Cancer Research

VEGFR-1 Expressed by Malignant Melanoma-Initiating Cells Is Required for Tumor Growth

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Abstract

Melanoma growth is driven by malignant melanoma-initiating cells (MMIC) identified by expression of the ATP-binding cassette (ABC) member ABCB5. ABCB5$^+$ melanoma subpopulations have been shown to overexpress the vasculogenic differentiation markers CD144 (VE-cadherin) and TIE1 and are associated with CD31$^-$ vasculogenic mimicry (VM), an established biomarker associated with increased patient mortality. Here we identify a critical role for VEGFR-1 signaling in ABCB5$^+$ MMIC-dependent VM and tumor growth. Global gene expression analyses, validated by mRNA and protein determinations, revealed preferential expression of VEGFR-1 on ABCB5$^+$ tumor cells purified from clinical melanomas and established melanoma lines. *In vitro*, VEGF induced the expression of CD144 in ABCB5$^+$ subpopulations that constitutively expressed VEGFR-1 but not in ABCB5$^-$ bulk populations that were predominantly VEGFR-1$^-$. *In vivo*, melanoma-specific shRNA-mediated knockdown of VEGFR-1 blocked the development of ABCB5$^+$ VM morphology and inhibited ABCB5$^+$ VM-associated production of the secreted melanoma mitogen laminin. Moreover, melanoma-specific VEGFR-1 knockdown markedly inhibited tumor growth (by >90%). Our results show that VEGFR-1 function in MMIC regulates VM and associated laminin production and show that this function represents one mechanism through which MMICs promote tumor growth. *Cancer Res; 71(4); 1474–85. ©2011 AACR.*

Introduction

Human malignant melanoma is a highly aggressive and drug-resistant cancer that is usually refractory to systemic therapy. Identification of undifferentiated subpopulations with embryonic-like plasticity within this malignancy has pointed to the existence of aggressive cell subsets that may be responsible for melanoma initiation, tumor progression, and resistance to chemotherapy (1–4). Recently our laboratory identified tumorigenic malignant melanoma-initiating cells (MMIC) capable of self-renewal and differentiation that can be prospectively enriched on the basis of preferential expression of the ATP-binding cassette (ABC) member ABCB5 (5–8), a chemoresistance gene (5, 9, 10). ABCB5$^+$ tumor cells, which were found to range in frequency from 1.6% to 20.4% in human melanomas (7), correlate with malignant disease initiation and metastatic progression in tumor xenotransplantation models involving either NOD/SCID or NOD/SCID interleukin-2 receptor (IL-2R) γ-chain null (NSG) murine recipients (7, 11, 12) and in clinical studies of human melanoma patients (7, 13–15) according to results from several laboratories. Consistent with these findings, the *ABCB5* gene is also preferentially expressed by *in vitro* self-renewing, clonogenic melanoma subpopulations (16), melanoma cell lines of metastatic as opposed to primary tumor origin (17), and melanomas with high *in vivo* tumorigenic capacity in human to murine xenotransplantation models (18, 19). Furthermore, ABCB5 is downregulated in human melanoma cells upon induction of terminal differentiation (20). Importantly, ABCB5$^+$ melanoma cells can be therapeutically targeted in experimental tumor xenotransplantation models, with specific killing of this tumor subpopulation resulting in inhibition of tumor growth (7).

MMIC-enriched ABCB5$^+$ melanoma subpopulations trigger tumorigenesis and promote neoplastic progression through enhanced self-renewal and proliferative capacity (7). Preferential evasion of host antitumor immunity, a determinant of tumor growth (4, 21–25), represents an additional mechanism

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responsible for the enhanced tumorigenicity of ABCB5$^+$ melanoma subpopulations (8). Tumor functions relevant to the generation of a protumorigenic microenvironment through differentiation (4, 26) could represent additional mechanisms for the preferential tumorigenicity of ABCB5$^+$ melanoma subpopulations. In this regard, we recently found that ABCB5$^+$ human melanoma cells are specifically associated with vasculogenic mimicry (VM), a phenomenon whereby more primitive and aggressive melanoma cells express endothelial genes and related proteins [TIE1 and CD144 (VE-cadherin), but not CD31] and *in situ* develop patterned networks composed of periodic acid–Schiff (PAS) and laminin-reactive basement membranes lined by tumor cells (27). Our previous results showed that subpopulations of ABCB5$^+$ human melanoma cells preferentially express the vasculogenic differentiation markers TIE1 and CD144 (7). Expression of TIE1 and CD144 by ABCB5$^+$ melanoma subpopulations was confirmed by gene and protein expression analyses of genetically tracked fluorescent melanoma xenografts (7). Furthermore, human tumor cells that coexpressed ABCB5 and TIE1/CD144 were distinct from mature CD31$^+$ tumor endothelium in clinical patient tumors and experimental melanomas in serial xenotransplantation experiments (7). Although it remains currently unknown whether VM might represent, in addition to conventional angiogenesis, a related yet independent mechanism of tumor perfusion, it has been established that VM characterized by expression of TIE1 and CD144 relates to melanoma aggressiveness (28) and that associated PAS-positive VM networks that also express laminin represent a biomarker in human melanomas associated with increased clinical mortality (29).

The molecular mechanisms governing melanoma VM, and their potential relationship to MMIC and melanoma growth, are largely unknown. We hypothesized that global gene expression analyses of tumorigenic, MMIC-enriched ABCB5$^+$ melanoma subpopulations, capable of VM, vis-à-vis ABCB5$^-$ melanoma bulk populations, might serve to identify molecular signaling pathways responsible for VM and, as a result, allow investigation of their potential relevance to melanoma growth.

Materials and Methods

Melanoma cells and culture methods

Authenticated human melanoma cell lines were obtained from the NCI/NIH Developmental Therapeutics Program or American Type Culture Collection (ATCC) and melanoma cell lines were cultured and passaged for fewer than 6 months as described (5–7). Clinical cutaneous melanoma cells were derived from surgical specimen according to Institutional Review Board–approved human subjects research protocols as described previously (7). Human umbilical vein endothelial cells (HUVEC) were purchased from Invitrogen and cultured according to the supplier's protocol.

Cell isolation

ABCB5$^+$-purified (ABCB5$^+$) or VEGFR-1$^+$-purified (VEGFR-1$^+$) cells were isolated by positive selection and ABCB5$^+$-depleted (ABCB5$^-$) or VEGFR-1$^+$-depleted (VEGFR-1$^-$) cell populations were generated by removing ABCB5$^+$ cells or VEGFR-1$^+$ cells by magnetic bead cell sorting with anti-ABCB5 (6) or anti-VEGFR-1 monoclonal antibody (mAb; R&D Systems) as described (7). Assessment of purity of melanoma cell isolates and determination of cell viability following magnetic cell sorting were carried out and yielded results as described previously (7).

RNA extraction and real-time quantitative PCR

Total RNA was isolated from ABCB5$^+$ and ABCB5$^-$ human melanoma cells or HUVEC cultures with the RT^2 qPCR Grade RNA isolation kit (SABiosciences). Standard cDNA synthesis reactions were carried out and the reverse transcriptase product was amplified by gene-specific primer pairs; β-actin was used as a normalizing control. The primers for ABCB5 detection were as described previously (5), primers for VEGFR-1 (Genbank accession no. NM_002019) detection were 5′-GACCTGGAGTTACCCTGATGAAA-3′ (forward) and 5′-GGCATGGGAATTGCTTTGG-3′ (reverse) and for β-actin detection 5′-CCTGGCACCCAGCACAAT-3′ (forward) and 5′-AGTACTCCGTGTGGATCGGC-3′ (reverse). Samples were assayed by Sybergreen chemistry and kinetic PCR (ABI 7300 Sequence Detector; Applied Biosystems). The relative amounts of transcripts were analyzed by the $2^{(-\Delta\Delta Ct)}$ method as described previously (5, 7, 30). Statistical differences between mRNA expression levels were determined by the nonparametric Mann–Whitney test. A 2-sided value of $P < 0.05$ was considered significant.

Global gene expression microarray analyses

Microarray analyses were carried out on purified ABCB5$^+$ ($n = 5$) and ABCB5$^-$ ($n = 5$) cell subsets derived from the established human melanoma cell lines G3361 and A375 and from 3 distinct clinical melanoma specimen previously characterized in our laboratory with regard to ABCB5 expression and MMIC phenotype in human melanoma xenotransplantation assays (7). Total RNA was extracted, processed, and hybridized as described previously (30) onto Affymetrix human HG-U133Plus2 GeneChip microarrays (Affymetrix). Statistical analysis of microarray results was carried out as described previously (30). The expression data set in its entirety is available through GEO (Gene Expression Omnibus) DataSets (accession number GSE26569). Functional gene networks were generated by Ingenuity Pathways Analysis (Ingenuity Systems; www.ingenuity.com) by mapping each gene identifier to its corresponding gene object in the Ingenuity Pathways Knowledge Base. These focus genes were overlaid onto a global molecular network developed from information contained in the Ingenuity Pathways Knowledge Base. Focus gene networks were then algorithmically generated on the basis of their connectivity and subsequently analyzed to identify the biological functions that were most significant to the genes in the network.

Western analysis

Total cell lysates were harvested from logarithmically growing cultures of the human melanoma cell lines MALME-3M, SK-MEL-2, SK-MEL-5, SK-MEL-28, UACC-62, UACC-257, M14, and MDA-MB-435 and analyzed by 8% SDS-PAGE and

Western assay to detect relative levels of ABCB5 (mAb 3C2-1D12; ref. 6) and α-tubulin (mAb clone DM1A; Sigma-Aldrich) by LI-COR Odyssey IR imaging system densitometry. Western blots for the analysis of VEGFR-1 expression were carried out with rabbit anti-VEGFR-1 antibody (Epitomics) or mouse anti-β-actin antibody as a control (Cell Signaling Technology) and horseradish peroxidase–linked secondary goat anti-mouse antibody or goat anti-rabbit antibody (Sigma), respectively. The reactive bands were detected by the addition of chemoluminescent substrate (ECL; GE Healthcare Bio-Sciences Corp.).

Flow cytometry

The analysis of cell surface coexpression of ABCB5 with VEGFR-1 was carried out by dual-color flow cytometry as described (7), using anti-ABCB5 mAb or isotype control mAb, followed by counterstaining with APC-conjugated donkey anti-mouse IgG secondary Ab as above, and phycoerythrin (PE)-conjugated anti-VEGFR-1 mAb (R&D Systems) or PE-conjugated isotype control mAb (BD PharMingen). Statistical differences between expression levels of markers were determined by the nonparametric Mann–Whitney test. A 2-sided value of $P < 0.05$ was considered significant.

In vitro vasculogenic differentiation and tube formation assays

VEGF-dependent induction of CD144 and of von Willebrand factor (VWF) expression and formation of capillary-like tube structures by human melanoma cells was assayed on growth factor–reduced Matrigel, a basement membrane matrix preparation (BD Biosciences). Purified $ABCB5^+$ or $ABCB5^-$ or unsegregated human melanoma cells were seeded into culture slide wells in medium 199 containing 5% fetal calf serum (31) in the presence or absence of VEGF (100 ng/mL). After 48-hour incubation, cells were fixed and then incubated with rabbit anti-CD144 polyclonal Ab (Bethyl Laboratories) or rabbit anti-VWF polyclonal Ab (Dako) overnight at 4°C. Subsequently, the cells were incubated with goat anti-rabbit Texas red–conjugated secondary Ab (Jackson ImmunoResearch Laboratories), washed and mounted in Vectashield (Vecta Laboratories) supplemented with 4′,6-diamidino-2-phenylindole and then analyzed by fluorescent microscopy as described previously (8). For tube formation assays, melanoma cells were seeded into culture slide wells as above and then pretreated with medium alone, rabbit anti-VEGFR-1 Ab (10 μg/mL; Santa Cruz Biotechnology), or rabbit isotype control Ab (10 μg/mL; BD Biosciences) prior to stimulation with VEGF (100 ng/mL). Tube formation was detected by phase-contrast microscope (Nikon Eclipse TE 300 microscope) after 24 hours of incubation. For quantitative analysis of tube formation and length and for the determination of CD144 and VWF expression at 48 hours, 3 randomly selected microscopy fields were photographed per experimental condition. Tube formation was analyzed by Image J software available from the NIH Web site as described previously (32). For quantification of CD144 and VWF expression, positive cells were counted by Neurolucida 8.10 software (MBF Bioscience). Differences among groups were analyzed by one-way ANOVA followed by Bonferroni *post hoc* tests. Differences with $P < 0.05$ values were considered statistically significant.

Immunohistochemistry and immunofluorescence

The following primary Abs were used: rat anti-laminin B2 (Abcam), mouse anti-ABCB5 (5–7), rabbit anti-CD271 (Ray-Biotech), and goat anti-VEGFR-1 (R&D Systems). Isotype-matched irrelevant Abs served as negative controls. The secondary Abs were goat anti-rat IgG-HRP and horse anti-mouse IgG-HRP (Biolegend) for immunohistochemistry and Alexa Fluor 594 goat anti-mouse IgG1, Alexa Fluor 488 goat anti-mouse IgG2a, Alexa Fluor 594 donkey anti-mouse IgG, Alexa Fluor 488 donkey anti-goat IgG3, Alexa Flour 488 donkey anti-mouse IgG, and Alexa Fluor 594 donkey anti-rabbit IgG (Invitrogen) for immunofluorescence staining. Immunohistochemistry was carried out by the 2-step horseradish peroxidase method as described previously (7). Immunofluorescence double staining was carried out as described previously (7). Quantitative analysis of laminin expression (percent positively staining area of sections) was carried out by the ImageJ program, and statistical differences between expression levels were determined by the nonparametric Mann–Whitney test.

In situ hybridization

ABCB5 RNA probes were prepared as follows: PCR-derived RNA probe templates were synthesized by introducing the T7 promoter into the antisense strand and the SP6 promoter into the sense strand. The primer pair (5′-TAATACGACTCACTA-TAGGGATGTCTGGCTTTTTCCCTTCTTGAC-3′) and (5′-GATTT AGGTGACACTATAGAAATTCAAGCTGGACGAATGA CCCCA-3′) was used to generate the DNA template for antisense and sense RNA probes spanning 200 base pairs of human ABCB5 cDNA. RNA probe labeling with digoxigenin (DIG) and *in situ* hybridizations (ISH) were carried out as described previously (33).

Melanoma transfection with VEGFR-1 short hairpin RNA

Two distinct shRNA plasmids targeting human VEGFR-1 mRNA, and negative control short hairpin RNA (shRNA) plasmids without homology to human mRNA, also containing the green fluorescent protein (GFP) marker, were purchased from SuperArray (SureSilencing; SuperArray). Human melanoma cells were transfected with the SuperFECT transfection reagent (SuperArray) according to the manufacturer's instructions. For determination of VEGFR-1 knockdown efficiency by real-time PCR or Western blotting, cells were harvested and total RNA was purified 48 hours posttransfection or cell lysates were prepared 96 hours posttransfection, respectively. Cells were harvested for use in xenotransplantation assays 24 hours posttransfection, when flow cytometric GFP fluorescence measurements at the Fl1 spectrum showed more than 90% transfection efficiency in both VEGFR-1 shRNAs- and control shRNA-transfected melanoma cultures.

Animals

BALB/c nude mice, NOD/SCID mice, and NSG mice were purchased from The Jackson Laboratory. Mice were

maintained in accordance with the institutional guidelines of Children's Hospital Boston and Harvard Medical School and experiments were carried out according to the approved experimental protocols.

Human to mouse melanoma xenotransplantation

Human to mouse melanoma xenografts were established by subcutaneous injection of human G3361 or A375 or of clinical patient-derived human melanoma cells in NOD/SCID or NSG mice as described previously (7, 11). Equal numbers of ABCB5$^+$ or ABCB5$^-$ cells derived from 3 distinct patients were xenografted into NSG mice (equal number of replicates for 10^4 or 10^3 cells per recipient). VEGFR-1$^+$ or VEGFR$^-$ melanoma cells were xenografted into NOD/SCID mice at 10^6 cells per recipient. VEGFR-1 shRNA–transfected (two distinct shRNAs) or control shRNA-transfected melanomas were xenografted into NOD/SCID mice at 10^7 cells per recipient (A375) or 10^6 cells per recipient (A375, G3361, specimens derived from $n = 3$ distinct patients). Tumor xenografts were harvested for histologic analysis in their entirety at 7 weeks (VEGFR-1$^+$ or VEGFR-1$^-$ melanoma cell-derived xenografts) or 3 weeks (shRNA-transfected xenografts) after tumor cell inoculation. Differences in tumor volume (TV), determined as described previously (7), were statistically compared by the nonparametric Mann–Whitney test, with a 2-sided value of $P < 0.05$ considered significant.

Results

VEGFR-1 is preferentially expressed by ABCB5$^+$ human melanoma cells

Melanoma growth is driven by MMIC identified by expression of ABCB5, as previously shown in human melanoma to NOD/SCID mouse tumor xenotransplantation models (7). Higher frequencies of cells capable of tumor initiation have been observed in side-by-side comparative studies in more severely immunocompromised NSG xenograft recipients (22). These findings pointed to heterogeneity among melanoma cells with regard to evasion of host antitumor immune responses, consistent with the recently shown existence of immunoevasive subpopulations of ABCB5$^+$ or CD271 (NGFR)$^+$ MMIC (8, 25, 34). The existence of MMIC has recently been confirmed in more immunocompromised xenotransplantation models, including NSG mice, based on CD271 expression (34). Likewise, we also find ABCB5$^+$ melanoma subpopulations enriched for MMIC in comparative tumorigenicity assays involving ABCB5$^+$ versus ABCB5$^-$ melanoma cell grafts to NSG recipients. Of 12 aggregate mice injected with ABCB5$^-$ melanoma cells (derived from 3 distinct patients), only one mouse xenografted with the highest cell dose (10^4 cells per inoculum) generated a tumor, consistent with the previously observed low rate of tumor formation by ABCB5$^-$ or CD271$^-$ melanoma bulk populations in primary xenograft recipients (refs. 7, 34; Fig. 1A). In contrast, 7 of 12 mice injected with ABCB5$^+$ cells (at identical doses of 10^4 or 10^3 cells per inoculum derived from the same patients) formed tumors (Fig. 1A), showing significant enrichment of tumorigenic capacity of ABCB5$^+$ MMIC even in more immunocompromised NSG mice ($P < 0.05$). Remarkably, we found the MMIC markers ABCB5 (7) and CD271 (34) preferentially coexpressed on the same tumor subpopulation in clinical human melanoma specimens (Fig. 1B). Moreover, preferential coexpression of ABCB5 and CD271 in human melanomas has also been documented by the Weissman Laboratory at Stanford University (Alexander Boiko and Irving Weissman, personal communication), indicating significant overlap of these MMIC populations.

To identify differentially expressed genes that might contribute to tumorigenic growth in MMIC-enriched melanoma subpopulations compared with tumor bulk populations, we first carried out microarray analyses on purified ABCB5$^+$ ($n = 5$) and ABCB5$^-$ ($n = 5$) cell subsets derived from 3 distinct patient-derived melanoma specimens or the established human melanoma cell lines G3361 and A375, all previously characterized in our laboratory by human melanoma xenotransplantation assays with regard to ABCB5 expression and MMIC phenotype (7). Using this approach (30), 399 genes were identified that were differentially expressed ($P < 0.05$) between ABCB5$^+$ and ABCB5$^-$ melanoma subpopulations (Supplementary Table 1), in addition to *ABCB5* itself, shown overexpressed in ABCB5$^+$ purified populations by real-time PCR ($P < 0.05$). One identified functional gene network, validated by PCR-based gene expression analyses in ABCB5$^+$ melanoma cell subsets, showed key molecules of vasculogenesis (the ability of tumor cells to differentiate along endothelial lines), and of angiogenesis (the ability of tumor cells to induce in-growth and proliferation of mature stromal blood vessels), specifically VEGFR-1, PTK2 (FAK), MET (HGFR), NRP2, and ETS1, to be significantly overexpressed in ABCB5$^+$ melanoma subsets (Fig. 1C). Preferential expression of VEGFR-1 by ABCB5$^+$ versus ABCB5$^-$ subpopulations was confirmed by real-time PCR at the mRNA level (Fig. 1D, 1.9 $\pm$ 0.5-fold vs. 0.3 $\pm$ 0.1-fold expression compared with detection in HUVEC, mean $\pm$ SE, $P < 0.001$) and was also shown by dual-color flow cytometry at the protein level (9.6% $\pm$ 2.2% vs. 0.9% $\pm$ 0.2% of cells, respectively, mean $\pm$ SE, $n = 10$, $P < 0.001$; Fig. 1E). In addition, immunofluorescence double staining analysis of clinical human melanoma specimens, clinical human melanoma xenografts, and human melanoma cell line xenografts, using human VEGFR-1-specific mAb, also revealed specific coexpression of VEGFR-1 on ABCB5$^+$ melanoma cells *in situ* (Fig. 1F).

VEGFR-1 signaling is required for CD144$^+$ VM by human melanoma cells

Because VEGFR-1 is associated with tumor vasculogenesis and considering that VM implicates primitive, pluripotential tumor cells (27), we next sought to determine whether VEGFR-1 is functionally expressed by MMIC-enriched ABCB5$^+$ melanoma cells and whether signaling through VEGFR-1 is required for CD144$^+$ VM differentiation of human melanoma cells. First, we evaluated the effects of VEGF treatment on CD144 and VWF expression by purified ABCB5$^+$ or ABCB5$^-$ melanoma subpopulations. VEGF (100 ng/mL; ref. 31) significantly and preferentially induced

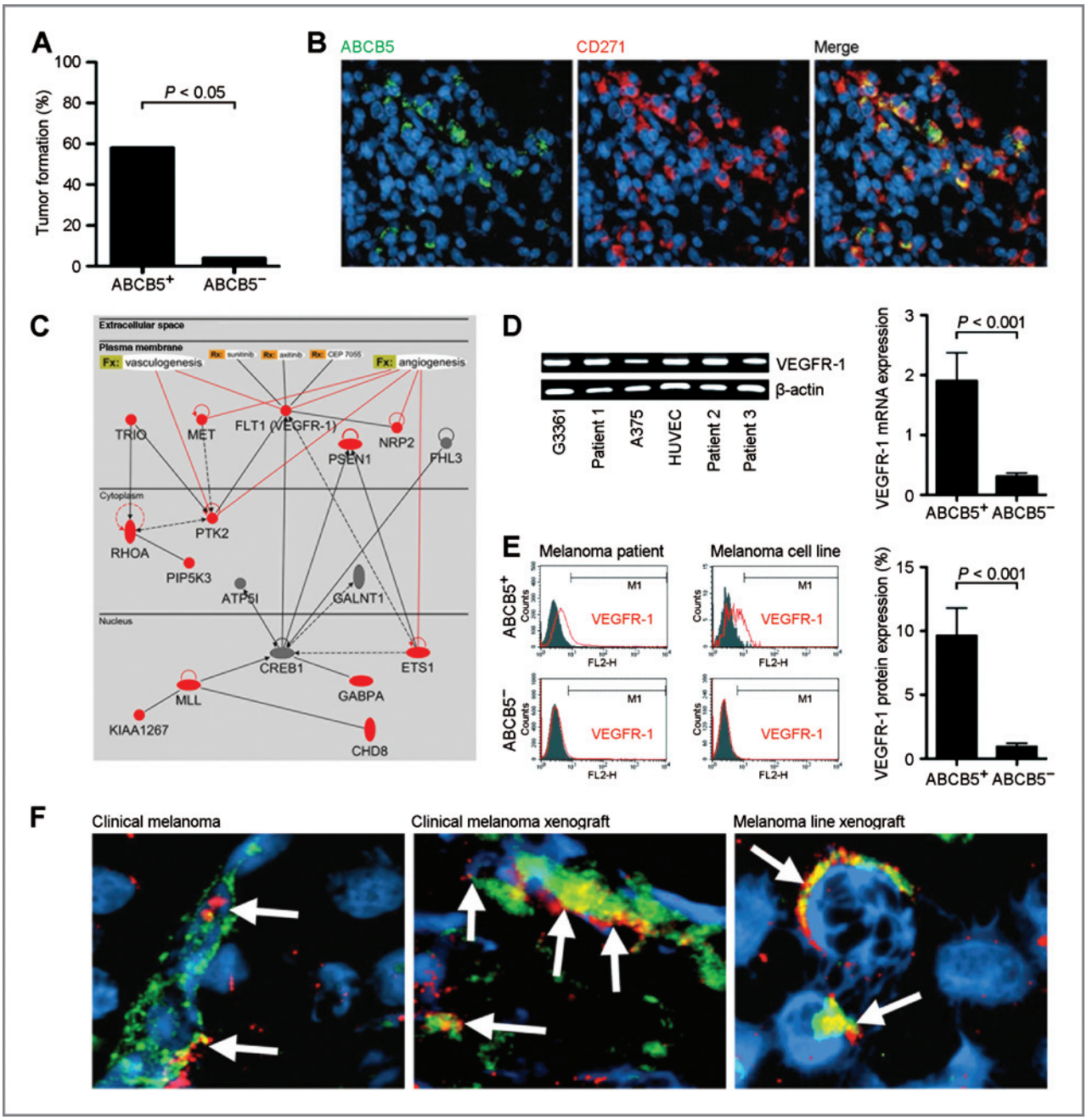

Figure 1. Vasculogenic/angiogenic pathways in human melanoma. A, tumorigenicity of ABCB5$^+$ versus ABCB5$^-$ melanoma cells in human to NSG mouse xenotransplantation experiments. B, representative immunofluorescence staining of ABCB5 (green) and CD271 (red) expression in clinical melanoma specimens; nuclei (blue). C, pathway activation across ABCB5$^+$ MMIC. Genes represented by red nodes (circles) are overexpressed in ABCB5$^+$ relative to ABCB5$^-$ human melanoma cells; those represented by black nodes are expressed at lower levels. Black lines show known gene interactions, and gene functions in vasculogenesis/angiogenesis or as drug targets are annotated (red lines). Gene relationships are based on Ingenuity Pathway Analysis. D, VEGFR-1 mRNA expression determined by real-time PCR in ABCB5$^+$ versus ABCB5$^-$ human melanoma cells. E, representative flow cytometric plots of VEGFR-1 protein expression on ABCB5$^+$ MMIC (top) and ABCB5$^-$ melanoma cells (bottom). Aggregate mean percentages are shown on the right. F, representative immunofluorescence double staining of ABCB5 (red) and VEGFR-1 (green) expression in melanoma specimens, with nuclei counterstained in blue. Arrows indicate zones of membrane coexpression (yellow).

expression of CD144 and VWF in 40.4% ± 5.5% and 53.7% ± 4.3% of VEGFR-1–expressing ABCB5$^+$ melanoma cells, respectively (mean ± SE, $n = 6$) compared with minimal or absent induction in ABCB5$^-$ melanoma cells with low to negative VEGFR-1 expression ($P < 0.0001$) (Fig. 2A). Moreover, preincubation with a blocking mAb to VEGFR-1 abrogated the ability of VEGF to induce CD144 expression in human melanoma cells (CD144 positivity 1.8% ± 1.3% in

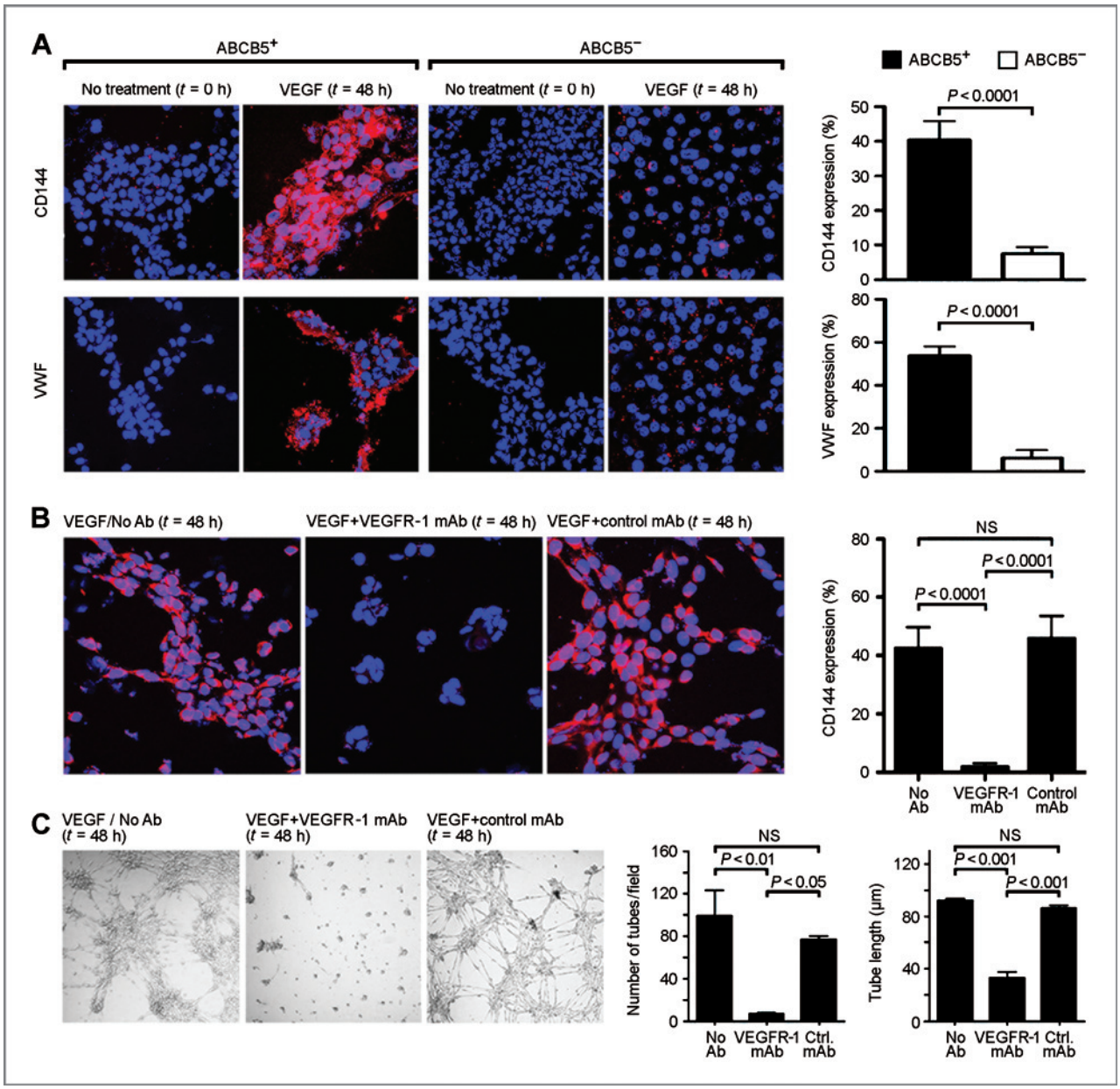

Figure 2. VEGF/VEGFR-1 signaling in human melanoma cells. A, representative immunofluorescence staining for CD144 (top) and VWF (bottom) expression (red) by purified ABCB5$^+$ or ABCB5$^-$ melanoma cells before and after VEGF treatment; nuclei (blue). Aggregate analysis of 6 distinct melanoma specimens is shown on the right. B, representative immunofluorescence staining for CD144 expression (red) by melanoma cells treated with VEGF as above but in the presence or absence of anti-VEGFR-1 blocking mAb or isotype control mAb; nuclei (blue). Aggregate analysis of 6 distinct melanoma specimens is shown on the right. C, tube formation of melanoma cells treated with VEGF in the presence or absence of anti-VEGFR-1 blocking mAb or isotype control mAb. Aggregate analyses of numbers of tubes per microscopy field and tube lengths (means ± SE, $n = 3$ replicate experiments) are shown on the right. NS, nonsignificant.

VEGFR-1 mAb-treated cultures vs. 42.5% ± 7.2% or 45.8% ± 7.8% in untreated or isotype control mAb-treated cultures, respectively, mean ± SE, $n = 6$, $P < 0.0001$; Fig. 2B). VEGFR-1 mAb also strongly inhibited VEGF-induced formation of multicellular tube-like growth by human melanoma cells in established *in vitro* vasculogenic differentiation assays that recall similar morphologic changes seen in differentiating endothelial cells (31), with significantly reduced numbers of tubes formed per microscopy field (6.7 ± 0.9 in VEGFR-1 mAb-treated vs. 99.0 ± 24.0 or 76.7% ± 3.3% in untreated or isotype control mAb-treated cultures, respectively, mean ± SE, $n = 3$, $P < 0.05$), and significantly lower average tube length (33.2 ± 4.5 μm in VEGFR-1 mAb-treated vs. 92.1 ± 1.6 or 86.5 ± 1.7 μm in

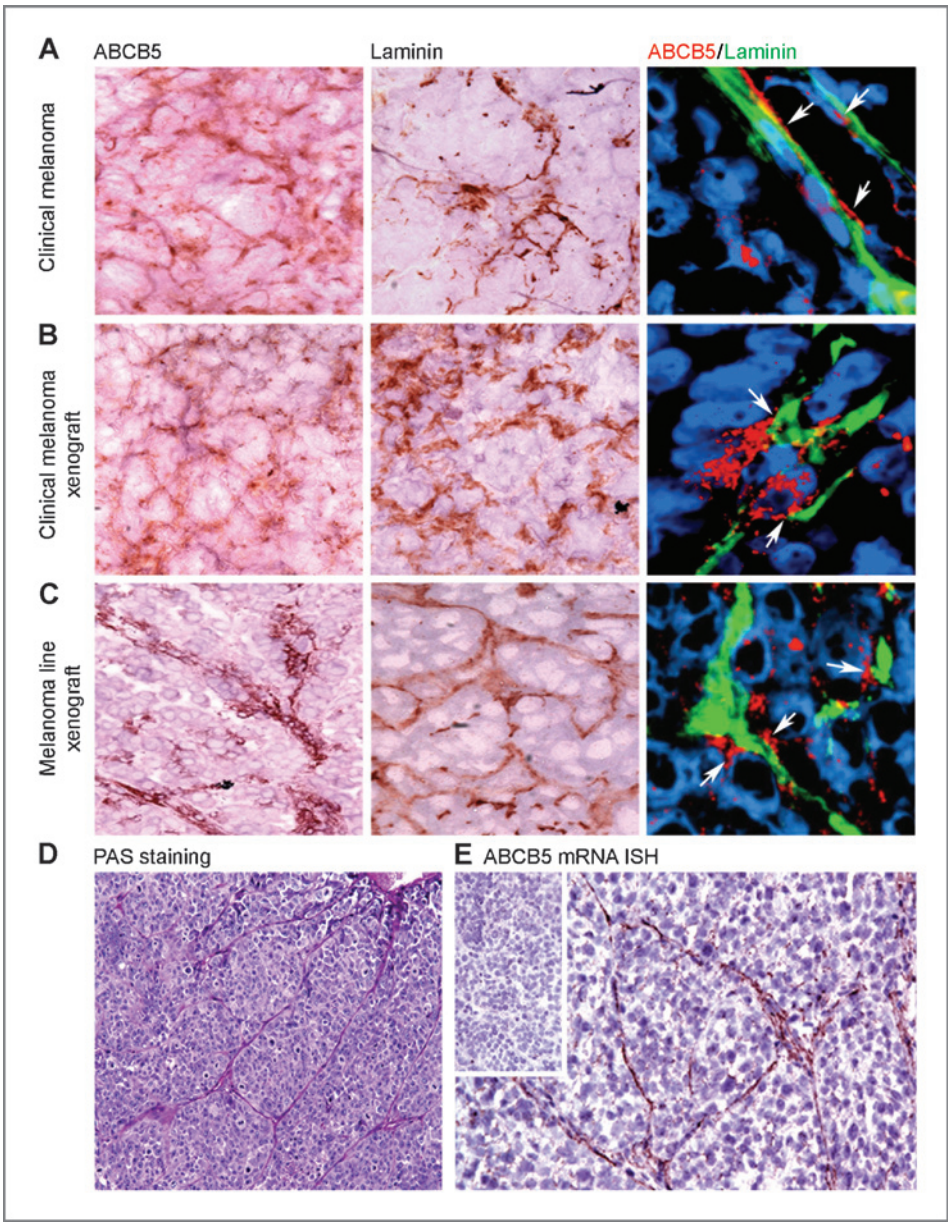

Figure 3. *In vivo* expression of the VM-associated markers ABCB5 and laminin. A–D, immunohistochemistry for ABCB5 protein (left), laminin (middle), and ABCB5 (red)/laminin (green) immunofluorescence double staining (right) in (A) clinical melanoma, (B) clinical melanoma xenografts, and (C) melanoma line xenografts, detecting identical patterns of VM-associated reticular channel-like reactivity (also detected in D) by PAS staining. Zones of close spatial association between ABCB5 and human laminin are indicated by arrows (A–C, right). E, ISH for ABCB5 mRNA (inset is sense control).

untreated or isotype control mAb-treated cultures, respectively, mean ± SE, $n = 3$, $P < 0.001$; Fig. 2C).

Detection of ABCB5$^+$ VM morphology in clinical and experimental human melanomas

Laminin-positive, PAS-positive patterned network VM morphology represents a biomarker associated with increased mortality in human melanoma patients (29). We found that ABCB5, previously shown preferentially coexpressed with the VM markers CD144 and TIE1 in distributions distinct from CD31$^+$ mature tumor vessels (7), can be detected in clinical human melanomas (Fig. 3A) and in experimental xenografts established from patient-derived melanoma specimens (Fig. 3B) or melanoma cell lines (Fig. 3C). The patterned channel-like networks (33) were highlighted via immunochemical laminin reactivity, preferential cellular and spatial association of ABCB5 expression with secreted laminin reactivity in patterned networks as shown in immunofluorescence double-labeling studies (Fig. 3A–C, right), and histochemical PAS reactivity (Fig. 3D) *in vivo*, consistent with established features of VM (27, 29). Identical VM patterned networks of ABCB5 protein expression determined by protein immunohistochemistry (Fig. 3A–C) were also detected by ABCB5 mRNA ISH (Fig. 3E). ABCB5 protein and mRNA expression also correlated significantly when assayed across a panel

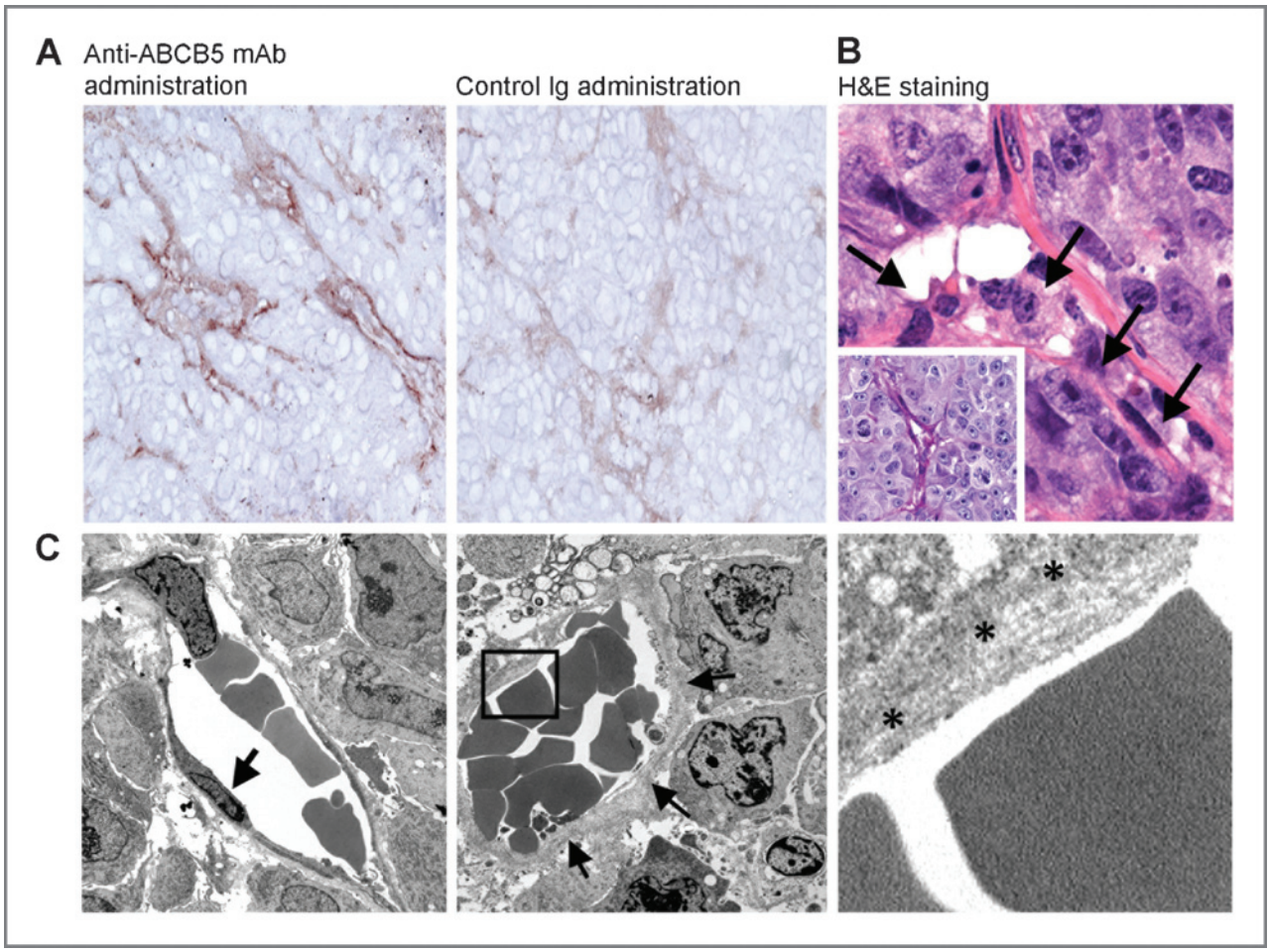

Figure 4. Detection of communicating VM patterned networks. A, immunohistochemistry of tumor xenografts after intravenous administration of anti-ABCB5 antibody (left) or control Ig (right) to melanoma xenograft-bearing mice. B, conventional histology discloses channels to be associated with linear lamella of PAS-positive extracellular matrix (inset) intimately associated with tumor cells (arrows). H&E, hematoxylin and eosin. C, transmission electron microscopy of a melanoma specimen depicting conventional tumor angiogenesis involving formation of spaces lined by flattened endothelial cells, containing erythrocytes, and surrounded by tumor cells (left) and other channels (middle and right), also containing erythrocytes, which are lined by extracellular matrix consistent with basement membrane (middle, arrows; right at high magnification, asterisks) and surrounded by tumor cells.

of human melanoma cell lines *in vitro* (Supplementary Fig. 1).

In further support of the preferential association of ABCB5 with VM, ABCB5 mAb, but not control Ig, administered intravenously to human melanoma xenograft-bearing mice showed specific binding to cell membranes of tumor cells in the pattern of anastomosing channels (Fig. 4A), confirming the intimate association of ABCB5$^+$ melanoma cells with apparently communicating VM channel lumens. Conventional histology (Fig. 4B) disclosed channels to be associated with linear lamellae of PAS-positive extracellular matrix (ECM; depicted in the inset, Fig. 4B) intimately associated with tumor cells (highlighted with arrows, Fig. 4B). Transmission electron microscopy of patient-derived melanoma showed that conventional tumor angiogenesis (Fig. 4C, left) involved the formation of spaces lined by flattened endothelial cells, containing erythrocytes, and surrounded by tumor cells whereas other channels also containing erythrocytes were lined by ECM consistent with basement membrane [Fig. 4C, middle (arrows), Fig. 4C, right (asterisks)] and surrounded by tumor cells and thus were structurally consistent with "vasculogenic mimicry," as defined by Maniotis and colleagues (27).

VEGFR-1 is required for *in vivo* VM and efficient tumor growth

First, to confirm that VEGFR-1 is expressed on a tumorigenic subpopulation of ABCB5$^+$ melanoma cells, we investigated the tumorigenicity of purified VEGFR-1$^+$ and VEGFR-1$^-$ melanoma subpopulations *in vivo*. VEGFR-1–dependent cell sorting was carried out by immunomagnetic selection as described (7) and groups of mice ($n = 14$ replicates per group) were xenografted subcutaneously with VEGFR-1$^+$ or VEGFR-1$^-$ melanoma cells representing 2 distinct patients and 1

melanoma cell line at 10^6 cells per inoculum, a dose previously shown to consistently initiate tumor formation when ABCB5$^+$ cells were used (7). Purified VEGFR-1$^+$ melanoma cells were consistently capable of tumor initiation, showing that VEGFR-1 is preferentially expressed on a tumorigenic subpopulation of ABCB5$^+$ melanoma cells. In addition, consistent with the highly selective expression of VEGFR-1 on ABCB5$^+$ melanoma subpopulations, we found that purified VEGFR-1$^+$ cells enriched for ABCB5$^+$ MMIC (Fig. 1E) gave rise to significantly larger tumors than VEGFR-1$^-$ melanoma cells, which contained lower proportions of ABCB5$^+$ MMIC (TV 131.0 $\pm$ 34.5 vs. 49.87 $\pm$ 21.0 mm^3, respectively; mean $\pm$ SE, $P < 0.01$; Fig. 5A). Intriguingly, despite a capacity of ABCB5$^+$-containing VEGFR-1$^-$ melanoma cell populations for tumor initiation, VEGFR-1$^-$ melanoma populations did not, unlike VEGFR-1$^+$ melanoma cell-derived xenografts, exhibit tumor cell–derived human laminin production (Fig. 5B). These results indicate that VEGFR-1$^+$/ABCB5$^+$ MMICs are primarily responsible for VM.

To examine the functional role of VEGFR-1 in melanoma VM and to dissect mechanistically whether VEGFR-1 expressed by human melanoma cells is required for more efficient tumor growth, we next investigated the effects of selective VEGFR-1 knockdown *in vivo*. VEGFR-1 shRNA transfection (2 distinct shRNAs) of human melanoma cells derived from 3 melanoma patients and 2 melanoma cell lines inhibited VEGFR-1 mRNA expression by up to 93% ($P < 0.01$; Fig. 5C, top) and significantly blocked VEGFR-1 protein expression (Fig. 5D, bottom). Patterned networks of ABCB5$^+$ VM expression were detected in tumors that formed from control shRNA-transfected melanoma inocula (Fig. 5D, top) but were not found, despite detectable, nonpatterned areas of ABCB5 positivity, in tumors that resulted from xenotransplantation of VEGFR-1 shRNA–transfected melanoma cells (Fig. 5D, bottom). VM formation within tumors, also evaluated by quantitative image analysis technology (7) to assess the pixilated density of associated laminin immunoreactivity per cross-sectional area, was inhibited by 86% (Fig. 5E) in tumors that formed from VEGFR-1 shRNA–transfected melanoma inocula compared with those that originated from controls (laminin immunoreactivity 0.8% $\pm$ 0.2% ($n = 6$) versus 5.6% $\pm$ 1.9% ($n = 6$), respectively; mean $\pm$ SE, $P < 0.01$). In contrast, VEGFR-1 knockdown in human melanoma cells exerted no significant effects on tumor angiogenesis, as determined by immunohistochemical staining for the marker of mature (murine) endothelium CD31, as documented via detection of conventional tubular, occasionally branching blood vessels (Fig. 5D). Importantly, inhibition of VEGFR-1–dependent ABCB5$^+$laminin$^+$ VM formation resulted in marked inhibition of tumor growth, with mean TV inhibited by 93% in recipients of VEGFR-1 shRNA- versus control-transfected melanoma inocula (TV = 5.3 $\pm$ 2.3 mm^3 ($n = 28$) versus 56.9 $\pm$ 28.3 mm^3 ($n = 15$), respectively; mean $\pm$ SE, $P = 0.001$; Fig. 5F). The additional finding that VM laminin positivity correlated negatively with tumor size within the subset of untreated control tumors (Spearman rank correlation $r = -0.6$) was consistent with a cause-and-effect relationship between inhibition of VEGFR-1–dependent laminin production and inhibition of tumor growth.

Discussion

Our study reveals several novel insights: First, ABCB5$^+$ MMICs previously found to be responsible for VM differ from melanoma bulk populations by preferentially expressing VEGFR-1. Second, VEGFR-1 signaling is required for MMIC-dependent VM differentiation and VM-associated laminin production. Finally, VEGFR-1 expression is required for efficient tumor growth.

VEGFR-1 is a tyrosine protein kinase and *src* oncogene family member and receptor for VEGF-A. When expressed by endothelial cells, VEGFR-1 regulates physiologic and pathologic angiogenesis (35–37). In addition, VEGFR-1, when expressed by cells of the hematopoietic lineage, possesses angiogenesis-independent functions in malignant disease by enabling cancer host hematopoietic progenitors to establish premetastatic cell clusters and modulate ECM composition of the metastatic niche, resulting in enhanced tumor growth and metastasis (36, 38). VEGF/VEGFR-1 signaling has previously been shown to function as an autocrine mechanism that can regulate tumor growth (39). However, a malignant, growth-promoting role of preferentially tumor-initiating cell-expressed VEGFR-1 has not been described to date.

Consistent with previous reports (39, 40), we found VEGFR-1 to be expressed by human melanoma cells. Importantly, the present study revealed VEGFR-1 expression to be predominantly restricted to ABCB5$^+$ MMIC among human melanoma cells, which raised the possibility of a MMIC-related function of this receptor. Indeed, our study identifies VEGFR-1 signaling as a novel molecular mechanism responsible for MMIC-dependent VM and VM-associated laminin production (28, 41, 42), a previously identified biomarker in human melanomas associated with aggressiveness (28) and increased clinical mortality (29). Furthermore, our results show that functional VEGFR-1 expression by MMIC is required not only for melanoma VM and associated laminin production but also for more rapid tumorigenic growth, providing a potential explanation for the previously established correlation between VM and poor clinical prognosis in this malignancy. Mechanistically, our study indicates that VEGFR-1 knockdown-mediated inhibition of tumor growth resulted at least in part through blockade of MMIC-dependent laminin production, because this ECM constituent represents a potent melanoma mitogenic proliferative factor through defined signaling pathways (43). It is noteworthy in this regard that laminin is also a key component of ECM preparations that exert potent protumorigenic effects when exogenously added to human melanoma grafts in experimental xenotransplantation models (22, 25).

Our demonstration that MMIC-expressed VEGFR-1 is required for tumor growth extends the known repertoire of tumor-initiating cell functions responsible for initiating or sustaining malignant progression, which include the tumor-initiating cell-defining functions of self-renewal, differentiation, and sustained proliferative capacity, as well as proangiogenic functions (25, 44) and immunomodulatory functions related to the evasion of host antitumor immunity (8, 25).

Recently, proof-of-principle has been established for the potential therapeutic utility of targeting tumor-initiating cells,

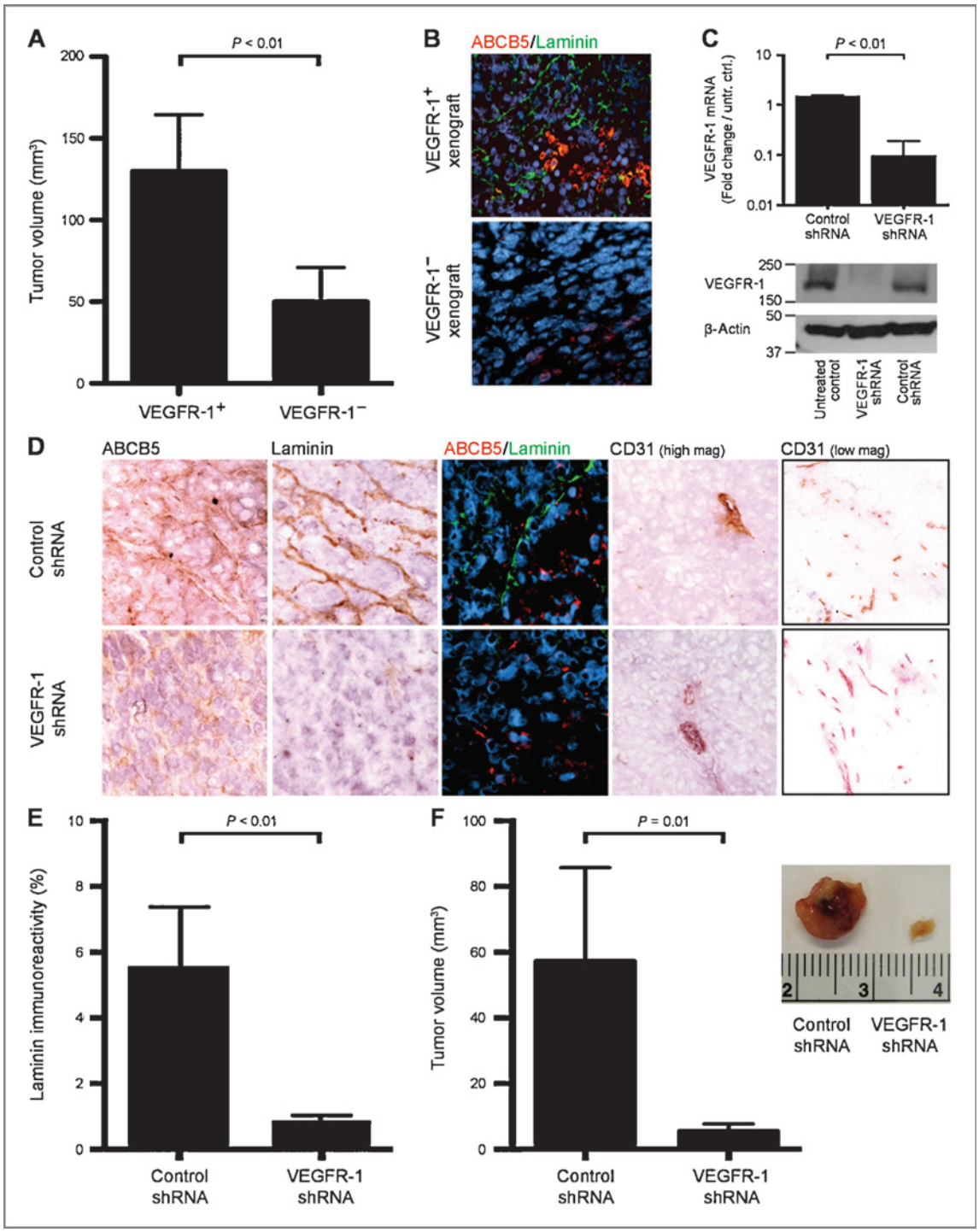

Figure 5. *In vivo* requirement for VEGFR-1 for efficient tumor growth. A, TV (mean ± SE) and (B) representative immunofluorescence double staining of ABCB5 (red) and laminin (green) expression with nuclear counterstaining (blue) of VEGFR-1$^+$ versus VEGFR-1$^-$ melanoma cell–derived xenografts. C, VEGFR-1 mRNA expression (top) and protein expression (bottom, molecular size unit: kD) in VEGFR-1 shRNA–transfected melanoma cells compared with controls; untr. ctrl., untreated control. D, representative immunohistochemistry (human ABCB5, human laminin, murine CD31) and immunofluorescence double staining of human ABCB5 and laminin (middle), revealing in the case of ABCB5 and laminin the extent of VM in melanomas that developed from control versus VEGFR-1 shRNA knockdown tumor xenografts or, in the case of CD31, the extent of the physiologic angiogenic response; mag, magnification. E, quantitative image analysis of laminin VM immunoreactivity for melanomas derived from control or VEGFR-1 shRNA–transfected melanoma xenografts ($n = 6$ recipient mice per experimental group). *Y*-axis, percentage of pixelated area with reactivity (mean ± SE). F, TVs (mean ± SE) 3 weeks following xenotransplantation of control or VEGFR-1 shRNA–transfected human melanoma cells (left). Typical macroscopic appearance of tumors dissected 3 weeks following transplantation of control shRNA- or VEGFR-1 shRNA–transfected melanoma cells (right).

including in human melanoma (7, 45, 46). Therefore, the newly discovered role of VEGFR-1 function in MMIC-dependent tumorigenic growth is relevant to the design of novel MMIC-targeted potential melanoma therapies. Specifically, our findings provide a rationale to investigate in future studies whether VEGFR-1-dependent, protumorigenic interactions of MMIC with VEGF-producing malignant or nonmalignant host cell populations in the tumor microenvironment can be specifically disrupted in a translationally relevant manner to inhibit tumor growth.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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