Cancer Research

Intracrine VEGF Signaling Mediates the Activity of Prosurvival Pathways in Human Colorectal Cancer Cells

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Abstract

The effects of vascular endothelial growth factor-A (VEGF-A/VEGF) and its receptors on endothelial cells function have been studied extensively, but their effects on tumor cells are less well defined. Studies of human colorectal cancer cells where the VEGF gene has been deleted suggest an intracellular role of VEGF as a cell survival factor. In this study, we investigated the role of intracrine VEGF signaling in colorectal cancer cell survival. In human colorectal cancer cells, RNAi-mediated depletion of VEGF decreased cell survival and enhanced sensitivity to chemotherapy. Unbiased reverse phase protein array studies and subsequent validation experiments indicated that impaired cell survival was a consequence of disrupted AKT and ERK1/2 (MAPK3/1) signaling, as evidenced by reduced phosphorylation. Inhibition of paracrine or autocrine VEGF signaling had no effect on phospho-AKT or phospho-ERK1/2 levels, indicating that VEGF mediates cell survival via an intracellular mechanism. Notably, RNAi-mediated depletion of VEGF receptor VEGFR1/FLT1 replicated the effects of VEGF depletion on phospho-AKT and phospho-ERK1/2 levels. Together, these studies show how VEGF functions as an intracrine survival factor in colorectal cancer cells, demonstrating its distinct role in colorectal cancer cell survival. *Cancer Res; 76(10); 3014–24. ©2016 AACR.*

Introduction

The VEGF family of ligands (VEGF-A, -B, -C, and -D) and the placenta growth factor are key mediators of tumor angiogenesis. Interactions of these factors with three tyrosine kinase receptors (VEGFR-1, -2, and -3) and two coreceptors [neuropilin (NRP)-1 and -2] have all been well characterized on endothelial cells (1, 2). The role of VEGF in vascular development is best demonstrated by the fact that heterozygous deletion of VEGF is embryonic lethal, primarily due to impaired formation of vascular structures (3, 4). VEGF-targeted therapies are used to treat a variety of cancers, including colorectal cancer, renal cell carcinoma, non–small cell lung cancer, pancreatic neuroendocrine tumors, and glioblastoma. At present, all FDA-approved antiangiogenic therapies use antibodies/traps to inhibit VEGF–VEGFR interactions or by inhibiting the activity of the VEGFRs. These approaches were developed based on the premise that VEGF-VEGFR signaling occurs in either a paracrine or autocrine fashion on endothelial cells (5). However, contrary to early expectations, these therapeutic approaches have led to only modest patient benefit, with rare exceptions, such as therapy for renal cell carcinoma (6).Thus, it is essential to enhance our understanding of the role of VEGF in tumor biology to improve upon current therapies targeting this essential protein.

Although VEGF and its receptors have been well characterized on endothelial cells, the roles of VEGF and its receptors in tumor cells are poorly understood. Our previous studies demonstrated the presence and function of VEGFRs in and on human colorectal cancer cells (7–10). Others (11) have shown that an intracellular VEGF axis might function in breast cancer cells to mediate cell survival. Prior studies from our laboratory have demonstrated that VEGF functions as a prosurvival factor in colorectal cancer cells and has a novel intracrine role that is distinct from its canonical paracrine or autocrine roles in angiogenesis (12). A better understanding of intracrine VEGF signaling is required to determine if targeting intracellular VEGF could be more effective than current methods that target the function of extracellular VEGF on membrane-bound VEGFRs.

In the present study, we elucidated the molecular mechanisms underlying the role of intracrine VEGF signaling in mediating colorectal cancer cell survival. Our findings clearly demonstrate that the depletion of intracellular VEGF decreases the activation of multiple signaling proteins that act as prosurvival or proliferation factors and that these effects are modulated by the activity of a VEGF-regulated tyrosine phosphatase. These findings provide mechanistic evidence for a novel and previously unidentified intracellular role of VEGF and support a paradigm shift in our understanding of VEGF's role in intracrine-mediated cancer cell survival.

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Materials and Methods

Cell culture

The colorectal cancer cell lines, HCT116, SW480, and HT29 (from ATCC) and HCP-1 (generated in our laboratory as described previously; ref. 13), were cultured as described previously (12, 13). All cell lines were authenticated by short tandem repeat analyses at the Characterized Cell Line Core facility at MD Anderson Cancer Center (MDACC) at the start and during the study.

Reagents

Reagents used were Recombinant human VEGF-A_{165} (R&D Systems, Inc.), VEGFR-1 antibody (18F1; ImClone Systems), SU5416 (Sigma-Aldrich), pazopanib (Selleck chemicals), Bevacizumab (Bev), and fluorouracil (5FU; MDACC pharmacy).

Western blotting

Western blotting experiments were performed as described in ref. 12. All cell lysates were prepared in radioimmunoprecipitation assay (RIPA) buffer with protease and phosphatase inhibitors. Approximately 50 to 100 μg proteins were analyzed to get clearly visible bands. All antibodies used are listed in Supplementary Data.

MTT assay

In vitro cell growth was assessed using a MTT assay (Sigma). Briefly, colorectal cancer cells were transfected with the required siRNAs for 24 hours and then replated into 96-well plates at 3,000 cells per well in 200 μL media. The cells were further incubated for 72 hours with or without fluorouracil (1 μg/mL for HT29 cells; 2 μg/mL for HCT116 and SW480 cells) followed by MTT assays according to the manufacturer's protocol.

siRNA knockdown

VEGF was depleted in colorectal cancer cells using a mixture of three different siRNAs targeting all isoforms of human VEGF. Similarly, VEGFR1 was depleted by a mixture of two siRNAs targeting the N-terminus of VEGFR1 mRNA. (All siRNA sequences are in Supplementary Data.) A validated nontargeting siRNA (control siRNA) was obtained from Sigma. The siRNA knockdown was performed in regular cell culture medium containing 10% FBS without antibiotics using the Lipofectamine 2000 siRNA transfection reagent (Invitrogen). Twenty-four hours after transfection, the cells were washed with PBS and cultured with Minimal Essential Media supplemented with 1% FBS and antibiotics. Forty-eight hours later, supernatant media were collected and assayed for VEGF depletion, or cells were lysed and lysates were used to assay for levels of different proteins and in reverse phase protein array (RPPA) and receptor tyrosine kinase (RTK) arrays.

RPPA analyses

RPPA analyses were performed at MD Anderson Cancer Center's Functional Proteomics RPPA Core facility. Briefly, serial dilutions of cell lysates were prepared following requisite treatments of colorectal cancer cells and arrayed on nitrocellulose-coated slides. Heatmaps were generated in Cluster 3.0 (http://www.eisenlab.org/eisen/) as a hierarchical cluster using Pearson correlation and a center metric.

RTK arrays

Changes in various RTKs were assayed using the Human Phospho-RTK Array Kit (R&D Systems), which can unbiasedly measure changes in a panel of about 50 RTKs. Briefly, colorectal cancer cell lysates were incubated with the membranes from the RTK array Kit according to the manufacturer's protocol. The membranes were then washed and treated with an anti-phosphotyrosine antibody for 2 hours, and the reacting protein signals were detected by exposing the membranes to autoradiography films using chemiluminescence substrates. Multiple exposures were taken and analyzed for changes in phospho-RTK levels.

Immunoprecipitation

Colorectal cancer cells stably expressing Myc-tagged $VEGF_{165}$ were transiently transfected with empty vector or a plasmid expressing FLAG-tagged VEGFR1. After 48 hours, cell lysates were prepared and were incubated with either EZ View Red anti-FLAG Gel or EZ View Red anti-Myc Gel (Sigma) for 14 to 16 hours at 4°C. Following three washes with RIPA buffer, the bound proteins were eluted by boiling the lysates in 1% sodium dodecyl sulfate. The eluted proteins were analyzed by Western blotting.

Phosphatase treatments

Colorectal cancer cells were transfected with VEGF-targeting siRNAs; 16 hours after transfection, the medium was replaced by MEM with 1% FBS and antibiotics, and the cells were further incubated for approximately 30 hours. Then, the cells were treated with either 4 nmol/L okadaic acid or 100 μmol/L Na_3VO_4 for approximately 16 hours. The cells were then lysed in RIPA buffer with protease and phosphatase inhibitors and examined for levels of various phospho-proteins. Untreated and control siRNA–transfected cells were used as controls.

Treatment with antiangiogenic agents or recombinant VEGF

Colorectal cancer cells were treated with bevacizumab (250 μg/mL; ref. 12), pazopanib (10 nmol/L; ref. 14), SU5416 (10 μmol/L; ref. 15), and 18F1 (20 μg/mL; ref. 8) for 14 to 16 hours and lysed to measure levels of various proteins. Cells grown in media containing 1% FBS were also treated with 20 ng/mL of recombinant VEGF for 5 to 30 minutes as required and assayed for alterations in signaling proteins.

Image analyses

Autoradiographs of Western blots were scanned, and the resulting TIFF images were analyzed using the ImageJ software program (http://imagej.nih.gov/ij). Band intensities were determined after subtracting background and selecting and plotting lanes for the required bands using the "Analyze" option. For measuring "spot" intensities in the RTK array, the "Oval" selection macro was used to draw circles large enough to cover the spots and measure their intensities. Intensities of areas without antigen reaction on the same scanned images using same-sized circles were measured as background and subtracted from all intensity measurements.

Immunofluorescence

Briefly, colorectal cancer cells were grown on chamber slides and fixed with 2% PFA in PBS. Following permeabilization in PBS with 1% Tween 20 and 3X washes with TBST, slides were blocked in 8% fish gelatin in TBST (blocking buffer). After 3X washes in TBST, slides were then incubated with primary antibodies diluted

in blocking buffer for 1 hour at room temperature. Slides were then washed again as above and incubated with secondary antibodies diluted in blocking buffer for 1 hour at room temperature. Stained cells were then visualized using a confocal microscope with a 60× oil objective, and images were taken with a CCD camera. DNA was visualized by staining with DAPI.

Graphical and statistical analyses

All graphical calculations and numerical data plotting were performed using Excel (Microsoft). All data are expressed as mean ± standard errors. The statistical significance of differences between different experimental groups was determined by the Student *t* test, and *P* values less than 0.05 were considered significant.

Results

VEGF depletion in colorectal cancer cells reduces cell survival and enhances chemosensitivity

In our previous studies, we investigated the effects of VEGF signaling in colorectal cancer cells by studying different colorectal cancer cells with homozygous deletion of VEGF genes (12). However, the effects of this depletion were subject to possible alterations in compensatory pathways, as is observed frequently in most studies involving the deletion of important genes. In the present study, therefore, to assess the effects on colorectal cancer cells that were truly due to VEGF depletion alone, we performed all experiments with VEGF-depleted cells soon after VEGF knockdown with siRNA (i.e., 72 to 96 hours following transfection). We initially designed three different siRNAs that target all VEGF isoforms; the combination of these three siRNAs appeared to elicit a stronger reduction of VEGF in multiple colorectal cancer cells than any of the three siRNAs individually (Supplementary Fig. S1). Transfection with this siRNA mixture led to strong and consistent decreases in the levels of all VEGF isoforms in different colorectal cancer cell lines, whereas transfection with a validated nontargeting control siRNA did not (Fig. 1A). HCT116, SW480, and HT29 colorectal cancer cells with siRNA-induced VEGF depletion had reduced proliferation (Fig. 1B) and increased apoptosis (Fig. 1C) compared with controls. 5FU treatment increased apoptosis in all colorectal cancer cell lines. However, VEGF depletion increased the cytotoxicity of 5FU compared with 5FU treatment alone (Fig. 1B and C). Bevacizumab treatment to inhibit paracrine/autocrine signaling did not reduce cell growth or alter chemosensitivity in the same colorectal cancer cells (Supplementary Fig. S2). The effects of this acute VEGF depletion in colorectal cancer cells (i.e., reduced survival and increased sensitivity to chemotherapy) were similar to those observed in colorectal cancer cells with somatic VEGF knockout, as described previously (12). These observations clearly indicate that VEGF is an intracrine/intracellular survival factor in colorectal cancer cells.

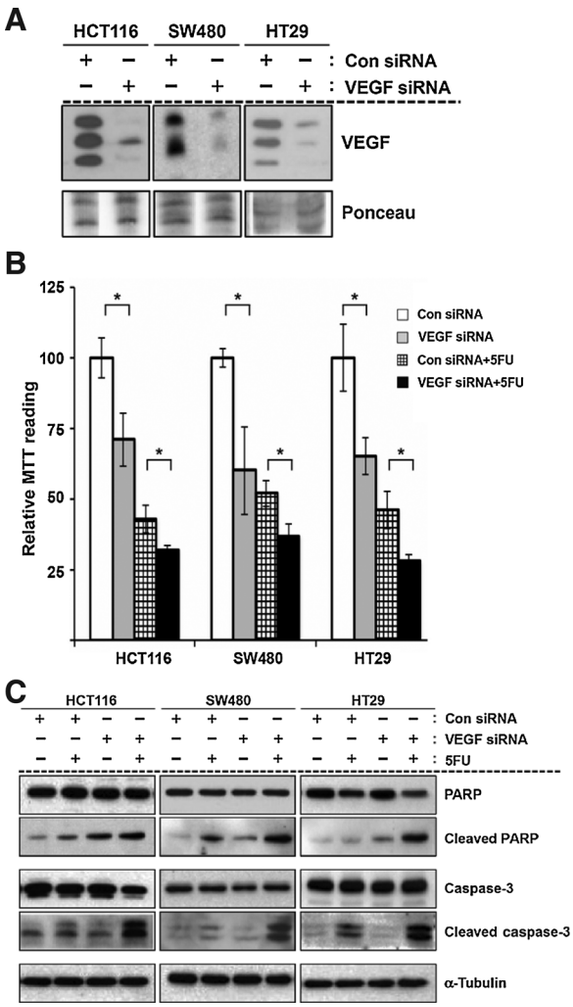

Figure 1.
siRNA-mediated depletion of VEGF in colorectal cancer cells inhibits cell growth and increases sensitivity to chemotherapeutic agents.
A, Western blotting shows levels of secreted VEGF in the supernatant media collected from control siRNA (Con siRNA)– and VEGF siRNA–treated HCT116, SW480, and HT29 cells. Ponceau S–stained membranes are shown for equal loading. B, colorectal cancer cells transfected with Con siRNA and VEGF siRNA and treated with or without 5FU were assayed for cell viability by MTT assay. *, $P < 0.05$. C, measurement of different apoptosis markers (PARP, cleaved PARP, caspase-3, and cleaved caspase-3) in colorectal cancer cells transfected with Con siRNA or VEGF siRNA and treated with or without 5FU. Note: Because cleaved proteins produced weaker signals than full-length proteins did on the same blot, equal proteins were loaded in two different gels and assayed for either full-length or cleaved proteins with their specific antibodies. One representative α-tubulin blot (loading control) is shown.

VEGF depletion reduces the activity of cell survival molecules

To investigate the molecular mechanisms by which VEGF depletion affects colorectal cancer cell survival, we used an unbiased approach to identify alterations in signaling molecules that are important to cell survival. Screening HCT116 cells by RPPA, which detects changes in approximately 150 signaling proteins (Supplementary Fig. S3A), identified AKT and ERK1/2 as having measurable decreases in their phosphorylation levels in response to acute VEGF depletion (Fig. 2A). The decrease in AKT phosphorylation (~50%) was of greater magnitude (~2 fold) compared with twice that in phospho-ERK1/2 phosphorylation (~25% decrease; Fig. 2A). These preliminary screening results were verified in HCT116, SW480, and HT29 colorectal cancer cell lines; all cell lines studied demonstrated a significant decrease in pAKT Ser473, as well as decreases in pERK1/2 Thr202/Tyr204 in response to VEGF depletion (Fig. 2B). [Note: Although we found pERK1/2 levels were diminished with VEGF depletion in multiple

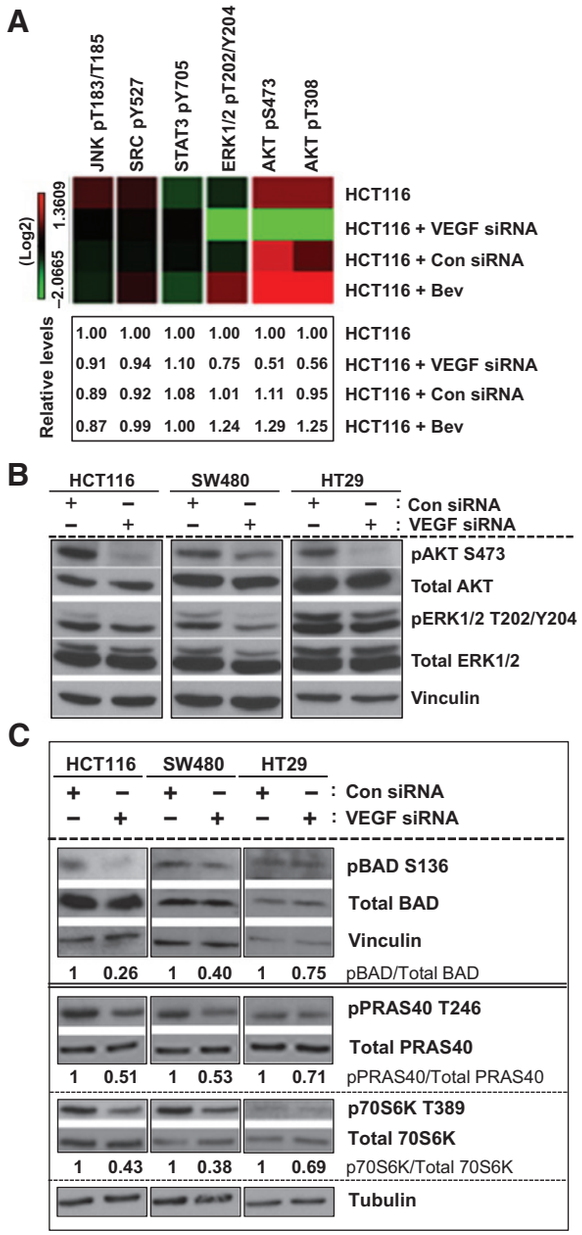

Figure 2.
VEGF depletion in colorectal cancer cells reduces the activity of prosurvival factors and their downstream signaling. A, heatmap from RPPA analysis of HCT116 cells treated with control siRNA (Con siRNA), VEGF siRNA, or bevacizumab (Bev). Untreated cells were used as the control. Selected signaling molecules are shown. (The complete results of the RPPA analyses are provided in Supplementary Figures.) The table below the heatmap indicates fold changes in the levels of the analyzed proteins. B, validation of RPPA analyses by Western blotting in HCT116, SW480, and HT29 colorectal cancer cell lines. The lysates from the cells treated with either Con siRNA or VEGF siRNA were analyzed for levels of pAKT (Ser 473), pERK1/2 (Thr 202/Tyr 204), total AKT, total ERK1/2, and vinculin. C, Western blot analyses to validate alterations in the activity of proteins downstream of AKT. Phosphorylated and total protein levels are shown. Vinculin was used for detecting equal loading in the blots measuring BAD, whereas tubulin was measured to ensure equal loading in blots detecting PRAS40 and 70S6K. The numbers below the blots denote the phospho-protein levels relative to total protein levels in each sample (Con siRNA–treated cells standardized to 1).

experiments (Fig. 2B), the effects were not as robust as for pAKT in all experiments. Thus, we assume that the effects on cell growth are most likely due to changes in pAKT levels with smaller contributions from pERK1/2.] Similar effects were observed in other colorectal cancer cell lines, including CaCo2, RKO, and HCP1 [a cell line newly isolated in our laboratory (13)] following VEGF depletion by siRNA treatment (data not shown), indicating a common VEGF-mediated regulation of prosurvival signaling in colorectal cancer cell lines.

The initial RPPA analysis of HCT116 cells also demonstrated alterations in the phosphorylation levels of PRAS40 and 70S6K, which are downstream of the AKT pathway (Supplementary Fig S3). To validate the effects of VEGF depletion on AKT signaling, we assessed representative downstream molecules in the AKT signaling pathway that are involved in apoptosis and proliferation. We found significant reductions in phosphorylation levels of BAD, PRAS40, and 70S6K in HCT116 and SW480 cell lines following VEGF depletion, whereas such reductions were minimal in HT29 cells (Fig. 2C), indicating that the effects of VEGF on colorectal cancer cell survival are mediated mostly through the prosurvival molecule AKT.

VEGF regulates AKT by an intracellular mechanism

To definitively determine whether the effects of VEGF depletion on AKT and ERK1/2 phosphorylation occur through a novel intracellular activity of VEGF, rather than through the paracrine/autocrine signaling described in endothelial cells, we treated colorectal cancer cells with bevacizumab, an antibody that inhibits the binding of VEGF to its receptors on the cell surface (1). The pAKT and pERK1/2 levels of the bevacizumab-treated colorectal cancer cells were no different from those of the untreated colorectal cancer cells (Fig. 3A). This indicated that the inhibition of extracellular VEGF-VEGFR1/R2 binding did not correlate to the effects of VEGF depletion that were most likely due to intracellular events.

Others have reported that bevacizumab does not inhibit VEGF-NRP1/2 binding (16, 17). Thus, VEGF depletion may affect colorectal cancer cell survival through a VEGF-NRP signaling cascade that is perturbed in the absence of VEGF but not affected by bevacizumab. To test this possibility, we incubated colorectal cancer cells treated with VEGF siRNAs in the presence of recombinant VEGF, which can bind to NRP1/2 on the colorectal cancer cell surface, and assessed the cells for rescue of pAKT levels. Adding recombinant VEGF to the cell culture media failed to increase the cells' levels of pAKT (Fig. 3B), clearly demonstrating that VEGF exerts its effects on various signaling molecules through a novel intracellular mechanism. Control siRNA–treated colorectal cancer cells exposed to recombinant VEGF also failed to demonstrate any significant increase in pAKT or pERK1/2 (Fig. 3B) levels, suggesting that VEGF, as a paracrine factor, has limited effects on these cells.

VEGFR1 depletion reduces pAKT levels in colorectal cancer cells

Most of VEGF's known effects are mediated through its binding to its receptors or coreceptors on cell membranes (1, 2, 18). Because the effects of VEGF depletion on prosurvival molecules appeared to be independent of cell membrane VEGF–VEGFR/NRP interactions, we sought to determine whether the intracellular signaling of VEGF required its interaction with intracellular VEGFRs. Indeed, depleting VEGFR1 in HCT116 cells decreased pAKT and pERK1/2 levels (Fig. 4A). We also found significant

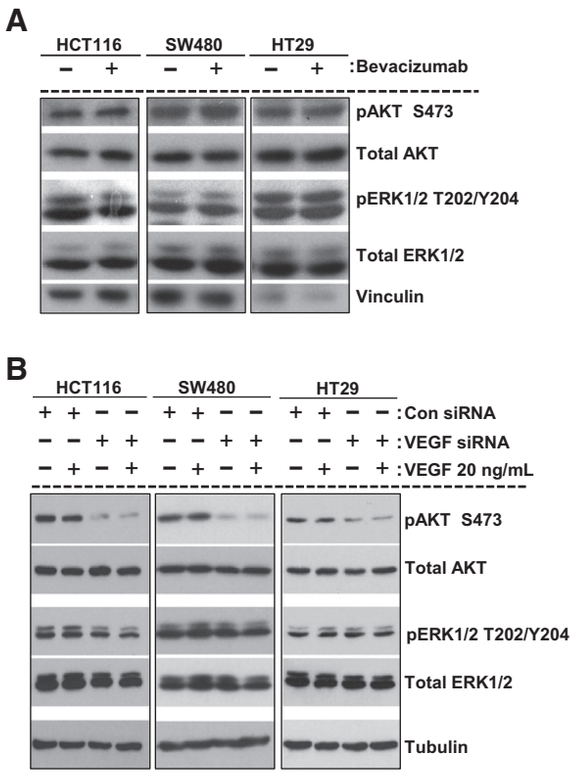

Figure 3.
Depletion of VEGF in colorectal cancer cells reduces pAKT and pERK by an intracellular mechanism. A, HCT116, SW480, and HT29 cells were treated with bevacizumab (250 μg/mL) for 24 hours, and the protein lysates of the treated cells were assayed for levels of pAKT, pERK1/2, total AKT, total ERK1/2, and vinculin. B, the protein lysates of HCT116, SW480, and HT29 cells transfected with VEGF siRNA and then treated with or without recombinant VEGF protein were analyzed for levels of phosphorylated and total AKT and ERK1/2 proteins. Con siRNA–transfected cells were utilized as controls.

decreases in phosphorylation levels of PRAS40, 70S6K, and BAD in colorectal cancer cells following VEGFR1 depletion (Fig. 4B) that were very similar to the effects of VEGF depletion. As expected, inhibiting VEGF-VEGFR1 binding on cell membranes with bevacizumab or a VEGFR1-binding antibody (18F1) did not alter pAKT or pERK1/2 levels compared with those in untreated control colorectal cancer cells (Fig. 4C). Interestingly, inhibition of the kinase function of VEGFR1 by pazopanib or SU5416 also had no effect on pAKT or pERK1/2 levels (Fig. 4C). [All the reagents were tested for efficacy by measuring their effects on VEGFR1 phosphorylation (Supplementary Fig. S4).] Together, these data indicate that intracellular VEGFR1 plays an important role in the intracellular VEGF-mediated regulation of pAKT and pERK1/2 that is independent of its kinase activity. Similar results were also obtained in experiments with SW480 cells (Supplementary Fig. S5). VEGFR1 is alternately spliced to produce a secreted version of VEGFR1, soluble VEGFR1 (sVEGFR1; ref. 19), that lacks both of the membrane binding and kinase domains, but has very strong binding affinity for VEGF. To determine if colorectal cancer cells expressed sVEGFR1 and if this protein could possibly form a complex with VEGF intracellularly, we analyzed cell lysates from different colorectal cancer cells for the presence of sVEGFR1 (Supplementary Fig. S6). HUVEC cell lysates were analyzed simultaneously as positive controls for sVEGFR1. However, there were no detectable intracellular sVEGFR1 in colorectal cancer cells, indicating the full-length VEGFR1 to be the major mediator in this intracellular signaling mechanism. Our recent studies have also demonstrated a complex pseudogene-mediated regulation of full-length VEGFR1 in colorectal cancer cells (20). Because the VEGFR1 pseudogene can regulate VEGFR1

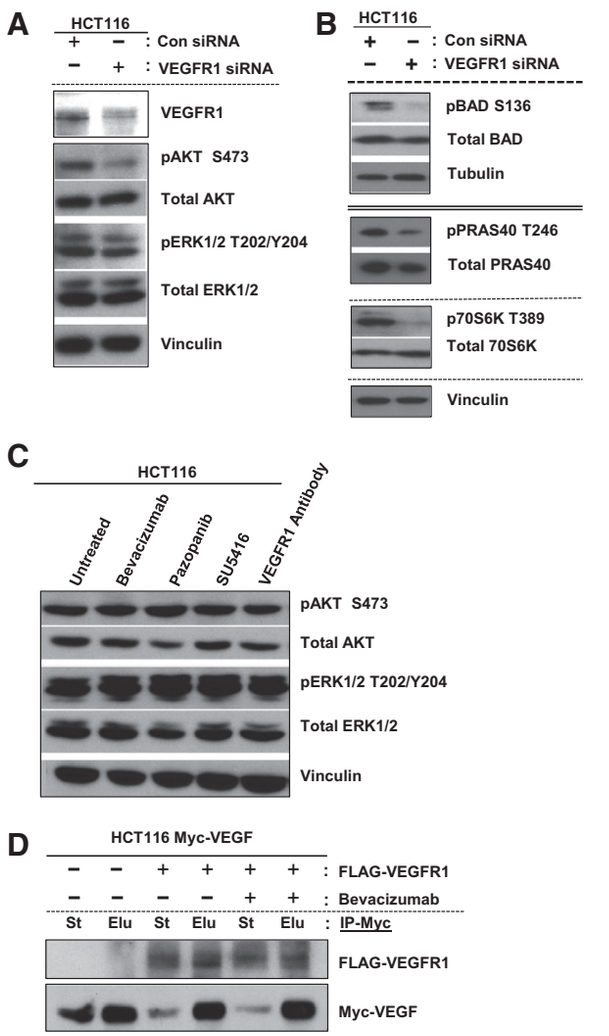

Figure 4.
Depletion of VEGFR1, but not inhibition of its kinase activity, produces effects similar to those of VEGF depletion in colorectal cancer cells. A, HCT116 colorectal cancer cells were transfected with either control siRNA (Con siRNA) or VEGFR1 siRNA and assayed for changes in pAKT and pERK1/2 levels. B, Western blots to validate alterations in the activity of proteins downstream of AKT were performed with lysates from control siRNA or VEGFR1 siRNA–treated HCT116 cells. C, HCT116 cells were incubated with antibodies that inhibit VEGF-VEGFR1 binding or VEGFR1 kinase inhibitors and were assayed for changes in pAKT and pERK1/2 levels. D, HCT116 cells stably expressing Myc-tagged VEGF were transiently transfected with empty vector or a plasmid-expressing FLAG-tagged VEGFR1. After 24 hours, the cells were treated with or without bevacizumab for another 24 hours. Protein lysates from all experimental sets were incubated with anti-Myc resin, and the immunoprecipitated proteins were analyzed by Western blotting for FLAG-VEGFR1 and Myc-VEGF. St, starting material; Elu, eluted material.

expression, but does not modify its function, effects of the VEGFR1 pseudogene on VEGF/VEGFR1 intracrine signaling were not further studied. Since our previous studies showed that colorectal cancer cells express very low to nondetectable levels of VEGFR2 and VEGFR3 (8, 12), VEGF–VEGFR2 and VEGF–VEGFR3 interactions were not investigated in the present study.

VEGF and VEGFR1 interact intracellularly

To determine whether VEGF and VEGFR1 interact to form a receptor–ligand complex, we performed coimmunoprecipitation experiments. As most antibodies against VEGF or VEGFR1 are weak, have bindings sites possibly hindering VEGF–VEGFR interactions, and have cross-reactivity to other proteins, as is observed in Western blots, these experiments were performed with epitope-tagged recombinant proteins. HCT116 cells stably expressing Myc-tagged $VEGF_{165}$ (Myc-VEGF) were transiently transfected with plasmids expressing FLAG-tagged VEGFR1 (FLAG-VEGFR1). Empty vector–transfected HCT116 cells expressing Myc-VEGF and FLAG-VEGFR1–transfected HCT116 cells not expressing Myc-VEGF were used as controls. Immunoprecipitation assays demonstrated that VEGFR1 and VEGF interact and form a complex in colorectal cancer cells (Fig. 4D; Supplementary Fig. S7). To determine whether this interaction was intracellular, rather than occurring on the cell membrane, we performed similar coimmunoprecipitation experiments with and without bevacizumab. FLAG-VEGFR1 coimmunoprecipitated with Myc-VEGF in both untreated HCT116 cells and HCT116 cells treated with bevacizumab for extended periods (~16 hours; Fig. 4D). As prolonged bevacizumab treatment should inhibit any VEGF-VEGFR1 binding at the cell surface, these observations strongly support the formation of an intracellular VEGF–VEGFR1 complex.

We also performed immunostaining of colorectal cancer cells to visualize intracellular localization of VEGF and VEGFR1 (Fig. 5). Using antibodies specific to VEGF (rabbit polyclonal), VEGFR1 (mouse monoclonal), and Lamin B (goat polyclonal), we performed confocal microscopy and obtained images. Both VEGF and VEGFR1 were observed in the cytoplasm and also on the nuclear membrane (Fig. 5). Some intranuclear localization of both proteins was also observed (Fig. 5). These observations are consistent with our studies that indicate the presence of both VEGF and VEGFR1 in both cytoplasm and the nucleus by Western blotting of fractionated protein extracts (data not shown). The strongest colocalization of VEGF and VEGFR1 was observed on and near the nuclear membrane, although cytoplasmic colocalization was also observed. We used a different set of antibodies (bevacizumab: anti-VEGF, rabbit polyclonal anti-VEGFR1 and goat polyclonal anti-Lamin B) to perform similar immunostaining and found very similar results (Supplementary Fig. S8), thus validating our observations.

A tyrosine phosphatase mediates VEGF's regulation of pAKT and pERK1/2

Activation of AKT through a VEGF-VEGFR1 signaling pathway in hepatic stellate cells (21) or through VEGF-VEGFR2 signaling in endothelial cells (22) has been reported previously. These signaling events are initiated through ligand–receptor interactions on the cell membrane. However, we sought to identify the mechanism by which intracellular VEGF signaling regulates AKT phosphorylation. There are two possible mechanisms that explain reduced pAKT levels in colorectal cancer cells with VEGF depletion: (i) the activation of a VEGF-regulated phosphatase causes pAKT to be dephosphorylated at a higher rate, and (ii) the VEGF-mediated regulation of upstream signaling factors inhibits AKT phosphorylation (shown pictorially in Supplementary Fig. S9). To identify the mechanism by which VEGF regulates AKT phosphorylation, we incubated colorectal cancer cells treated with VEGF siRNAs in the presence of phosphatase inhibitors and assayed for rescued pAKT levels. Inhibition of serine/threonine phosphatases by okadaic acid did not rescue pAKT levels in VEGF-depleted cells to the levels in cells with normal VEGF (Fig. 6). However, treating VEGF-depleted cells with a tyrosine phosphatase inhibitor, Na_3VO_4, rescued pAKT levels to similar or higher than that observed in cells with normal levels of VEGF (Fig. 6). pERK1/2 levels appeared to be rescued by both the serine/threonine phosphatase inhibitor and the tyrosine phosphatase inhibitor (Fig. 6). Because a tyrosine phosphatase inhibitor and not a serine/threonine phosphatase inhibitor rescued AKT phosphorylation at the serine 473 residue, it clearly indicates that the VEGF-mediated activation of a serine/threonine phosphatase is not responsible for decreased pAKT. However, this finding did raise the possibility that pAKT levels may be regulated by the inactivation of upstream signaling molecules, e.g., RTKs, which can in turn be regulated by the activity of tyrosine phosphatases.

VEGF depletion affects the phosphorylation of multiple RTKs

Tumor cells express a variety of RTKs on their cell surface. It is possible that VEGF-mediated regulation of one or multiple RTKs can lead to altered pAKT levels in colorectal cancer cells. We took an unbiased approach to determine whether VEGF depletion inactivates single or multiple RTKs in colorectal cancer cells. Protein lysates from colorectal cancer cells treated with control siRNA and VEGF siRNA were analyzed on a human phospho-RTK array that enables detection of the phosphorylation status of approximately 50 phospho-RTKs. The phosphorylation levels of multiple RTKs in VEGF siRNA–treated HCT116 cells were significantly different from those in control siRNA–treated cells (Fig. 7A and B). Reduced phosphorylation in EGFR, c-MET, MSP-R, IR, c-RET, AXL, and DTK receptors was observed in the VEGF-depleted HCT116 cells (Fig. 7A and B). Similar effects were observed in SW480 cells, which had lower levels of phosphorylated EGFR, IR, and DTK (Supplementary Fig. S10). Alterations in a variety of RTKs in colorectal cancer cells indicated that VEGF depletion has a broad and significant effect on cell signaling. We further validated these observations by measuring the levels of phosphorylated EGFR and c-MET individually in HCT116 cells treated with either control siRNA or VEGF siRNA (Fig. 7C; Supplementary Fig. S10). To further validate our hypothesis that VEGF depletion enhances the activity of one or more tyrosine phosphatases, we treated colorectal cancer cells with Na_3VO_4 and assayed for rescue of pEGFR and pc-MET levels (Fig. 6C). If VEGF depletion inhibited RTK phosphorylation, Na_3VO_4 treatment would not significantly increase phosphorylated RTK levels. However, if phosphorylation of RTKs is not affected, but VEGF depletion enhances phosphatase activity, Na_3VO_4 treatment would easily rescue phosphorylated RTKs to normal or higher than normal levels. Na_3VO_4 treatment significantly increased pEGFR and pc-MET levels in VEGF-depleted cells, supporting a mechanism involving a VEGF-mediated phosphatase (Fig. 7C). Similar results were also obtained for SW480 cells (Supplementary

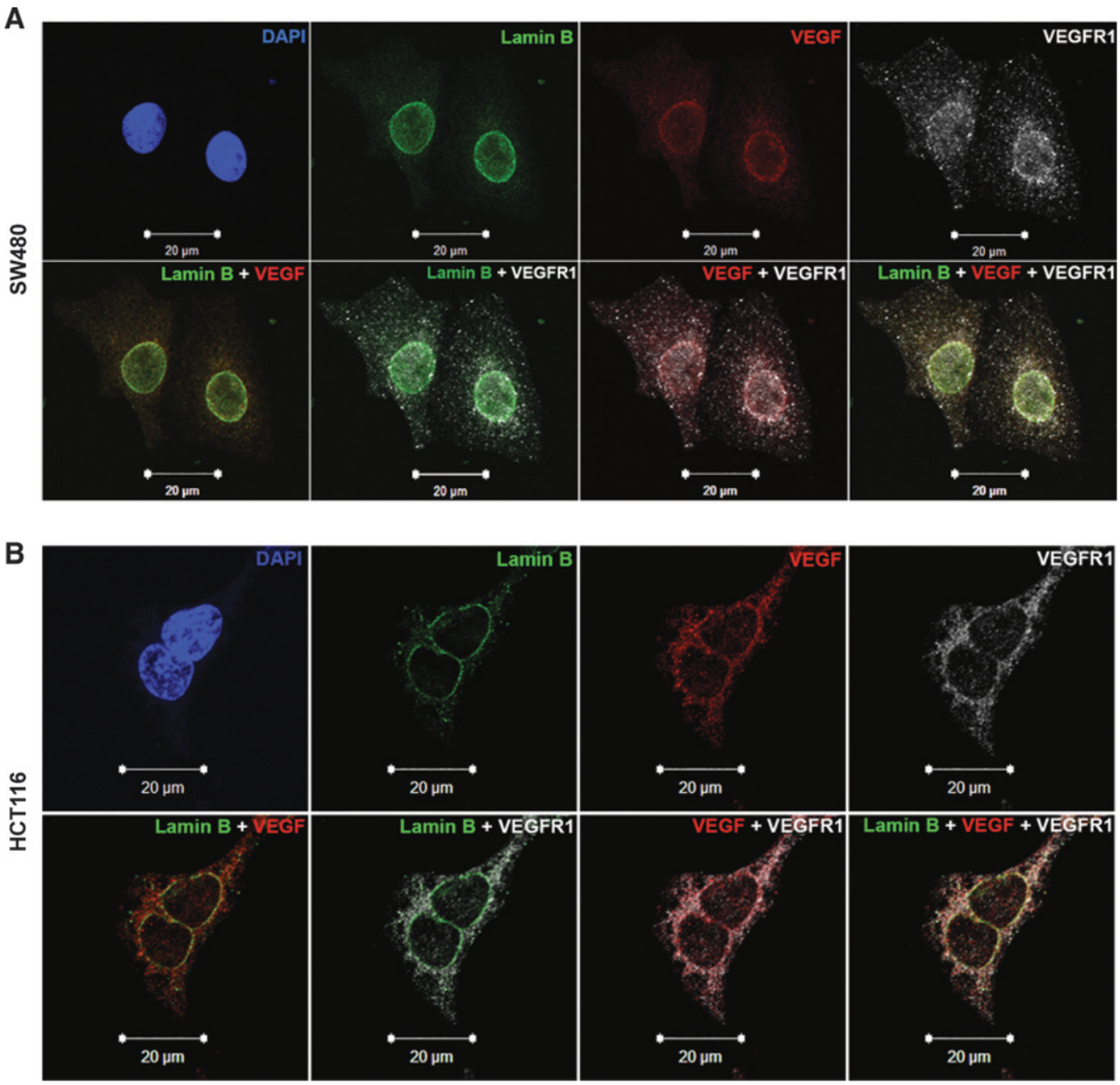

Figure 5.
Localization of VEGF and VEGFR1 in colorectal cancer cells. A and B, SW480 (A) and HCT116 (B) cells were grown on chamber slides, fixed with paraformaldehyde, and stained with DAPI (blue), goat polyclonal anti-Lamin B (green), rabbit polyclonal anti-VEGF (red), and mouse monoclonal anti-VEGFR1 (white) antibodies. Overlay of various markers is also shown for both sets.

Fig. S10), validating our studies in different colorectal cancer cell lines.

Overexpression of RTKs rescues reduced pAKT levels in VEGF-depleted colorectal cancer cells

Overexpression of RTKs could possibly rescue the effects of increased phosphatase activity in VEGF-depleted colorectal cancer cells. To test this hypothesis, HCT116 and SW480 cells were doubly transfected with VEGF siRNAs and FLAG-tagged EGFR plasmids. FLAG-EGFR–expressing cells had higher pEGFR and increased pAKT levels compared with empty vector controls in VEGF-depleted colorectal cancer cells validating our previous observations (Supplementary Fig. S11).

Discussion

The biology behind tumor-derived VEGF's regulation of the functions of endothelial cells and the tumor vasculature has been well characterized (1, 18, 23). However, the roles and effects of VEGF signaling in or on tumor cells are still not well understood. Multiple studies indicate that VEGF has a direct effect on the survival and growth of a variety of cancer cells (24–28), mostly through an autocrine signaling mechanism. Lee and colleagues (11) showed that the depletion of VEGF or VEGFR1 in breast cancer cells resulted in reduced tumor cell survival and postulated that VEGF functions as an intracellular autocrine or "intracrine" survival factor. Our previous study using VEGF-deficient human

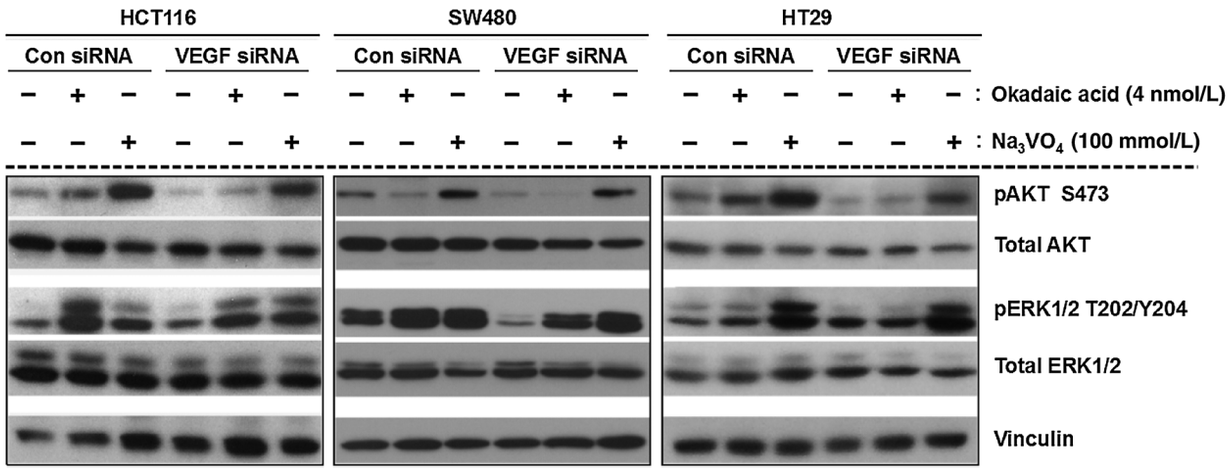

Figure 6.
Intracellular VEGF signaling is mediated through a tyrosine phosphatase. Levels of total and phosphorylated AKT and ERK1/2 were measured in HCT116, SW480, and HT29 cells transfected with control siRNA (Con siRNA) or VEGF siRNA and then treated with okadaic acid (4 nmol/L) or Na_3VO_4 (100 μmol/L) for 16 hours.

colorectal cancer cell lines generated by genetic deletion (29) showed that VEGF acts as a survival factor for human colorectal cancer cells (12). These effects were not mediated in an autocrine or paracrine fashion, suggesting that VEGF has an intracrine function in colorectal cancer cells.

In this study, we present further evidence for the novel intracrine signaling pathway regulated by intracellular VEGF and provide insights into the molecular mechanisms underlying this signaling pathway in colorectal cancer cell survival. Depletion of VEGF by siRNAs recapitulated the effects of somatic VEGF depletion in colorectal cancer cells, i.e., reduced survival and enhanced chemosensitivity, suggesting that VEGF depletion combined with chemotherapy may prove beneficial to patients where conventional therapy, including VEGF-targeted therapies, has failed. RPPA analyses and validation experiments indicated that VEGF depletion reduced the activity of survival molecules AKT and ERK1/2. These pathways were unaffected when colorectal cancer cells were treated with antibodies inhibiting VEGF–VEGFR interactions or with VEGFR kinase inhibitors. In addition, supplementing the media of VEGF siRNA–treated colorectal cancer cells with recombinant VEGF also failed to rescue the effects of VEGF depletion on AKT and ERK1/2.

Interestingly, we found that tyrosine phosphatase inhibitors, rather than Ser/Thr phosphatase inhibitors, rescued effects of VEGF depletion on AKT. Through unbiased assays, we also show that phosphorylation of multiple RTKs is decreased in VEGF-depleted colorectal cancer cells in comparison with colorectal cancer cells with normal VEGF levels. Treating VEGF-depleted colorectal cancer cells with a tyrosine phosphatase inhibitor rescued the reduced phosphorylation of the RTKs. Based on our findings, we propose that VEGF depletion activates a tyrosine phosphatase to reduce activity of multiple RTKs, which in turn leads to reduced activity of AKT and ERK1/2, resulting in decreased cell survival and enhanced chemosensitivity (Fig. 7). Future studies, including identification of the phosphatase and depletion of the phosphatase, are required to elucidate the mechanism underlying VEGF/VEGFR's role in cell survival.

Another key finding from our study is that VEGFR1 depletion, similar to VEGF depletion, reduces pAKT levels. Inhibition of VEGF-VEGFR1 binding by bevacizumab or 18F1, or targeting the kinase activity using two different receptor kinase inhibitors, failed to produce similar effects. Also, *in vitro* immunoprecipitation experiments indicated the intracellular formation of a VEGF–VEGFR1 complex. These observations strongly suggest that a VEGF–VEGFR1 complex is functional in the intracrine signaling mechanism. The possibility of a VEGF–VEGFR1 intracellular complex has been postulated before in breast cancer cells (11). A separate study in mouse skin tumors also indicated that VEGF-VEGFR1 is required for tumor cell proliferation in a cell-autonomous manner (30). Put together, these findings strongly support a VEGF-VEGFR1–mediated intracrine signaling in multiple cancer types and suggest a new kinase-independent function for VEGFR1 in regulating signaling pathways in cancer cell survival. However, to better understand the role of VEGFR1 in this intracrine signaling process, studies using kinase-dead mutants of VEGFR1 and identifying possible interacting partners are warranted.

The discovery of VEGF's importance in angiogenesis, a process essential to tumor growth (31), led to VEGF's importance as a therapeutic target. Although targeting VEGF has proven effective against certain tumor types, such as renal cell carcinoma (32, 33), the overall benefits of blocking VEGF signaling have not been as beneficial as initially expected (6, 23, 34–36), and multiple mechanisms of resistance to anti-VEGF therapy have been proposed (5, 37, 38). However, understanding the mechanisms of intracrine VEGF signaling and its effects on tumor cell survival presents new possibilities for targeting VEGF not only in tumor cells but also in endothelial cells that are susceptible to the depletion of intracellular VEGF (39). Such targeting can be achieved with advances in the delivery of VEGF-targeting siRNAs using liposomal formulations. One such study targeting VEGF and kinesin spindle protein in human patients has shown some interesting findings, including a patient with a complete response to therapy (40). In fact, findings from our studies (12) suggest that inhibiting intracrine VEGF signaling would have maximum benefit when combined with chemotherapy.

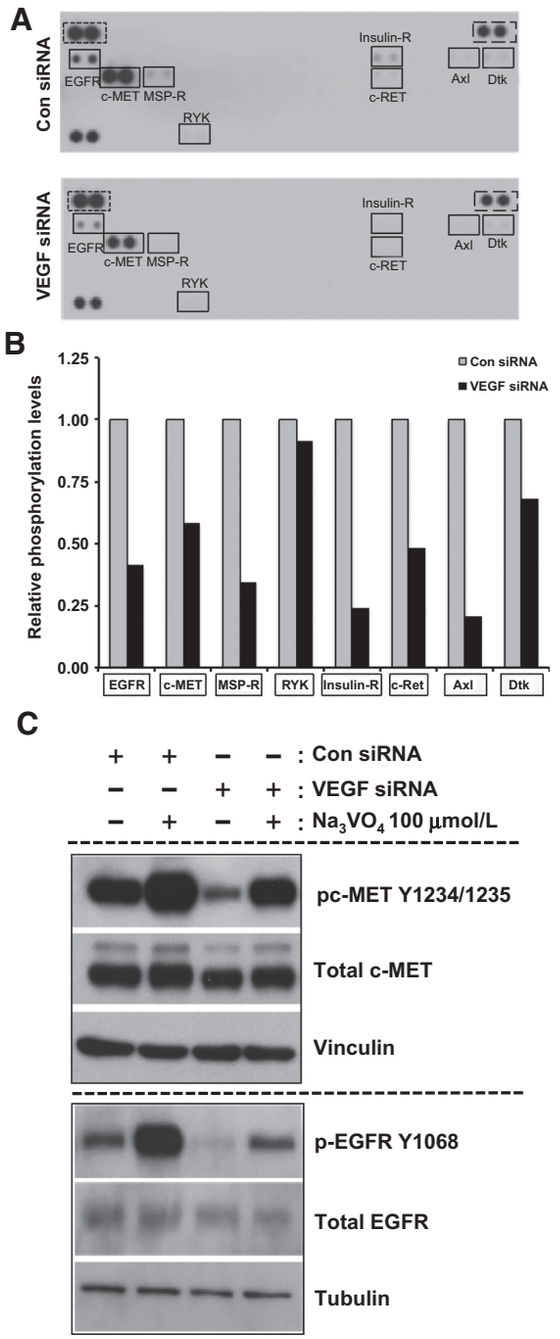

Figure 7.
VEGF depletion affects the phosphorylation of multiple RTKs in colorectal cancer cells. A, HCT116 cells were transfected with control siRNA (Con siRNA) or VEGF siRNA, and the cell lysates were analyzed for alterations in multiple phospho-RTK levels using a human phospho-RTK array. Various pRTK spots are shown enclosed in boxes, and their identities are indicated adjacent to the box. Broken boxes indicate internal control spots that were used for normalizing and measuring intensity differences. B, bar diagram representing phosphorylation levels of RTKs in Con siRNA– or VEGF siRNA–treated HCT116 cells. Gray and black bars indicate Con siRNA and VEGF siRNA, respectively. All results are from a single experimental set. C, pc-MET and pEGFR levels in HCT116 cells transfected with Con siRNA or VEGF siRNA following VEGF depletion in the presence or absence of a tyrosine phosphatase inhibitor (Na_3VO_4).

The roles of VEGF-VEGFR signaling and the effects of inhibiting VEGF and/or VEGFR in various cancers are quite complex. Some recent studies of glioblastoma and pancreatic neuroendocrine tumors in mouse models have indicated that antiangiogenesis therapy may induce tumor invasiveness and increase metastasis (41, 42). Similar results have been observed in human breast cancer cells in mice (43). However, the implications of these studies in humans are not well understood. These effects were shown to result from increased c-MET activation due to VEGF blockade (42, 44), where blocking paracrine VEGF–VEGFR2 interaction inactivated the PTP1B phosphatase to increase pc-MET levels (44). Inversely, our findings suggest that an intracellular VEGF–VEGFR1 complex interacts and inactivates an as-yet unidentified tyrosine phosphatase in colorectal cancer cells. Depletion of either VEGF or VEGFR1 results in activation of this phosphatase, resulting in reduced RTK activation. Our previous studies (8, 12) indicate that colorectal cancer cells predominantly express VEGFR1 in contrast to VEGFR2 and VEGFR3. Also, our studies and the previous study in breast cancer cells (11) indicate that VEGFR1 is mostly intracellular and found in both, the cytoplasm and the nucleus. Immunofluorescence and colocalization studies indicate that most of the VEGF and VEGFR1 interaction is at the nuclear membrane and in other parts of the cytoplasm. However, based on the functional consequences (reduction of RTK, AKT activity), we hypothesize that the intracellular signaling is mainly due to the cytoplasmic or perinuclear VEGF–VEGFR1 complexes. Based on these findings, intracellular VEGF–VEGFR1 complexes and VEGF–VEGFR2 complexes formed at cell membrane appear to have opposing effects on RTK activation and thus on cell viability and migration. Thus, the VEGF receptor status and their location in the tumor cells may dictate the function of VEGF signaling and their effects on tumor cells.

In conclusion, our study reveals a novel mechanism of intracrine VEGF signaling that mediates the survival of colorectal cancer cells. This study also demonstrates that antiangiogenic therapies targeting VEGF–VEGFR interactions on the cell membrane or inhibiting VEGFR kinase activity do not affect colorectal cancer cell survival through the mechanisms by which targeting VEGF inside the cell does. Depleting intracellular VEGF appears to effectively induce colorectal cancer cell death and enhance the efficacy of chemotherapy and thus provides new opportunities to therapeutically manipulate the VEGF-dependent survival of these cells.

Disclosure of Potential Conflicts of Interest

L.M. Ellis is a consultant/advisory board member for Genentech/Roche. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

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Development of methodology: R. Bhattacharya, X.-C. Ye, R. Wang, M. McManus
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): R. Bhattacharya, X. Ling, F. Fan
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): R. Bhattacharya, L.M. Ellis
Writing, review, and/or revision of the manuscript: R. Bhattacharya, D. Boulbes, L.M. Ellis
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): R. Wang
Study supervision: L.M. Ellis

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