in an autocrine manner as well as angiogenesis induction in a paracrine manner.

In the present study, we first examined the expression of VEGF-A, VEGFR1, and VEGFR2 in melanoma cells as well as their participation in the motility and proliferation of these cells. All melanoma cells tested secreted $VEGF_{165}$ into the culture media and expressed VEGFR1 and VEGFR2, suggesting that the $VEGF_{165}$ produced by melanoma cells might regulate the proliferation and motility of these cells in an autocrine manner. In addition, radio-receptor assay using [^{125} I]-labeled $VEGF_{165}$ confirmed that SK-MEL-28 cell line expressed high-affinity binding sites with a dissociation constant of 130 pM with 1300 binding sites per cell while the low-affinity sites with a dissociation constant of 4.1 nM with 20,000 binding sites per cell (data not shown). $VEGF_{165}$ also facilitated the motility of melanoma cells in both a chemotactic and chemokinetic manner, although it did not alter the proliferation of melanoma cells.

PlGF, which is about 40% homologous to VEGF-A at the amino acid level, binds specifically to VEGFR1 and induces various signaling pathways (Tammela *et al*. 2005; Shibuya 2006). In the current study, both PlGF and $VEGF_{165}$ enhanced the migration of melanoma cells, suggesting that the $VEGF_{165}$-induced migration of these cells is regulated via VEGFR1.

VEGFR1 and VEGFR2 belong to the receptor tyrosine kinase (RTK) subfamily and are known to induce the activation of several intracellular signaling molecules, including PI3K, Akt, Erk1/2, and p38 mitogen-activated protein kinase (MAPK), when they bind VEGF-A (Zhang *et al*. 2010; Szabo *et al*. 2016; Roskoski 2017). To determine whether the motility of melanoma cells is regulated by VEGFR1 or VEGFR2, the effects of inhibiting the tyrosine kinase activity of VEGFR1 or VEGFR2 on $VEGF_{165}$-induced cell motility were investigated. TKIs of both VEGFR1 and VEGFR2 suppressed the migration of melanoma cells induced by $VEGF_{165}$, although VEGFR2 TKI did not affect $VEGF_{165}$-induced cell migration. The neutralizing antibody against VEGFR1 also suppressed $VEGF_{165}$-induced cell migration. Furthermore, the transfection of an ASO targeting VEGFR1 markedly reduced the migration of melanoma cells, and the addition of $VEGF_{165}$ did not increase the migration of melanoma cells transfected with this VEGFR1-targeting ASO. However, the migration of melanoma cells transfected with a VEGFR2-targeting ASO was not suppressed. Additionally, $VEGF_{165}$ enhanced the migration of melanoma cells transfected with this VEGFR2-targeting ASO. Collectively, these findings suggest that the $VEGF_{165}$-induced migration of melanoma cells is mediated though signaling involving VEGFR1.

The MAPK pathway is a canonical signaling pathway triggered by several RTKs (McKay and Morrison 2007; Tarcic and Yarden 2010) including VEGF receptors (Yu and Sato 1999). Therefore, we investigated whether $VEGF_{165}$ induces the activation of the MAPK cascade in melanoma cells. We found that Erk is constitutively phosphorylated in SK-MEL-28 cells and $VEGF_{165}$ did not affect the phosphorylation of Erk, indicating that $VEGF_{165}$ is not involved in the MAPK pathway of melanoma cells. In addition to the MAPK cascade, the PI3K/Akt pathway is activated through RTKs (Matsuoka and Yashiro 2014; Mayer and Arteaga 2016; Nozhat and Hedayati 2016) including VEGF receptors (Yu and Sato 1999). In the melanoma cells tested in the present study, Akt was phosphorylated by $VEGF_{165}$. To clarify whether $VEGF_{165}$-induced Akt phosphorylation is regulated via VEGFR1 or VEGFR2, we investigated the effects of VEGFR1 and VEGFR2 TKIs on $VEGF_{165}$-induced Akt phosphorylation. VEGFR1/2 TKIs suppressed the induction of Akt phosphorylation by $VEGF_{165}$ in SK-MEL-28 cells, but the VEGFR2 TKI did not affect $VEGF_{165}$-induced Akt phosphorylation. These findings show that $VEGF_{165}$ induces phosphorylation of Akt via VEGFR1 in melanoma cells. We also examined the participation of PI3K in the $VEGF_{165}$-induced Akt phosphorylation of melanoma cells, finding that the PI3K inhibitor wortmannin suppressed $VEGF_{165}$-induced Akt phosphorylation in melanoma

cells. Wortmannin also suppressed the $VEGF_{165}$-induced migration of melanoma cells. These findings indicate that $VEGF_{165}$ promotes the migration of melanoma cells through the activation of PI3K/Akt signaling via VEGFR1. Using recombinant human VEGFR1 shows that PI3-kinase binds directly to phosphorylated tyrosine residue 1213, which resulted from an autophosphorylation event (Yu *et al*. 2001).

In conclusion, the melanoma cells examined in this study produced $VEGF_{165}$ and expressed RNAs encoding its receptors VEGFR1 and VEGFR2. However, these melanoma cell lines expressed VEGFR1 protein but not VEGFR2 protein. We found that $VEGF_{165}$ enhanced cell motility via VEGFR1 but not VEGFR2. Thus, the motility of melanoma cells may be regulated by a $VEGF_{165}$/VEGFR1-mediated autocrine signaling pathway. Moreover, we found that $VEGF_{165}$-induced melanoma cell motility is mediated by the PI3K/Akt pathway via VEGFR1. A survey of 167 melanoma specimens found that less than 10% of the tumors expressed VEGFR2, and they suggested that anti-VEGF proliferation therapy would not be an effective strategy for melanomas (Molhoek *et al*. 2011). Our results suggest that VEGF-A/VEGFR1 signaling could serve as a therapeutic target to prevent the invasion and metastasis of melanoma with inhibition of the associated signaling pathway being a therapeutic strategy to treat melanoma.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11626-022-00717-3.

Acknowledgements We would like to thank Drs. Masabumi Shibuya (Jobu University) and Kenya Shitara (Kyowa Hakko Kirin Co., Ltd.) for providing KM1570, a neutralizing antibody to VEGFR1.

Author contribution Conceptualization, T.O., Y.H., S.Y.; methodology, K.K., T.S., A.H., M.H., Y.Y., Y.H.; acquisition of data, K.K., T.S., Y.H., A.S.; statistical analysis, T.S., T.O., Y.H.; writing—review and editing, T.S., T.O., Y.H.; project administration, T.O.; funding acquisition, K.K., T.O.; supervision, T.O. All authors have read and agreed to the published version of the manuscript.

Funding Grants-in-Aid for Scientific Research (C) from the Japanese Ministry of Education, Culture, Sports, Science and Technology to K.K. (grant number: 24593033) and Grants-in-Aid for Scientific Research (B) to T.O. (grant number: 18H03000).

Data availability The data presented in this study are available on request from the corresponding author. Publicly available datasets were analyzed in this study.

Declarations

Ethics approval Not applicable.

Consent to participate Not applicable.

Conflict of interest The authors declare no competing interests.

References

Ahmed B, Qadir MI, Ghafoor S (2020) Malignant melanoma: skin cancer-diagnosis, prevention, and treatment. Crit Rev Eukaryot Gene Expr 30:291–297

Aoyagi Y, Iinuma H, Horiuchi A, Shimada R, Watanabe T (2010) Association of plasma VEGF-A, soluble VEGFR-1 and VEGFR-2 levels and clinical response and survival in advanced colorectal cancer patients receiving bevacizumab with modified FOLFOX6. Oncol Lett 1:253–259

Apte RS, Chen DS, Ferrara N (2019) VEGF in signaling and disease: beyond discovery and development. Cell 176:1248–1264

Borsotti P, Ghilardi C, Ostano P, Silini A, Dossi R, Pinessi D, Foglieni C, Scatolini M, Lacal PM, Ferrari R, Moscatelli D, Sangalli F, D'Atri S, Giavazzi R, Bani MR, Chiorino G, Taraboletti G (2015) Thrombospondin-1 is part of a Slug-independent motility and metastatic program in cutaneous melanoma, in association with VEGFR-1 and FGF-2. Pigment Cell Melanoma Res 28:73–81

Bravo-Cordero JJ, Hodgson L, Condeelis J (2012) Directed cell invasion and migration during metastasis. Curr Opin Cell Biol 24:277–283

de Santa Carrillo, Pau E, Arias FC, Caso Peláez E, Muñoz Molina GM, Sánchez Hernández I, Muguruza Trueba I, Moreno Balsalobre R, Sacristán López S, Gómez Pinillos A, del Val Toledo Lobo M (2009) Prognostic significance of the expression of vascular endothelial growth factors A, B, C, and D and their receptors R1, R2, and R3 in patients with nonsmall cell lung cancer. Cancer 115:1701–1712

Chen HC (2005) Boyden chamber assay. Methods Mol Biol 294:15–22

Clark AG, Vignjevic DM (2015) Modes of cancer cell invasion and the role of the microenvironment. Curr Opin Cell Biol 36:13–22

Dewerchin M, Carmeliet P (2012) PlGF: a multitasking cytokine with disease-restricted activity. Cold Spring Harb Perspect Med 2: a011056

Dvorak HF (2021) Reconciling VEGF with VPF: the importance of increased vascular permeability for stroma formation in tumors, healing wounds, and chronic inflammation. Front Cell Dev Biol 9:660609

Frank NY, Schatton T, Kim S, Zhan Q, Wilson BJ, Ma J, Saab KR, Osherov V, Widlund HR, Gasser M, Waaga-Gasser AM, Kupper TS, Murphy GF, Frank MH (2011) VEGFR-1 expressed by malignant melanoma-initiating cells is required for tumor growth. Cancer Res 71:1474–1485

Gitay-Goren H, Halaban R, Neufeld G (1993) Human melanoma cells but not normal melanocytes express vascular endothelial growth factor receptors. Biochem Biophys Res Commun 190:702–708

Graziani G, Ruffini F, Tentori L, Scimeca M, Dorio AS, Atzori MG, Failla CM, Morea V, Bonanno E, D'Atri S, Lacal PM (2016) Antitumor activity of a novel anti-vascular endothelial growth factor receptor-1 monoclonal antibody that does not interfere with ligand binding. Oncotarget 7:72868–72885

Guo X, Chen M, Cao L, Hu Y, Li X, Zhang Q, Ren Y, Wu X, Meng Z, Xu K (2021) Cancer-associated fibroblasts promote migration and invasion of non-small cell lung cancer cells via miR-101-3p mediated VEGFA secretion and AKT/eNOS pathway. Front Cell Dev Biol 9:764151

Hayashido Y, Hamana T, Ishida Y, Shintani T, Koizumi K, Okamoto T (2007) Induction of alpha2-antiplasmin inhibits E-cadherin processing mediated by the plasminogen activator/plasmin system, leading to suppression of progression of oral squamous cell carcinoma via upregulation of cell-cell adhesion. Oncol Rep 17:417–423

Hayashido Y, Lucas A, Rougeot C, Godyna S, Argraves WS, Rochefort H (1998) Estradiol and fibulin-1 inhibit motility of human ovarian- and breast-cancer cells induced by fibronectin. Int J Cancer 75:654–658

Hennequin LF, Thomas AP, Johnstone C, Stokes ES, Plé PA, Lohmann JJ, Ogilvie DJ, Dukes M, Wedge SR, Curwen JO, Kendrew J, Lambert-van der Brempt C (1999) Design and structure-activity relationship of a new class of potent VEGF receptor tyrosine kinase inhibitors. J Med Chem 42:5369–5389

Hlobilkova A, Ehrmann J, Knizetova P, Krejci V, Kalita O, Kolar Z (2009) Analysis of VEGF, Flt-1, Flk-1, nestin and MMP-9 in relation to astrocytoma pathogenesis and progression. Neoplasma 56:284–290

ICLAC Databases (2021) International Cell Line Authentification Committee. https://iclac.org/databases/

Iversen K, Robins RE (1980) Mucosal malignant melanomas. Am J Surg 139:660–664

Jia LQ, Osada M, Ishioka C, Gamo M, Ikawa S, Suzuki T, Shimodaira H, Niitani T, Kudo T, Akiyama M, Kimura N, Matsuo M, Mizusawa H, Tanaka N, Koyama H, Namba M, Kanamaru R, Kuroki T (1997) Screening the p53 status of human cell lines using a yeast functional assay. Mol Carcinog 19:243–253

Kasuga T, Nojiri I, Furuse K, Kubo E, Noda Y, Sugano H, Sakamoto G (1976) Establishment of human melanoma cell like (HMV-II). Proceedings of the Japanese Cancer Association 35th annual meeting

Lacal PM, Failla CM, Pagani E, Odorisio T, Schietroma C, Falcinelli S, Zambruno G, D'Atri S (2000) Human melanoma cells secrete and respond to placenta growth factor and vascular endothelial growth factor. J Invest Dermatol 115:1000–1007

Lacal PM, Graziani G (2018) Therapeutic implication of vascular endothelial growth factor receptor-1 (VEGFR-1) targeting in cancer cells and tumor microenvironment by competitive and non-competitive inhibitors. Pharmacol Res 136:97–107

Martins SF, Garcia EA, Luz MA, Pardal F, Rodrigues M, Filho AL (2013) Clinicopathological correlation and prognostic significance of VEGF-A, VEGF-C, VEGFR-2 and VEGFR-3 expression in colorectal cancer. Cancer Genomics Proteomics 10:55–67

Matsuoka T, Yashiro M (2014) The role of PI3K/Akt/mTOR signaling in gastric carcinoma. Cancers (basel) 6:1441–1463

Mayer IA, Arteaga CL (2016) The PI3K/AKT pathway as a target for cancer treatment. Annu Rev Med 67:11–28

McKay MM, Morrison DK (2007) Integrating signals from RTKs to ERK/MAPK. Oncogene 26:3113–3121

Molhoek KR, Erdag G, Rasamny JK, Murphy C, Deacon D, Patterson JW, Slingluff CL Jr, Brautigan DL (2011) VEGFR-2 expression in human melanoma: revised assessment. Int J Cancer 129:2807–2815

Motwani J, Eccles MR (2021) Genetic and genomic pathways of melanoma development, invasion and metastasis. Genes (Basel) 12:1543

Myoken Y, Kayada Y, Okamoto T, Kan M, Sato GH, Sato JD (1991) Vascular endothelial cell growth factor (VEGF) produced by A-431 human epidermoid carcinoma cells and identification of VEGF membrane binding sites. Proc Natl Acad Sci U S A 88:5819–5823

Nozhat Z, Hedayati M (2016) PI3K/AKT pathway and its mediators in thyroid carcinomas. Mol Diagn Ther 20:13–26

Okamoto T, Tanaka Y, Kan M, Sakamoto A, Takada K, Sato JD (1996) Expression of fibroblast growth factor binding protein HBp17 in normal and tumor cells. In Vitro Cell Dev Biol Anim 32:69–71

Pasquali S, Hadjinicolaou AV, ChiarionSileni V, Rossi CR, Mocellin S (2018) Systemic treatments for metastatic cutaneous melanoma. Cochrane Database Syst Rev 2:Cd011123

Peebles PT, Trisch T, Papageorge AG (1978) Isolation of four unusual pediatric solid tumor cell lines. Pediatr Res 12:485

Roskoski R Jr (2017) Vascular endothelial growth factor (VEGF) and VEGF receptor inhibitors in the treatment of renal cell carcinomas. Pharmacol Res 120:116–132

Sato JD, Kawamoto T, Okamoto T (1987) Cholesterol requirement of P3–X63-Ag8 and X63-Ag8.653 mouse myeloma cells for growth in vitro. J Exp Med 165:1761–1766

Shibuya M (2006) Vascular endothelial growth factor receptor-1 (VEGFR-1/Flt-1): a dual regulator for angiogenesis. Angiogenesis 9:225–230

Shibuya M (2011) Vascular endothelial growth factor (VEGF) and its receptor (VEGFR) signaling in angiogenesis: a crucial target for anti- and pro-angiogenic therapies. Genes Cancer 2:1097–1105

Shiku H, Takahashi T, Oettgen HF (1976) Cell surface antigens of human malignant melanoma. II. Serological typing with immune adherence assays and definition of two new surface antigens. J Exp Med 144:873–881

Sopo M, Anttila M, Hämäläinen K, Kivelä A, Ylä-Herttuala S, Kosma VM, Keski-Nisula L, Sallinen H (2019) Expression profiles of VEGF-A, VEGF-D and VEGFR1 are higher in distant metastases than in matched primary high grade epithelial ovarian cancer. BMC Cancer 19:584

Streit M, Detmar M (2003) Angiogenesis, lymphangiogenesis, and melanoma metastasis. Oncogene 22:3172–3179

Szabo E, Schneider H, Seystahl K, Rushing EJ, Herting F, Weidner KM, Weller M (2016) Autocrine VEGFR1 and VEGFR2 signaling promotes survival in human glioblastoma models in vitro and in vivo. Neuro Oncol 18:1242–1252

Tammela T, Enholm B, Alitalo K, Paavonen K (2005) The biology of vascular endothelial growth factors. Cardiovasc Res 65:550–563

Tarcic G, Yarden Y (2010) MAP Kinase activation by receptor tyrosine kinases: in control of cell migration. Methods Mol Biol 661:125–135

Vaisman N, Gospodarowicz D, Neufeld G (1990) Characterization of the receptors for vascular endothelial growth factor. J Biol Chem 265:19461–19466

von Marschall Z, Cramer T, Höcker M, Burde R, Plath T, Schirner M, Heidenreich R, Breier G, Riecken EO, Wiedenmann B, Rosewicz S (2000) De novo expression of vascular endothelial growth factor in human pancreatic cancer: evidence for an autocrine mitogenic loop. Gastroenterology 119:1358–1372

Yde SS, Sjoegren P, Heje M, Stolle LB (2018) Mucosal melanoma: a literature review. Curr Oncol Rep 20:28

Yu Y, Sato JD (1999) MAP kinases, phosphatidylinositol 3-kinase, and p70 S6 kinase mediate the mitogenic response of human endothelial cells to vascular endothelial growth factor. J Cell Physiol 178:235–246

Yu Y, Hulmes JD, Herley MT, Whitney RG, Crabb JW, Sato JD (2001) Direct identification of a major autophosphorylation site on vascular endothelial growth factor receptor Flt-1 that mediates phosphatidylinositol 3'-kinase binding. Biochem J 358:465–472

Zhang Z, Neiva KG, Lingen MW, Ellis LM, Nör JE (2010) VEGF-dependent tumor angiogenesis requires inverse and reciprocal regulation of VEGFR1 and VEGFR2. Cell Death Differ 17:499–512