Supporting Information

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SI Materials and Methods

Animals. C57BL/6 and SCID male 6- to 8-wk-old mice were purchased from the animal facility of the Department of Microbiology, Tumor and Cell Biology at Karolinska Institute. *Vegfr1 tk*$^{-/-}$ mice were kindly provided by Masabumi Shibuya, Tokyo University, Tokyo. Zebrafish embryos of the Tg(*fli1:EGFP*)y1 strain (Zebrafish Model Organism Database) were used for the tumor cell dissemination assay. All animal studies were approved by the North Stockholm Experimental Animal Ethical Committee.

Cell Culture. Various transfected and nontransfected tumor cell lines were cultured in DMEM (HyClone; catalog no. SH30243.01) supplemented with 10% (vol/vol) heat-inactivated FBS (HyClone; catalog no. SH30160.03), 100 U/mL penicillin, and 100 μg/mL streptomycin (HyClone; catalog no. SV30010). The 528ras cell line was provided by Janusz Rak, McGill University, Montreal, and the other tumor cell lines were bought from ATCC.

Antibodies. Antibodies used in this study include rat anti-mouse CD31 antibody (BD-Pharmingen; catalog no. 553370); rabbit anti-mouse NG2 antibody (Millipore; catalog no. AB5320); mouse anti-human/mouse α-SMA antibody (Dako; clone: 1A4); rat anti-mouse CD206 antibody (BioLegend; catalog no. 141702); goat anti-mouse CD31 antibody (R&D Inc.; catalog no. AF3628); rabbit anti-mouse VE-cadherin antibody (Abcam; catalog no. 33168); rabbit anti-mouse collagen IV antibody (Abcam; catalog no. 19808); rabbit anti-mouse fibronectin antibody (Abcam; catalog no. 23750); rabbit anti-RFP antibody (Invitrogen; catalog no. R10367); rabbit anti-mouse cleaved caspase-3 (Asp175) antibody (Cell Signaling; catalog no. 9661); Alexa Fluor-647–conjugated anti-mouse F4/80 antibody (BioLegend; catalog no. 123121); Alexa Fluor-488–conjugated anti-mouse CD206 antibody (BioLegend; catalog no. 141709); FITC-conjugated mouse anti-pimonidazole adducts antibody (Hypoxyprobe; catalog no. HP2-1000 kit); Cy5-conjugated goat anti-rat antibody (Chemi-Con; catalog no. AP183S); Alexa-555–conjugated donkey anti-goat antibody (Life Technologies; catalog no. A21432); Alexa-488–conjugated donkey anti-rabbit antibody (Life Technologies; catalog no. A21206) Cy5-conjugated goat anti-rabbit antibody (Chemi-Con; catalog no. AP132S); and Alexa Fluor-488–conjugated donkey anti-rat antibody (Invitrogen; catalog no. A21202).

Mouse Tumor Models. Cultured tumor cells were suspended at concentrations of 1×10^7/mL or 1×10^6 cells in 100 μL of PBS and were s.c. injected into the dorsal back along the midline of each mouse. Tumor sizes were measured with a caliper every other day and were calculated according to a standard formula (length × width squared × 0.52). A rabbit anti-mouse VEGF-A–specific neutralizing antibody and a nonimmune rabbit IgG were i.p. administered two times per week at a dose of 2.5 mg/kg starting at day 1 after tumor injection. At the end of the experiments, mice were killed by cervical dislocation, and tumor tissues were fixed immediately with 4% (wt/vol) paraformaldehyde (PFA) at 4 °C overnight or were freshly frozen in a −80 °C freezer until further use.

Hypoxia Probe Labeling, Vascular Permeability, and Blood Perfusion. The hypoxia probe pimonidazole hydrochloride (Hypoxyprobe; catalog no. HP2-1000 kit) was i.v. injected through the tail vein at a dose of 60 mg/kg. The injected mice were killed 30 min later, and tumor tissues were dissected and then were immediately fixed in 4% (wt/vol) PFA and frozen at −70 °C until further use. Some PFA-fixed samples were paraffin embedded. The fluorescein-labeled 70-kDa and 2,000-kDa fixable dextran molecules (Invitrogen; catalog no. D1818 and D7139) were i.v. injected into each of the tumor-bearing mice, which were killed 15 min (for 70-kDa dextran) or 5 min (for 2,000-kDa dextran) after injection. Tumors were dissected and fixed with 4% (wt/vol) PFA immediately after tumor removal.

Metastasis. At the size of 1.5 cm^3, primary tumors were removed surgically, and the incisions were sutured under anesthesia. For pain relief, operated mice received Temgesic (RB Pharmaceuticals) (0.1 mg/kg) twice daily for 2 d. Treatments with neutralizing antibodies were terminated after primary tumor removal. Mice bearing T241 and MDA-MB-435 tumors were killed at the end of week 6 for the detection of metastasis. Mice bearing VEGF-A–null and UACC-62 tumors were kept for additional 2 wk before being killed. Organs and tissues including lung, axillary lymph nodes, liver, spleen, and kidney were examined for RFP$^+$ tumor metastases by gross examination and fluorescence microscope (Nikon Eclipse 90i).

Zebrafish Model of Tumor Cell Dissemination. At 24 h postfertilization (hpf), transgenic *Fli1:EGFP* zebrafish embryos were incubated with aquarium water containing 0.2 mM 1-phenyl-2-thio-urea (PTU; Sigma-Aldrich; catalog no. P7629) to prevent pigmentation. Zebrafish embryos were dechorionated at 48 hpf and anesthetized with 0.04 mg/mL of tricaine (MS-222; Sigma-Aldrich; catalog no. E10521). Embryos were transferred to an agarose gel mode to receive microinjection. Approximately 300–500 tumor cells labeled with DiI (Invitrogen; catalog no. D3899) were resuspended in serum-free DMEM, followed by injection into the perivitelline space of each embryo with an Eppendorf microinjector (FemtoJet 5247; Eppendorf; catalog no. 5247000.013). Nonfilamentous borosilicate glass capillary needles (1.0 mm in diameter; World Precision Instruments; catalog no. 1B100-4) were used for microinjection. The zebrafish embryos were transferred immediately to the PTU-containing water after injection and were kept at 28 °C for the indicated time points. Tumor growth and invasion were examined at days 0 and 4 using a fluorescent microscope. Disseminated tumor cells per embryo were quantified in 10 zebrafish embryos per group.

Whole-Mount Staining. Tumor whole-mount staining was performed as described previously (1–4). In brief, fresh tumor tissues were fixed with 4% (wt/vol) PFA at 4 °C overnight, followed by washing with PBS. Tissues were cut into small, thin pieces by scalpel, digested with proteinase K (20 mM in Tris buffer, pH 7.5) for 5 min, permeabilized with 100% (vol/vol) methanol for 30 min, blocked in 3% (wt/vol) milk in 0.3% Triton X-100 (Sigma-Aldrich; catalog no. X100) in PBS, and incubated with primary antibodies at 4 °C overnight. The primary antibody-stained tumor tissues were blocked further with 3% (wt/vol) milk, followed by incubation for 2 h with fluorescein-labeled secondary antibodies at room temperature. Tissues were washed thoroughly with PBS at 4 °C overnight before mounting in Vectashield mounting medium (Vector Laboratories; catalog no. H-1000), and then were stored at −20 °C in the dark until examination under a confocal microscope. Consecutive scannings of seven layers of each tissue sample were assembled to constitute a 3D-image dataset using a software program (Nikon, EZ-C1) analysis. Quantitative analyses were performed from the data of at least 24 different random fields (eight tumors; three random fields per tumor) of each tissue sample using an Adobe Photoshop CS software program.

Immunofluorescent and H&E Staining. For immunofluorescent staining, the PFA-fixed tissues were paraffin embedded and cut into 5-μm

thicknesses. Tissue slides were deparaffinized and hydrated by immersion in Tissue-Clear (Sakura; catalog no. 1466) and ethanol. The tissues were boiled for 15 min in an unmasking solution (Vector Labs; catalog nos. H3301 and H3300) to retrieve antigen. The samples were washed three times with PBS, followed by blocking with 4% (vol/vol) nonimmune goat (Vector; catalog no. S-1000) or donkey serum (Abcam; catalog no. ab166643), and were stained overnight at 4 °C with various primary antibodies. Tissue samples were incubated at room temperature for 30 min with species-matching fluorescein-labeled secondary antibodies. Tissue samples were mounted in Vectashield mounting medium and stored at −20 °C in the dark. For VE-cadherin staining, frozen tumor sections (5-μm thickness) were fixed with cold 100% (vol/vol) acetone for 10 min, followed by washing with PBS. The tissue samples were incubated with the primary antibody overnight at 4 °C. After rigorous washing, an Alexa-488–conjugated donkey anti-rabbit secondary antibody was incubated at room temperature for 45 min. Fluorescent signals were examined using a fluorescence microscope. For H&E staining, paraffin-embedded tissues were cut into thin (5-μm) sections. After deparaffinization and hydration, tissues were stained with hematoxylin (Sigma-Aldrich; catalog no. MHS16-500ML), rinsed with water for 10 min, and counterstained with rosin (Sigma-Aldrich; catalog no. HT110116-500ML). Stained tissues were examined under a light microscope (Nikon Eclipse TS100).

FACS Analysis. To detect CTCs, 300 μL of heparinized fresh blood from each animal was collected by heart puncture at the primary tumor size of 1.5 cm^3. RBCs were lysed with a lysis buffer (eBioscience; catalog no. 00-4333-57) and removed by centrifuging at 130 × *g* for 5 min. The remaining cell fractions were fixed with 4% (wt/vol) PFA for 15 min, followed by permeabilizing for 3 min with 0.15% Triton X-100. Cells were incubated for 1 h at 4 °C with a rabbit anti-RFP primary antibody, followed by washing with PBS. Samples were incubated at 4 °C for 30 min with a Cy5-conjugated anti-rabbit secondary antibody. For macrophage detection, fresh tumor tissues were cut into small (~1 mm^3) pieces and were digested at 37 °C for 1 h with a combination of 0.15% type I collagenase (Sigma-Aldrich; catalog no. C0130-500MG) and 0.15% type II collagenase (Sigma-Aldrich; catalog no. C6885-1G). Samples were filtered through a 40-μm cell strainer to obtain the single-cell suspension. Cells were counted, and the suspension was adjusted to a final cell density of 1×10^7 cells/mL and incubated at 4 °C for 1 h with a mixture of an Alexa Fluor-647–conjugated anti-mouse F4/80 antibody and an Alexa Fluor-488–conjugated anti-mouse CD206 antibody. The stained samples were scanned on a FACScans (Becton Dickinson) and analyzed with CellQuestPro software (BD Biosciences).

ELISA and Western Blotting. VEGF-B protein was detected using commercially available ELISA kits (Mybiosource; catalog nos. MBS268048 and MBS704262). ELISAs were performed according to the manufacturer's instructions. Each experiment was calibrated with the standard amounts of known proteins, and a linear concentration-dependent curve was established before measurement of samples. Each sample was used in triplicate, and the experiments were repeated at least three times. To detect the hypoxia level in tumor tissue by immunoblotting, total tissue proteins were extracted with a CelLytic MT cell lysis reagent (Sigma-Aldrich; catalog no. C3228) from pimonidazole hydrochloride-perfused tumor tissues. In each sample the same amount of proteins was subjected to SDS/PAGE (Invitrogen; catalog no. NP0321/NP0323), and the gel was transferred onto nitrocellulose membranes (Thermo Scientific; catalog no. 88018). The membranes were blocked at room temperature for 1 h with 5% (wt/vol) BSA (Sigma-Aldrich; catalog no. A7030-100MG), followed by incubation at 4 °C overnight with an anti-pimonidazole antibody (Hypoxyprobe; catalog no. HP2-1000 kit). Membranes were incubated with an anti-mouse secondary antibody conjugated with IRDye 800 (LI-COR; catalog no. 926-32212; 1:15,000 dilution). Stained membranes were visualized and quantified using an Odyssey CLx detection system (LI-COR).

Stable Expression of VEGF-B. The full-length cDNA sequence coding for human *VEGFB* was cloned into a pLVX-IRES-tdTomato lentivirus vector (Clontech; catalog no. 631238). Lentiviral particles were packaged by transfecting the *VEGFB* vector or control vector into 293T cells with a Lenti-X HTX Packaging Mix (Clontech; catalog no. 631247). The lentiviral supernatant was collected by centrifuging the cultured medium at 500 × *g* for 10 min. Transduction was performed by adding 80% (vol/vol) lentiviral supernatant into the culture medium of subconfluent T241 VEGF-A–null and UACC-62 cells. Medium was replaced with fresh medium 72 h after infection. RFP^+ (tdTomato) cells were sorted by FACS (FACSVantage/DiVa, BD Bioscience).

***VEGFB*-shRNA and Quantitative PCR.** Human MDA-MB-435 cells were used as target cells for the VEGF-B knockdown experiment. The packaging 293T cells were used to prepare viral particles that carry different shRNA constructs (GeneCopoeia) encoding mCherry and a puromycin resistance gene that subsequently were incubated with target cells for 24 h. Cells then were subjected to puromycin selection, followed by FACS sorting for selection of the brightest RFP population. Knockdown efficiency was validated on mRNA and protein levels by quantitative PCR (qPCR) and ELISA. For the real-time qPCR assay, total RNAs from cells were extracted using a 2-mercaptoethanol–supplemented (Sigma-Aldrich; catalog no. 3148) lysis buffer supplied in a RNA extraction kit (Thermo Scientific; catalog no. K0732). Ethanol was added to the lysates before purification thorough a column according to the manufacturer's protocol. The total amount of RNA was measured and used for reverse transcription (Thermo Scientific; catalog no. K1632) to obtain cDNA for subsequent qPCR analysis. qPCR was performed with a Power SYBR Green master mix (Life Technologies; catalog no. 4367659) reaction in a Step-One Plus machine with cycles according to the manufacturer's instructions (Applied Biosystems). The following primers were used to detect specific mRNA expression: human *VEGFB* forward primer: 5′-AGTGCTGTGAAGCCAGACA-3′; reverse primer: 5′-GGAGTGGGATGGGTGATG-3′; human GAPDH forward primer: 5′-CATTTCCTGGTATGACAACGA-3′; and reverse primer: 5′-GTCTACATGGCAACTGTGAG-3′. GAPDH was used as the internal control, and all data are presented as relative quantification.

TUNEL Staining. Apoptotic cells in tumor tissue were detected by an in situ cell-death detection kit (Roche; catalog no. 11684795910) according to the manufacturer's protocol. After staining, the mounted samples were examined with a fluorescent microscope, and at least 24 random areas were measured for each group using a Photoshop software program (Adobe).

Correlation of VEGF-B Expression in Human Cancer Patients. Survival data from the Cancer Genome Atlas (cancergenome.nih.gov/) of 411 patients with LSCC and 415 patients with skin cutaneous melanoma were analyzed for the VEGF-B–high (above median) and VEGF-B–low (below median) groups. The statistical difference was analyzed using the Kaplan–Meier survival method followed by log-rank test.

Statistic Analysis. Data were analyzed quantitatively as follows: three randomized fields of eight independent samples per group for quantification of immunohistochemistry staining image; tumors in 20 mice per group for drawing the tumor growth curve; 10 zebrafish per group for the tumor cell metastasis assay; and 12 samples per group for FACS data quantification. The Adobe Photoshop CS4 software

program was used to detect positive areas and to count or to measure the length with a color range tool, a count tool, and a ruler tool. Statistical differences between two groups were analyzed using the two-tailed Student *t*-test. Statistical *P* values were defined as follows: $P < 0.05$ = significant; $P < 0.01$ = highly significant; and $P < 0.001$ = extremely significant. Data are presented as means ± SEM.

1. Ji H, et al. (2014) TNFR1 mediates TNF-α-induced tumour lymphangiogenesis and metastasis by modulating VEGF-C-VEGFR3 signalling. *Nat Commun* 5:4944.
2. Xue Y, et al. (2012) PDGF-BB modulates hematopoiesis and tumor angiogenesis by inducing erythropoietin production in stromal cells. *Nat Med* 18(1):100–110.
3. Xue Y, et al. (2009) Hypoxia-independent angiogenesis in adipose tissues during cold acclimation. *Cell Metab* 9(1):99–109.
4. Dong M, et al. (2013) Cold exposure promotes atherosclerotic plaque growth and instability via UCP1-dependent lipolysis. *Cell Metab* 18(1):118–129.

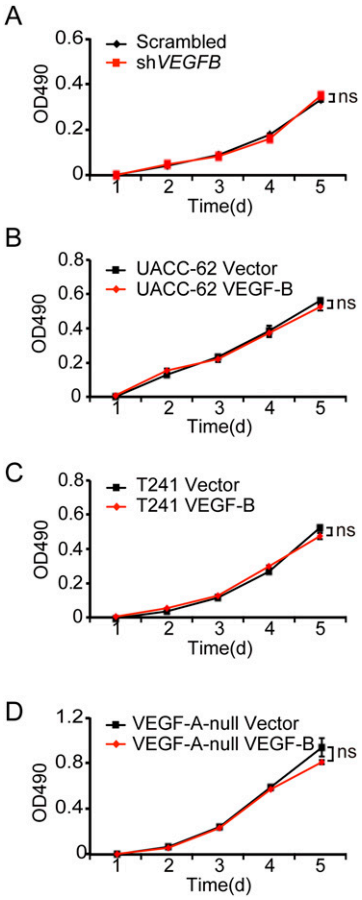

Fig. S1. Tumor-cell proliferation rates in vitro. (*A*) Proliferation curve of scrambled-shRNA- and *VEGFB*-shRNA-MDA-MB-435 cell lines in vitro ($n = 6$ samples per group). (*B*) Proliferation curve of vector- and VEGF-B-UACC-62 cell lines in vitro ($n = 6$ samples per group). (*C*) Proliferation curve of vector- and VEGF-B-T241 cell lines in vitro ($n = 6$ samples per group). (*D*) Proliferation curve of vector- and VEGF-B-528ras cell lines in vitro ($n = 6$ samples per group). All error bars represent SEM. All *P* values were analyzed according to Student's *t* test. ns, not significant.

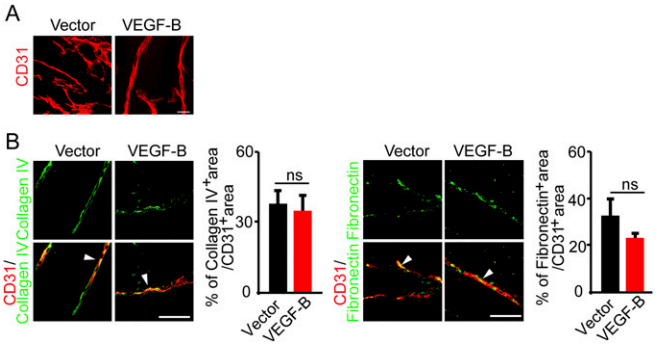

Fig. S2. Tumor vasculature of vector- and VEGF-B-UACC-62 melanomas. (*A*) Vector and VEGF-B tumor tissues were stained with CD31 and analyzed by confocal microscopy. (Scale bar, 50 μm.) (*B*) CD31 (red) and collagen IV (green) (*Left*) or fibronectin (green) (*Right*) double immunostaining of vector- and VEGF-B-UACC-62 melanomas. Arrowheads point to double immunopositive signals (yellow). (Scale bar, 50 μm.) Bar charts show quantification of collagen IV- or fibronectin-positive signals in tumor vessels (n = 7–10 random fields per group).

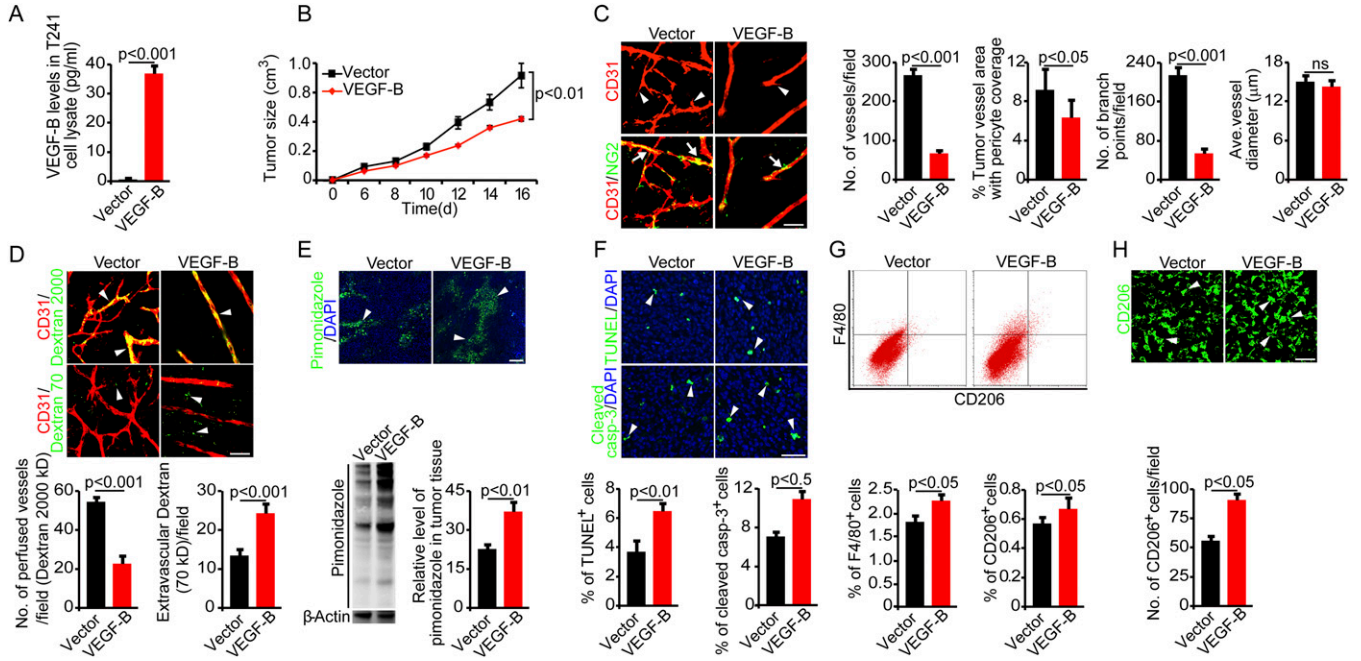

Fig. S3. Gain of VEGF-B function inhibits tumor growth by altering angiogenesis and vascular functions in mouse tumors. (*A*) Expression levels of VEGF-B protein in T241 fibrosarcoma tumor cells. (*B*) Tumor growth curves of vector- and VEGF-B-T241 tumors (n = 20 mice per group). (*C*, *Left*) Vasculature of vector- and VEGF-B-T241 tumors. Arrowheads point to vascular branches, and arrows indicate pericyte coverage. (Scale bar, 50 μm.) (*Right*) Vessel numbers, pericyte coverage, branch points, and diameters of tumor vessels were quantified (n = 24 random fields per group). (*D*, *Upper*) Blood perfusion (2,000-kDa dextran) and vascular permeability (70-kDa dextran) of vector- and VEGF-B-T241 tumors. Arrowheads indicate perfused or leaked dextran signals. (Scale bar, 50 μm.) (*Lower*) Quantification of blood perfusion and leakiness (n = 24 random fields per group). (*E*) Measurement of tissue hypoxia of vector- and VEGF-B-T241 tumors. Immunofluorescent staining (*Upper*) and Western blotting (*Lower Left*) were used to detect the hypoxia probe pimonidazole. Arrowheads indicate pimonidazole-positive signals. (Scale bar, 100 μm.) (*Lower Right*) The bar chart shows quantification of the pimonidazole signal (n = 4 samples per group). (*F*, *Upper*) Measurement of cellular apoptosis by TUNEL and cleaved caspase-3 of vector- and VEGF-B-T241 tumors. Arrowheads indicate apoptotic cells. (Scale bar, 50 μm.) (*Lower*) Quantification data are presented as percentages of apoptotic cells versus total DAPI$^+$ cells (n = 24 random fields per group). (*G*, *Upper*) FACS measurement of F4/80$^+$ and CD206$^+$ macrophages of vector- and VEGF-B-T241 tumors. (*Lower*) Quantification is presented as the percentage of positive signals versus the total gated events (n = 12 samples per group). (*H*, *Upper*) Immunohistochemical analysis of CD206$^+$ macrophages of vector- and VEGF-B-T241 tumors (n = 24 fields per group). Arrowheads indicate CD206$^+$ macrophages. (Scale bar, 50 μm.) (*Lower*) Quantification of the number of CD206$^+$ cells per field. All error bars represent SEM. All P values were analyzed according to Student's t test. ns, not significant.

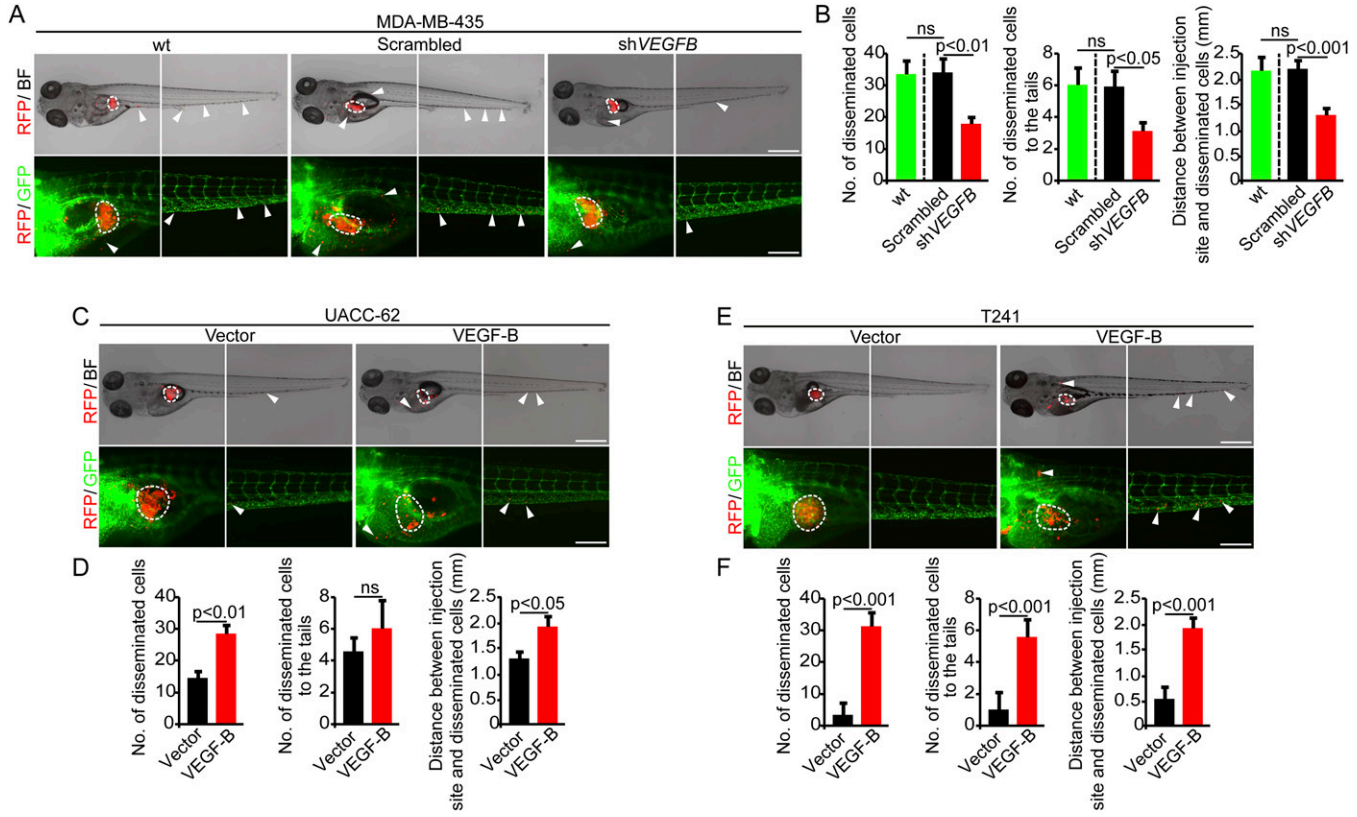

Fig. S4. VEGF-B promotes cancer metastasis in a zebrafish model. (*A*) Dissemination of DiI-labeled human nontransfected WT, scrambled-shRNA-, and *VEGFB*-shRNA-MDA-MB-435 melanoma cells in zebrafish. Dashed lines encircle primary tumor sites. Arrowheads indicate the disseminated tumor cells. [Scale bar, 500 μm (*Upper*); 200 μm (*Lower*).] (*B*) Quantification of disseminated WT, scrambled-shRNA-, and *VEGFB*-shRNA-MDA-MB-435 melanoma cells in the whole body of zebrafish embryos (n = 10 zebrafish embryos per group). (*C*) Dissemination of DiI-labeled human vector- and VEGF-B-UACC-62 melanoma cells in zebrafish. Dashed lines encircle primary tumor sites. Arrowheads indicate the disseminated tumor cells. [Scale bar, 500 μm (*Upper*); 200 μm (*Lower*).] (*D*) Quantification of disseminated vector- and VEGF-B-UACC-62 melanoma cells in the whole body of zebrafish embryos (n = 10 zebrafish embryos per group). (*E*) Dissemination of DiI-labeled vector- and VEGF-B-T241 tumor cells in zebrafish. Dashed lines encircle primary tumor sites. Arrowheads indicate the disseminated tumor cells. [Scale bar, 500 μm (*Upper*); 200 μm (*Lower*).] (*F*) Quantification of disseminated vector- and VEGF-B-T241 tumor cells in the whole body of zebrafish embryos (n = 10 zebrafish embryos per group). All error bars represent SEM. All *P* values were analyzed according to Student's *t* test. ns, not significant.

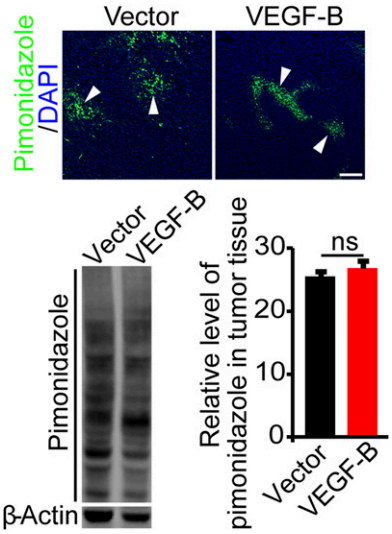

Fig. S5. Measurement of tissue hypoxia in VEGF-A–null tumors. Measurement of tissue hypoxia in vector- and VEGF-B-VEGF-A–null tumors. Immunofluorescent staining (*Upper*) and Western blotting (*Lower Left*) were used to detect the hypoxia probe pimonidazole. Arrowheads indicate the hypoxic area with pimonidazole-positive signals. (Scale bar, 100 μm.) (*Lower Right*) The bar chart shows the quantification of the pimonidazole signal (n = 3 samples per group). All error bars represent SEM. All *P* values were analyzed according to Student's *t* test. ns, not significant.

Table S1. *VEGFB* expression levels in various human cancer cell lines

Cell line	Relative mRNA level
Melanoma	
UACC-62	1.002 ± 0.027
UACC-257	8.089 ± 0.377
SK-MEL-2	0.689 ± 0.008
MAL-MEL-3M	2.119 ± 0.018
MDA-MB-435	5.137 ± 0.178
MUM 2C	1.211 ± 0.006
A375	0.882 ± 0.030
A375 R4	1.297 ± 0.005
BL	1.506 ± 0.012
MEL505	0.631 ± 0.046
Ovarian cancer	
OVCAR 3	4.465 ± 0.251
OVCAR 4	1.778 ± 0.119
OVCAR 8	1.758 ± 0.007
IGROV-1	1.260 ± 0.021
Breast cancer	
MDA-MB-231	1.051 ± 0.039
MCF7	0.570 ± 0.001
Renal cell carcinoma	
A498	0.752 ± 0.001
768/0	0.338 ± 0.015
Colorectal cancer	
HT29	1.945 ± 0.030
Nonsmall cell lung carcinoma	
H226	1.220 ± 0.031
Retinoblastoma	
RB355	2.550 ± 0.033
Glioblastoma	
U251 MG	1.662 ± 0.007
U-87 MG	0.650 ± 0.046
Neuroblastoma	
IMR-32	0.313 ± 0.000
Liposarcoma	
SW872	2.103 ± 0.337
Prostatic cancer	
DU145	1.477 ± 0.033
Cervical cancer	
HeLa	1.834 ± 0.034
Epidermoid cancer	
A431	0.265 ± 0.003

qRT-PCR was used to define *VEGFB* mRNA levels in various human cancer cell lines. The data are represented as mean ± SEM. A triplicate sample was analyzed in each cell line.