

# Human Melanoma Cytolysis by Combined Inhibition of Mammalian Target of Rapamycin and Vascular Endothelial Growth Factor/ Vascular Endothelial Growth Factor Receptor-2

Kerrington R. Molhoek,<sup>1</sup> Heinrich Griesemann,<sup>4</sup> Jianfen Shu,<sup>2</sup> Jeffrey E. Gershenwald,<sup>5</sup>  
David L. Brautigam,<sup>3</sup> and Craig L. Slingluff, Jr.<sup>1</sup>

<sup>1</sup>Department of Surgery, Division of Surgical Oncology, University of Virginia School of Medicine; <sup>2</sup>Department of Public Health Sciences, Division of Biostatistics and Epidemiology, University of Virginia; <sup>3</sup>Center for Cell Signaling, University of Virginia Health System, Charlottesville, Virginia; <sup>4</sup>Department of Immunology, Institute for Cell Biology, Eberhard Karls University, Tuebingen, Germany; and <sup>5</sup>Departments of Surgical Oncology and Cancer Biology, University of Texas M. D. Anderson Cancer Center, Houston, Texas

## Abstract

**Vascular endothelial growth factor (VEGF) plays a vital role in tumor angiogenesis. VEGF is produced by human melanomas, and the VEGF receptor 2 (VEGFR-2) is expressed by most advanced stage melanomas, suggesting the possibility of an autocrine loop. Here, we show that bevacizumab, an anti-VEGF antibody, inhibits proliferation of VEGFR-2<sup>+</sup> melanoma cell lines by an average of 41%; however, it failed to inhibit proliferation of VEGFR-2<sup>neg</sup> melanoma cell lines. The growth inhibitory effect of bevacizumab was eliminated by VEGFR-2 knockdown with small interfering RNA, showing that VEGF autocrine growth in melanoma is mediated through VEGFR-2. However, bevacizumab inhibition of autocrine signals did not completely inhibit cell proliferation nor cause cell death. Cell survival is mediated partially through mammalian target of rapamycin (mTOR), which is inhibited by rapamycin. Combination of bevacizumab with rapamycin caused loss of half of the VEGFR-2<sup>+</sup> melanoma cells, but no reduction in the number of VEGFR-2<sup>neg</sup> melanoma cells. The results show (a) an autocrine growth loop active in VEGFR-2<sup>+</sup> melanoma, (b) a nonangiogenic mechanism for inhibition of melanoma by blocking autocrine VEGFR-2 activation, and (c) a possible therapeutic role for combination of inhibitors of mTOR plus VEGF in selected melanomas. [Cancer Res 2008;68(11):4392–7]**

## Introduction

Malignant melanoma remains poorly responsive to systemic therapy. Treatments targeting molecular changes that underlie malignant behavior hold promise. Such approaches may target cell signaling pathways critical for cancer growth and survival or tumor angiogenesis and metastasis. However, the clinical benefit of targeted therapies as single agents has been less than desired. We are interested in enhancing antitumor effects in melanoma by combining targeted therapies that inhibit growth and survival of melanoma cells. We previously showed melanoma proliferation was inhibited by low-dose rapamycin (1), a drug that inhibits mammalian target of rapamycin (mTOR) in the phosphatidylinositol 3-kinase pathway and is a Food and Drug Administration–approved agent for immunosuppression posttransplant. The

Clinical Trials Evaluation Program of the NIH has initiated a program of clinical trials of combination therapies for selected malignancies.

Bevacizumab (Avastin) is a humanized anti-vascular endothelial growth factor (VEGF) monoclonal antibody approved for therapy of colorectal and lung cancers based on a significant increase in survival, when given in combination with cytotoxic chemotherapy (2). This agent was developed to block angiogenesis, a critical process for the survival of tumors as they increase in size (3, 4). Recognition of VEGF as an angiogenic factor was followed by the discovery that it is produced by both cancer cells and stromal cells, creating a microenvironment favorable for tumor growth (5–10). Production of VEGF seems to be an integral part of melanoma cancer progression because normal melanocytes do not produce it (11, 12), whereas tumor-derived melanoma cell lines express it (12–14). VEGF expression is up-regulated in melanoma cells (15), and elevated serum VEGF levels directly correlate with stage of disease progression in melanoma patients (16, 17). The VEGF receptor 2 (VEGFR-2) is the major mediator of mitogenic, angiogenic, and permeability-enhancing effects of VEGF (3). VEGFRs are not expressed on normal melanocytes (11, 15, 18), but VEGFR-2 expression is up-regulated in some human melanoma cells during malignant transformation (15). These results suggest a role of VEGF in the development and progression of melanomas. Expression of VEGF and VEGFR-2 by some human melanoma cells raises the possibility that VEGF may be an autocrine growth factor for some human melanoma cells. Therefore, bevacizumab might have an effect on melanomas, independent of its antiangiogenic effects. Here, we tested bevacizumab and rapamycin, singly and in combination, for their effects on proliferation of multiple tumor-derived human melanoma cell lines.

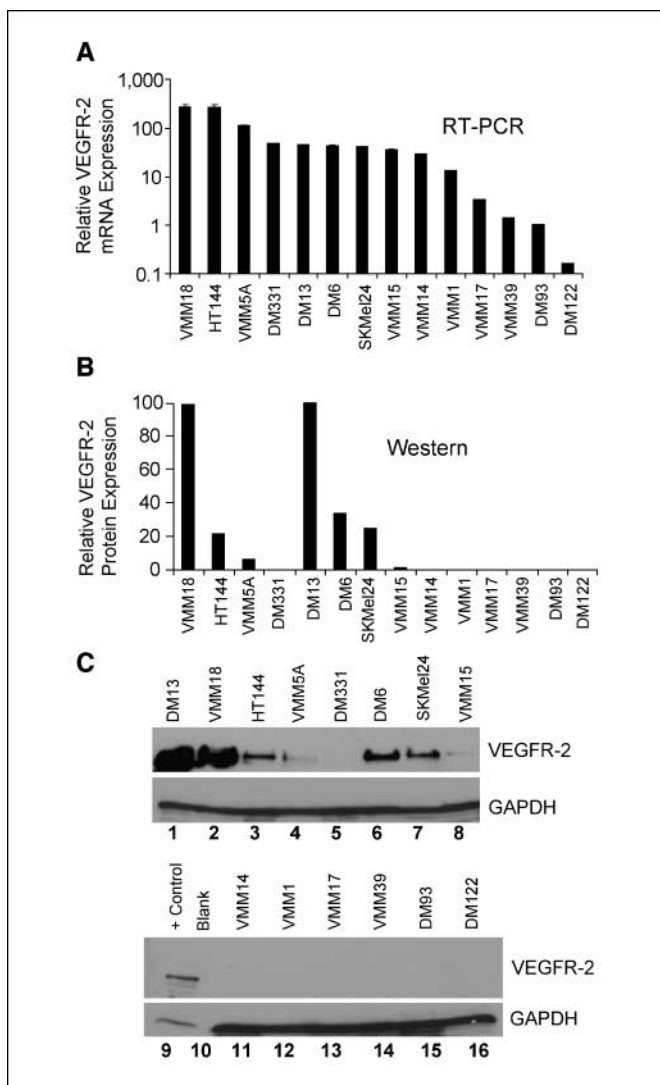
## Materials and Methods

**Cell culture.** Melanoma cell lines used in this study were cultured from tumor-involved lymph nodes surgically resected from patients at University of Virginia (VMM5A, VMM14, VMM15, VMM17, VMM18, and VMM39) or from patients at Duke University (DM6, DM13, DM93, DM122, and DM331) as described previously (1, 19–21). The VMM1 melanoma cell line was derived from a metastatic tumor in the brain surgically resected from a patient at University of Virginia (21). SKMel24 and HT144 were both obtained from American Type Culture Collection. All of the cell lines were cultured in RPMI 1640 supplemented with 5% fetal bovine serum (FBS), 2 mmol/L L-glutamine, penicillin (100 units/mL), and streptomycin (100 µg/mL) at 37 °C in 5% CO<sub>2</sub>, unless otherwise indicated.

**Reagents and inhibitors.** One hundred milligrams (25 mg/mL) of bevacizumab (Avastin; Genentech List No. 15734) was purchased from University of Virginia Hospital Pharmacy and used at 50 µg/mL in cell

**Requests for reprints:** Craig L. Slingluff, Jr., Joseph Helms Farrow Professor of Surgery, Human Immune Therapy Center, University of Virginia School of Medicine, Room 1352, Jordan Hall, P.O. Box 801457, Charlottesville, VA 22908. Phone: 434-243-2611; Fax: 434-982-3276; E-mail: cls8h@virginia.edu.

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**Figure 1.** Expression of VEGFR-2 mRNA and protein in human melanoma cell lines. **A**, qRT-PCR was performed as described in Materials and Methods, and the levels were normalized to the geometric mean of GAPDH and HPRT1 as controls. Results are plotted on a logarithmic scale to allow comparison of the 1,000-fold range in mRNA levels. *Error bars*, SD from triplicate samples. The melanoma cell lines are in rank order starting with the cell line that expressed the highest amount of VEGFR-2 mRNA. **B** and **C**, Western blot analysis of VEGFR-2 protein expression in various melanoma cell lines. Cell extracts were immunoblotted with VEGFR-2 antibody (**C**, top) and with anti-GAPDH as a loading control (**C**, bottom). Staining intensity was quantitated, normalized to the amount of GAPDH, and plotted (**B**) relative to DM13 (set as 100). The cell lines are arranged in the same order as in **A**.

number assays. Rapamycin (R-5000) was purchased from LC Laboratories, and a stock solution was made in DMSO and used at 1 nmol/L in cell number assays.

**Assay of cell number.** Melanoma cells (1,000 cells per well) were plated in triplicate in 96-well plates with 5% FBS and allowed to adhere overnight. After 12 to 16 h, the cells were washed and treated with serum alone or with inhibitors, as indicated. Cell numbers were assayed 48 h later (or 7 d later for Fig. 4C) using Cell Titer-Glo (Promega), according to the instructions provided by the manufacturer. This assay uses luciferase to measure ATP; because ATP levels are kept constant in living cells, the level is proportional to the number of viable cells (22). Relative light units (RLU) measured in that assay are used to determine the number of viable cells. The assay has been validated and used extensively in lieu of other assays, such as

reduction of chromogenic substrates [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] or pulse-labeling of DNA synthesis (23, 24).

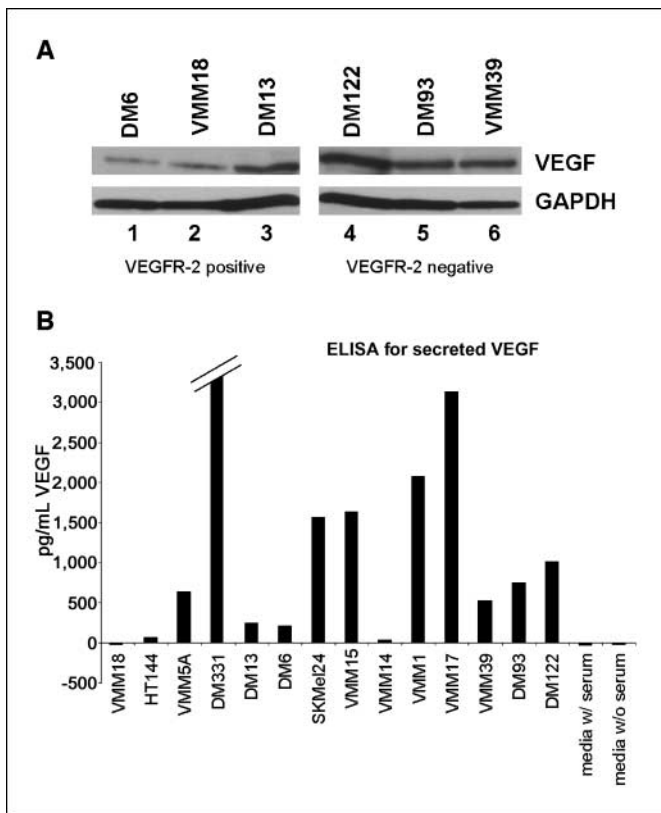
**Statistical analysis.** The logarithm transformation of the RLUs (raw data), instead of cell numbers calculated from RLUs, was the outcome for analysis, and linear regression was performed. The six cell lines were grouped into either high or low VEGFR-2 expression. Within either group, several comparisons were examined, including effects of individual drugs (in serum-containing media) versus the control serum, as well as the effects of both drugs given in combination versus the control serum, each drug alone, and the expected effects of the two drugs working in combination but acting independently. These comparisons were analyzed for significant differences between the VEGFR-2 high and low groups.

**Western blot analysis.** For analysis of proteins, cells from all 14 melanoma lines (VMM18, HT144, VMM5A, DM331, DM13, DM6, SKMel24, VMM15, VMM14, VMM1, VMM17, VMM39, DM93, and DM122) were plated in 100 mm Petri dishes and incubated for 24 h in RPMI medium plus 5% FBS. After 24 h, the medium was aspirated and the cells were washed twice with 10 mL PBS, harvested, and lysed, as described previously (1). Protein yields were determined by bicinchoninic acid analysis. Proteins were resuspended in SDS-containing sample buffer and heated for 10 min at 100°C, and 20 µg/lane were resolved by SDS-PAGE using 10% gels and transferred to Immobilon-P (Millipore). Membranes were blocked in 1% bovine serum albumin in 50 mmol/L Tris-Cl (pH 7.5), 0.9% NaCl, 0.05% Tween 20, and 0.01% antifoam A. Membranes were probed with antibodies listed below. Proteins were detected with Pierce SuperSignal West Pico Chemiluminescent substrate as recommended by the manufacturer and used to expose to Kodak BioMax film.

**Antibodies.** Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (used at 1:500) was purchased from Chemicon International. Anti-VEGFR-2 antibody (used at 1:1,000) was purchased from Cell Signaling. Anti-VEGF antibody (used at 1:1,000) was purchased from Abcam. This is an antibody to VEGF-A. Anti-phosphorylated mitogen-activated protein kinase [MAPK; extracellular signal-regulated kinase 1/2 (ERK1/2)], clone 12D4 antibody (used at 1:500), was purchased from Upstate. Anti-mouse IgG, peroxidase-linked species-specific whole antibody from sheep, secondary antibody (used at 1:5,000), and anti-rabbit IgG, peroxidase-linked species-specific whole secondary antibody from donkey (used at 1:5,000) were purchased from Amersham Biosciences.

**Quantitative real-time PCR.** Total RNA samples were obtained using the RNEasy mini kit according to manufacturer's instructions (Qiagen). Reverse transcription was done with MultiScribe reverse transcriptase (Applied Biosystems) and random hexamers, as per the manufacturer's instructions. The resulting cDNA was then subjected to quantitative real-time PCR (qRT-PCR) as described previously (25). The data collected from these quantitative PCRs defined a threshold cycle ( $C_t$ ) of detection for the target (VEGFR-2) or the control genes [GAPDH and hypoxanthine phosphoribosyltransferase 1 (HPRT1)] in each cDNA sample. To convert the  $C_t$  value into a relative abundance of target and control gene per sample, a standard curve was generated for the control gene using serial dilutions of cDNA sample: an arbitrary value of template was first assigned to the highest standard and then corresponding values were assigned to the subsequent dilutions, and these relative values were plotted against the  $C_t$  value determined for each dilution, resulting in the generation of the standard curve. The relative amount of target and control genes in each sample was then determined using the comparative  $C_t$  method (Applied Biosystems). The ratios of VEGFR-2 transcript are normalized by the geometric mean of the two control genes, GAPDH and HPRT1, and are plotted on the graph.

**ELISA.** For the quantitative determination of VEGF concentrations from each of the 14 melanoma cell cultures,  $2.5 \times 10^6$  melanoma cells were plated on 100-mm dishes in 5 mL and the medium was collected 24 h later. ELISA was performed with the Quantikine Immunoassay to Human VEGF kit (R&D Systems), according to the instructions provided by the manufacturer, with the exception that 100 µL of medium was used. A standard curve was generated using the supplied reagents, VEGF (pg/mL) was determined for each of the melanoma cell supernatants using the average value from triplicate samples, and medium with serum was used as a blank.



**Figure 2.** Expression of VEGF by human melanoma cell lines. A, Western blot analysis of VEGFR-2<sup>+</sup> and VEGFR-2<sup>neg</sup> melanoma cell lines with VEGF antibody (top) as described in Materials and Methods. GAPDH was immunoblotted as a loading control (bottom). B, ELISA for analysis of secreted VEGF collected from human melanoma cell lines after 24 h in culture (in pg/mL). DM331 was too high to measure. The melanoma cell lines are in the same rank order as Fig. 1A.

**Small interfering RNA experiments.** Small interfering RNA (siRNA) oligonucleotides that target VEGFR-2 and a nontargeting siRNA pool were purchased from Upstate and resuspended according to the manufacturer's instructions. For transfection,  $1 \times 10^6$  melanoma cells (DM6 and VMM18) were used in each condition (100 nmol/L SMARTpool or nonspecific negative control) with the Amaxa nucleofector kit, according to manufacturer's instructions, and plated into each well of a 12-well dish and

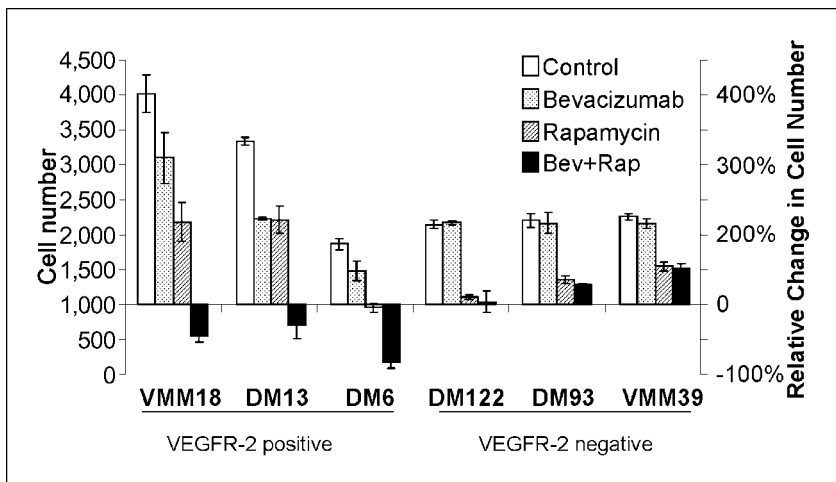
incubated for 18 to 24 h in culture medium before cell number assays and Western blot analysis.

**Human subjects.** All of the research involving human subjects was approved by the University of Virginia's institutional review board (Human Investigation Committee, HIC 5202, and HIC 10598) in accordance with assurances filed with and approved by the Department of Health and Human Services.

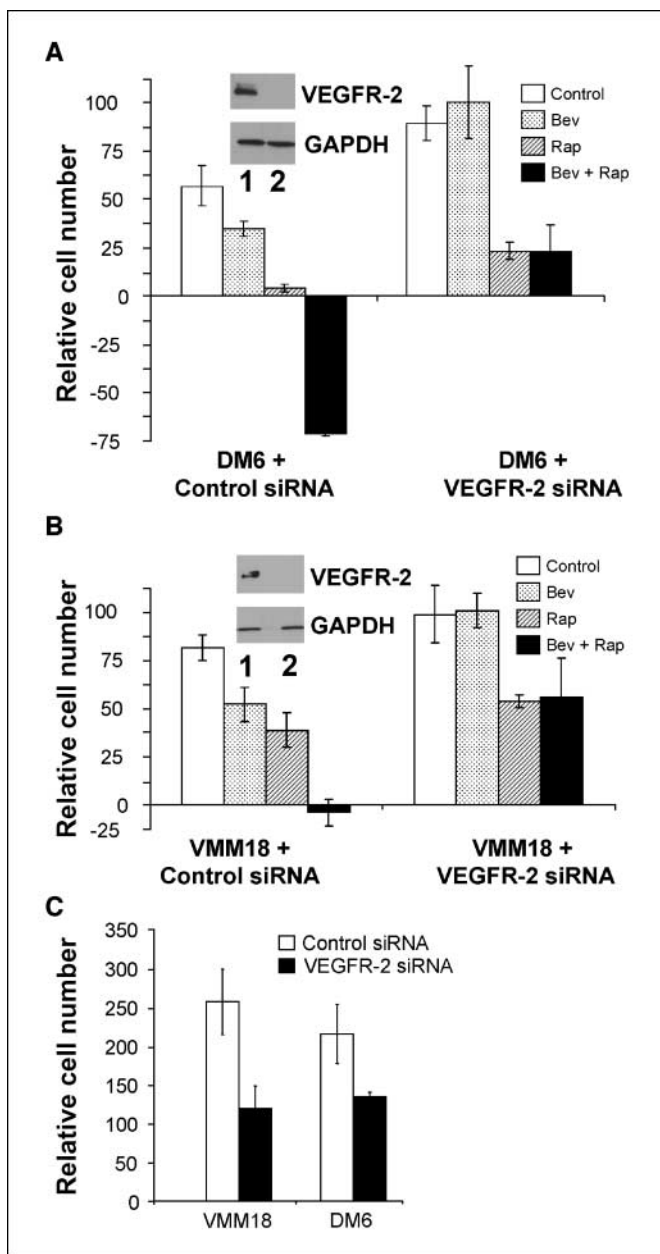
**Results**

**VEGFR-2 in melanoma cell lines.** Levels of VEGFR-2 mRNA and protein were analyzed in 14 melanoma cell lines (Fig. 1). qRT-PCR for VEGFR-2 expression revealed mRNA in all 14 cell lines; however, the expression varied over a 1,000-fold range, normalized with GAPDH and HPRT1 (Fig. 1A, note a logarithmic scale). Extracts of the 14 melanoma cell lines were analyzed by immunoblotting for VEGFR-2 protein and quantitated by densitometry normalized to GAPDH as a loading control (Fig. 1B and C). The results revealed that seven of the eight cell lines with the highest mRNA levels also expressed VEGFR-2 protein. DM331 was the exception in this group, without detectable VEGFR-2 protein. None of the six melanoma cell lines with the lowest levels of mRNA exhibited any VEGFR-2 protein (Fig. 1B). Among the cell lines that expressed VEGFR-2 protein, the levels of protein did not correspond to the relative levels of mRNA, suggesting that there are posttranscriptional mechanisms for regulation of VEGFR-2. From this set of 14 melanoma cell lines, we chose for further study the three lines with the highest levels of VEGFR-2 protein (VMM18, DM13, and DM6) and the three cell lines with the lowest levels of mRNA and no detectable VEGFR-2 protein (DM122, DM93, and VMM39).

**VEGF in melanoma cell lines.** Western blotting showed that melanoma cell lines expressed VEGF, whether or not they expressed VEGFR-2 (Fig. 2A). As expected, RT-PCR analysis showed VEGF mRNA was expressed in all six of these cell lines (not shown). We also analyzed, by ELISA, VEGF secreted from all 14 melanoma cell lines (Fig. 2B). There was greater than a 3,000-fold range in the amount of secreted VEGF in the 14 melanoma cell lines. The data for secreted VEGF are arranged in rank order based on the levels of the VEGFR-2 mRNA (Fig. 1A). This shows there is no correspondence between the levels of secreted VEGF and the levels of VEGFR-2 mRNA or VEGFR-2 protein. It seems that melanoma cell lines independently produce VEGF and VEGFR-2.



**Figure 3.** Effects of rapamycin and bevacizumab on proliferation of human melanoma cell lines. Numbers of human melanoma cells (left axis) and the relative change in cell number (right axis) are shown in a cell proliferation assay using Cell Titer-Glo starting with 1,000 cells per well. Cells were cultured in media plus serum, plus no treatment (white), bevacizumab (dotted), rapamycin (hatched), or a combination of bevacizumab and rapamycin (black), as indicated and described in Materials and Methods. The three cell lines on the left are labeled VEGFR-2 positive because of detection of the protein by Western blotting (Fig. 1). The three cell lines to the right are VEGFR-2 negative with no detectable VEGFR-2 protein.



**Figure 4.** Knockdown of VEGFR-2 prevents cell death in response to rapamycin and bevacizumab. DM6 (A) and VMM18 (B) cell lines were transfected with siRNA to VEGFR-2 (lane 2), as well as a nonspecific control (lane 1) and analyzed by Western blot (insets of graphs). Separate cultures of siRNA-transfected DM6 and VMM18 cells were maintained in media plus serum and treated without drug (white), bevacizumab (dotted), rapamycin (hatched), or a combination of bevacizumab and rapamycin (black), as indicated. C, relative cell number after 7 d of growth is plotted for VMM18 and DM6 melanoma cell lines transfected with a nonspecific control siRNA (white column) or siRNA to VEGFR-2 (black column). Error bars, SD from triplicate values.

**Bevacizumab and rapamycin effects on melanoma cell proliferation.** We examined proliferation of human melanoma cell lines *in vitro* and the effects of bevacizumab and rapamycin individually and in combination (Fig. 3). Cells were grown with or without bevacizumab at concentrations estimated to be achieved in serum with therapeutic human dosing (26) and with or without rapamycin below levels estimated to be achieved in serum with

therapeutic human dosing (27). Bevacizumab added alone significantly inhibited proliferation of the VEGFR-2<sup>+</sup> cell lines VMM18, DM13, and DM6 ( $P < 0.001$ ), but did not inhibit proliferation of cell lines without detectable VEGFR-2 protein DM122, DM93, and VMM39 ( $P = 0.18$ ). We concluded that there is an autocrine loop where VEGF, which is produced by all of the melanoma cell lines, promotes proliferation only of those melanoma cell lines that express VEGFR-2.

Rapamycin added alone inhibited proliferation of all six melanoma cell lines with a mean inhibition of 72% compared with control ( $P < 0.001$ ). Combination of bevacizumab plus rapamycin did not just inhibit cell proliferation, but caused a net loss of VEGFR-2<sup>+</sup> cells, consistent with an induction of cell death. In contrast, bevacizumab plus rapamycin had no additional effect compared with rapamycin alone (77% versus 73%) in the VEGFR-2–negative cell lines. Thus, bevacizumab does not have a significant additive effect on melanoma cells without VEGFR-2, whereas bevacizumab kills VEGFR-2<sup>+</sup> melanoma cells in combination with rapamycin.

**Knockdown of VEGFR-2 by siRNA prevents cell death from combination of bevacizumab plus rapamycin.** Bevacizumab only had effects on VEGFR-2<sup>+</sup> melanoma cells, and we wanted to test whether the observed effects were dependent on VEGFR-2. Using an siRNA SMARTpool for VEGFR-2 and a nonspecific siRNA control, we knocked down VEGFR-2 in both the DM6 and VMM18 cell lines (Fig. 4A and B). Immunoblotting showed that the VEGFR-2 siRNA resulted in the loss of detectable protein. Cell proliferation assays showed that bevacizumab alone did not inhibit the VEGFR-2 knocked down cell lines compared with the same cell lines transfected with a control siRNA (Fig. 4A and B). Combination treatment with bevacizumab plus rapamycin in the VEGFR-2 knocked down cell lines did not produce cell death and was no different than treatment with rapamycin alone. These results show that VEGFR-2 is necessary for the negative effects of bevacizumab on melanoma cell proliferation *in vitro* and for net cell death when used in combination with rapamycin.

If VEGF was promoting melanoma cell growth with VEGFR-2, then knockdown of the receptor would be expected to inhibit cell proliferation. We did not detect an effect on proliferation at 48 hours; so to test this hypothesis, VMM18 and DM6 melanoma cell lines were transfected in parallel with control siRNA or VEGFR-2 siRNA and evaluated at 7 days. Knockdown of VEGFR-2 reduced cell proliferation in both VMM18 and DM6 by ~50% (Fig. 4C).

Bevacizumab inhibited proliferation of VEGFR-2<sup>+</sup> melanoma cells; however, this did not involve significant reduction in ERK activation. Immunoblotting for phosphorylated ERK was not reduced in VMM18 cells treated with bevacizumab compared with untreated controls (data not shown). Therefore, ERK activation did not depend on VEGFR-2, and the VEGFR-2 receptor must have other intracellular effectors that contribute to proliferation.

## Discussion

Our results show that VEGFR-2 renders melanomas especially susceptible to combination therapy of bevacizumab plus rapamycin. The combination therapy is lethal to cells with VEGFR-2, and knockdown of VEGFR-2 by siRNA renders them insensitive. Thus, melanoma-derived VEGF can cause not only angiogenesis, but also autocrine activation of tumor cells that express VEGFR-2. This discovery may be useful to guide therapy for individual patients.

The melanoma cell lines examined fell into two groups, VEGFR-2<sup>+</sup> and VEGFR-2<sup>neg</sup>. All 14 melanoma cell lines expressed VEGFR-2 transcript. However, only about half had detectable levels of the VEGFR-2 protein, and these had high levels of mRNA. However, some cell lines with high levels of mRNA had no detectable VEGFR-2 protein; therefore, we suspect that there may be other modes of regulation, such as microRNAs, that limit accumulation of the protein even when the mRNA is expressed. Regardless of the VEGFR-2 protein expression levels, we observed that every melanoma cell line tested produced VEGF in secreted and cell-bound forms. This is consistent with reports of VEGF in melanoma patient biopsies, based on immunohistochemistry (12, 14, 28–31). It also is consistent with prior reports with epithelial cancers of a growth protective effect of VEGF induced by radiation exposure (32–34).

VEGF has three receptors: VEGFR-1 is thought to be a less active “decoy receptor” (35), VEGFR-2 is a protein tyrosine kinase that activates a variety of cell signaling pathways to promote cell proliferation (36), and VEGFR-3 is closely related to VEGFR-2, but its expression is primarily in lymphatic endothelial cells (35, 37). VEGFR-2 is believed to be the dominant effector of VEGF function on cells other than lymphatic endothelial cells (38). VEGFR-2 has been found in 50% to 80% of melanomas by immunohistochemistry, with incidence increasing with advanced disease stage (11, 12, 16, 17, 31, 39). Using a receptor tyrosine kinase antibody array, we detected selective activation of VEGFR-2 in VMM18 (a VEGFR-2<sup>+</sup> cell line), but not in DMI22 (a VEGFR-2<sup>neg</sup> cell line; data not shown). Our hypothesis was that melanoma cells expressing VEGFR-2 were dependent on VEGF for proliferation. We found that bevacizumab inhibited the proliferation of three of three VEGFR-2<sup>+</sup> melanoma cell lines tested. Knockdown of VEGFR-2 by siRNA prevented this inhibition. This showed that the response to VEGF was due to VEGFR-2. Furthermore, knockdown of VEGFR-2 reduced the proliferation of VEGFR-2<sup>+</sup> melanoma cell lines. Overall, these findings show that melanoma-derived VEGF interacts with cell-surface VEGFR-2 on melanoma cells, providing an autocrine growth signal (15, 39–41).

Melanoma cell proliferation is thought to be mediated in part through activation of the mTOR pathway. mTOR is an enzyme that

regulates translation and transcription during cell growth; thus, it is a logical therapeutic target, and its inhibitors, rapamycin (sirolimus) and CCI-779 (temsirolimus), may have therapeutic potential in cancer. However, in clinical trials to date, clinical activity of CCI-779 was low when it was used as a single agent in melanoma patients (42). Clinical efficacy of mTOR inhibition likely will require combination therapy that inhibits other growth pathways as well.

VEGFR-2 is believed to signal cell proliferation and survival through MAPK and AKT/mTOR pathways, but our prior results, recently confirmed in other cancer cell lines, showed crosstalk between these pathways (1, 43). We did not find evidence of decreased activation of ERK with inhibition of VEGF, which suggests that the effect of VEGFR-2 on melanoma cell proliferation is mediated through other pathways. It is also possible that VEGFR-2 signaling may differ in melanomas with or without BRAF or RAS mutations. Additional studies are planned for elucidating the downstream effects of blocking the VEGF/VEGFR-2 autocrine loop. Regardless, the combination of bevacizumab plus rapamycin causes not only synergistic inhibition of proliferation, but also death of human melanoma cells expressing VEGFR-2. These data provide rationale for combination therapy with inhibitors of VEGF and mTOR in patients with VEGFR-2<sup>+</sup> melanomas.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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