# Intracrine Vascular Endothelial Growth Factor Signaling in Survival and Chemoresistance of Human Colorectal Cancer Cells

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## Abstract

Although the effects of VEGF on angiogenesis and vascular function are well known, the effects of VEGF on tumor cell function remain to be elucidated. We studied phenotypic changes in human colorectal cancer (CRC) cells with homozygous deletion of VEGF alleles to determine the potential direct role of VEGF on tumor cell function. Loss of VEGF expression led to significantly decreased cell growth and increased spontaneous apoptosis in CRC cells (p<0.01). Loss of VEGF also increased the *in vitro* sensitivity of cells to the cytotoxic effects of the chemotherapeutic drug 5-fluorouracil, as shown by increased apoptosis (p<0.05). These effects were mediated via upregulation of the proapoptotic mediators caspase-3, cleaved PARP and Bax and downregulation of the pro-survival mediator survivin. Our findings suggest a novel and distinct function of VEGF in mediating autocrine/intracrine CRC cell survival.

### Keywords

VEGF; chemosensitivity; colorectal cancer; autocrine; intracrine; apoptosis

## Introduction

Vascular endothelial growth factor A (VEGF-A, or simply VEGF) was originally identified as a 'permeability factor' and was later shown to also mediate endothelial cell proliferation (Connolly et al., 1989; Leung et al., 1989; Senger et al., 1983; Senger et al., 1986). VEGF is the prototypical member of a family of VEGF ligands that includes placental growth factor

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**Conflict of interest**

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(PlGF) and VEGFs A–E (Ferrara et al., 2003; Houck et al., 1991; Tischer et al., 1991). VEGF expression is upregulated in the majority of human tumors, and its expression is inversely associated with survival. Standard models of VEGF biology focus on tumor cell secretion of VEGF, whereby it acts in a paracrine manner to attract and stimulate proliferation of endothelial cells (reviewed in (Brown et al., 1997)). It has since been shown that VEGF can also mediate a protective/survival effect on a number of cell types, including endothelial cells, embryonic stem cells and hematopoietic stem cells (Ferrara et al., 1996; Gerber et al., 2002; Gerber et al., 1998). In addition, recent studies have shown that VEGF acts as an autocrine/paracrine growth and survival factor for tumor cells that express VEGF receptor (Bachelder et al., 2001; Barr et al., 2008; Calvani et al., 2008; Dias et al., 2002; Pidgeon et al., 2001; Santos & Dias, 2004; Vincent et al., 2005).

Our laboratory previously demonstrated that expression of the VEGFR-1 receptor by colorectal cancer (CRC) cells mediates cell motility and invasion (Fan et al., 2005). The presence of VEGF receptors on CRC cells led us to hypothesize that VEGF has a direct effect on tumor cell function that may contribute to tumor cell survival. However, preliminary studies in our laboratory with the use of VEGF-neutralizing antibodies did not lead to changes in cell survival. Therefore, we sought to understand the role and mechanism of intracrine VEGF signaling in CRC. In this report, we demonstrate that loss of intracrine VEGF signaling leads to an increase in spontaneous apoptosis and chemosensitivity. These findings have implications for a better understanding of mechanisms of action (or lack thereof) of VEGF-targeted therapies when combined with chemotherapy in patients with metastatic CRC.

## Results

### Loss of autocrine VEGF decreases growth of CRC cells

To determine the role of autocrine VEGF in CRC cell growth and survival, we used two pairs of isogenic CRC lines in which the VEGF gene was disrupted. HCT116/VEGF$^{-/-}$ and LS174T/VEGF$^{-/-}$ cells were generated by homologous recombination–mediated deletion of the VEGF alleles as described previously (Dang et al., 2006). The loss of VEGF expression in the VEGF$^{-/-}$ cells was confirmed by western blot analysis. As shown in Figure 1A, VEGF expression was undetectable in conditioned medium from HCT116/VEGF$^{-/-}$ and LS174T/VEGF$^{-/-}$ cells compared with expression in the corresponding parental cells.

To determine the biological effect of loss of autocrine/intracrine VEGF on CRC cells *in vitro,* we first assessed cell growth using the 3-(4,5-dimethyl-2-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. To exclude the effects of exogenous VEGF (fetal bovine serum (FBS)–derived), the cells were grown for the assay under low-serum (1%) conditions. As shown in Figure 1B, loss of VEGF expression led to significant inhibition in cell growth compared with the control at days 2 and 3 in HCT116 cells (top panel, p< 0.01) and at days 1, 2 and 3 in LS174T cells (bottom panel, p< 0.01). Since decreased cell number could result from a combination of deregulated cell proliferation and cell death, we investigated, using propidium iodide (PI)/fluorescence-activated cell-sorting (FACS) analysis, whether loss of VEGF affected cell cycle progression in the cells. As shown in Figure 2A, cell cycle analysis revealed a significantly higher percentage of HCT116/VEGF$^{-/-}$ cells (12.3% ±

0.8%) with sub-G0/G1 DNA content, compared with parental cells (6.1% ± 0.4%, p = 0.002) (Figure 2A); these results represent the mean ± standard error of three independent experiments). A similar trend was observed in the LS174T/VEGF$^{-/-}$ cells, although the difference was not statistically significant.

## Loss of VEGF increases spontaneous apoptosis in CRC cells

Having observed decreased survival of the VEGF$^{-/-}$ cells, we sought to determine whether this result was due to an increase in spontaneous apoptosis. HCT116/VEGF$^{+/+}$ and VEGF$^{-/-}$ cells were grown under low-serum conditions for 48h, stained with Annexin V/FITC and analyzed by flow cytometry. As shown in Figure 2B, there was a significant increase in the spontaneous apoptotic rate of the HCT116/VEGF$^{-/-}$ cells (17.2% ± 0.6%) compared with VEGF$^{+/+}$ cells (11.5 ± 0.7%, p = 0.004). The Annexin V assay could not be performed on the LS174T cells because these cells tend to grow in clumps and require extensive trypsinization and manipulation to obtain a single-cell suspension; these processes damage the membranes of the cells and cause nonspecific binding of the dye, resulting in false-positive staining.

To determine whether the increased rate of spontaneous apoptosis in the VEGF$^{-/-}$ cells could be overcome by the addition of exogenous VEGF-A, HCT116 VEGF$^{-/-}$ cells were grown in serum-reduced medium (1% fetal calf serum) and stimulated with VEGF-A for 24 hr. Cells were then stained with Annexin V/FITC and analyzed by flow cytometry. However, this treatment did not rescue the rate of spontaneous apoptosis in the VEGF–/– cells, suggesting an intracrine function of VEGF (data not shown).

To examine the mechanism by which loss of VEGF contributes to increased spontaneous cell death, we analyzed the VEGF$^{+/+}$ and VEGF$^{-/-}$ CRC cells for expression of apoptotic mediators. The caspases are key mediators of apoptosis (Cohen, 1997; Cryns & Yuan, 1998). Caspase 3, the effector caspase in the apoptotic cascade, is activated through cleavage by caspase-9 as a result of an increase in mitochondrial permeability and release of cytochrome C. Cleaved caspase 3 was assessed by western blot analysis. As shown in Figure 2C, we noticed an increased level of cleaved caspase-3 in the VEGF$^{-/-}$ cells. In addition, we observed increased constitutive expression of the proapoptotic protein Bax and decreased expression of the pro-survival factor survivin in the VEGF$^{-/-}$ cells (Figure 2C).

## Loss of autocrine VEGF increases the chemosensitivity of CRC cells to 5-fluorouracil

Since loss of VEGF led to an increase in spontaneous cell death and apoptosis, we investigated whether it would also affect cells' response to chemotherapeutic drugs, such as 5-fluorouracil (5FU). Treatment with a clinically relevant dose of 5FU significantly decreased survival of both HCT116/VEGF$^{-/-}$ and LS174T/VEGF$^{-/-}$ cells compared with survival of the parental cells. As shown in Figure 3A, we observed a significantly higher percentage of dead cells among 5FU-treated HCT116/VEGF$^{-/-}$ cells (23.2% vs. 7.5%, p<0.05) and LS174T/VEGF$^{-/-}$ cells (24.8% vs. 8.0%, p<0.05) than among parental cells.

To further validate the above-described findings, HCT116/VEGF$^{+/+}$ and VEGF$^{-/-}$ cells treated with 5FU for 48h were stained with Annexin V/FITC and analyzed by flow cytometry. As shown in Figure 3B, there was a significant increase in the 5FU-induced

apoptotic rate of the HCT116/VEGF$^{-/-}$ cells (11.1% ± 0.6%) compared with the VEGF$^{+/+}$ cells (4.8 ± 0.6%, p=0.004).

To confirm the mechanism of 5FU-induced cell death, we compared the activation of apoptotic mediators in the VEGF$^{+/+}$ and VEGF$^{-/-}$ CRC cells treated with 5FU. The cleavage of caspase-3 and PARP, were assessed by immunoblotting using antibodies that specifically recognize their cleaved products. Consistent with the increased apoptosis in the 5FU-treated VEGF$^{-/-}$ cells, we observed a marked increase in the levels of active caspase-3 in these cells (Figure 3C). PARP is cleaved by activated caspase-3, and since PARP cleavage is the signature event in apoptosis, we rationalized that analysis of cleaved PARP level should indicate an increase in spontaneous apoptosis in the VEGF$^{-/-}$ cells. Both HCT116/VEGF$^{-/-}$ and LS174T/VEGF$^{-/-}$ cells were found to have increased PARP cleavage following 5FU treatment compared with parental cells. To further confirm the specificity of 5FU-induced apoptosis in VEGF$^{-/-}$ cells, we analyzed the activation of PARP in these cells in response to increasing concentration of 5FU. As shown in Figure 3D, HCT116/VEGF$^{-/-}$ cells demonstrated a dose-dependent increase in PARP cleavage when treated with increasing concentrations of 5FU.

To further validate these findings in an alternate system using an additional knockout/knockdown approach, we transiently downregulated VEGF expression in HCT116/VEGF$^{+/+}$ cells using VEGF specific siRNA. As shown in Supplementary figure 1, treatment with 5FU led to a marked increase in the levels of the apoptotic markers, cleaved caspase 3 and cleaved PARP and decrease in level of antiapoptotic protein, surviving, in the siVEGF-A cells compared to siControl cells.

To examine the effect of VEGF loss on apoptosis *in vivo,* we stained xenograft tissue samples derived from HCT116/VEGF$^{+/+}$ and VEGF$^{-/-}$ CRCcells for cleaved caspase 3. Tumors derived from VEGF$^{-/-}$ cells exhibited more cleaved caspase 3 positive cells compared to the VEGF$^{+/+}$ derived tumors.

## Intracrine VEGF regulates CRC cell survival

To further explore the mechanism of VEGF signaling–mediated survival in these cells, we examined the receptor status of the cells. Transcripts corresponding to VEGFR-1, VEGFR-2, NRP-1 and NRP-2 were detectable by reverse transcription (RT)–PCR analysis in both cell lines (Figure 4A). However, only VEGFR-1, NRP-1 and NRP-2 were detected at the protein level by western blot analysis in both cell lines. We did not detect VEGFR-2 protein in these cell lines by western blot analysis even after repeated efforts using several different antibodies to the protein. The fact that VEGFR-2 transcripts were detected (albeit at very low levels) in these cells suggests that perhaps the VEGFR-2 transcripts are relatively unstable or translated at very low levels (below the threshold for detection by western blot) in these cells. VEGFR-3 was undetectable by both RT-PCR and western blot analysis in these cells. No significant differences were noted in the expression of the receptors between the VEGF$^{+/+}$ and VEGF$^{-/-}$ cells.

To examine the effect of VEGF loss on VEGFR1 receptor activation status, we used immunoblot analysis to examine VEGFR1 receptor phosphorylation. As shown in Figure

4B, there was a marked increase in VEGFR1 phosphorylation in the VEGF$^{-/-}$ cells compared to parental cells. To determine whether the changes in VEGFR1 phosphorylation observed in the VEGF$^{-/-}$ cells had resulted from potential changes in ligand expression, we next examined the levels of PlGF, a known ligand for VEGFR-1, in these cells. PlGF level, as determined by ELISA, was significantly higher in the VEGF$^{-/-}$ compared to parental cells suggesting that absence of intracrine VEGF survival signaling in CRC cells results in the activation of a compensatory pathway mediated through the PlGF/VEGFR1 axis.

To gain further insight into the role of autocrine VEGF signaling in mediating survival of CRC cells, we compared the effect of loss of VEGF expression (intracellular and secreted, as in the case of the VEGF$^{-/-}$ cells) and that of extracellular inhibition of VEGF (with a blocking antibody) on spontaneous cell death in HCT116 and LS174T cells. HCT116 and LS174T cells were incubated in the presence of bevacizumab (a monoclonal anti-VEGF antibody) for 48h at 37°C, stained with PI and analyzed by flow cytometry. Treatment with bevacizumab had no significant effect on the rate of spontaneous cell death in either cell line (Figure 4C). To further dissect the autocrine VEGF signaling pathway, we studied the induction of spontaneous cell death in HCT116 and LS174T cells following treatment with SU5416, an intracellular inhibitor of VEGF receptor activation. PI/FACS analysis revealed no significant change in the rate of apoptosis in either cell line following treatment with SU5416 (Figure 4D), suggesting that VEGFR-1 kinase activity does not mediate survival of VEGF$^{-/-}$ cells.

## Discussion

In contrast to the role of tumor-derived VEGF in mediating the functions of endothelial cells in the tumor microenvironment, relatively little is known regarding the role of autocrine/intracrine VEGF signaling in tumor cells. An increasing body of evidence supports the notion that VEGF has a direct effect on tumor cells, acting as a growth and survival factor (Bachelder et al., 2001; Barr et al., 2008; Calvani et al., 2008; Dias et al., 2002; Lichtenberger et al., 2010; Pidgeon et al., 2001; Santos & Dias, 2004; Vincent et al., 2005). In a recent study, Lee et al (Lee et al., 2007b) reported that VEGF functions as an internal autocrine survival factor in human breast cancer cells through internally expressed VEGFR-1. Additionally, Masuda et al (Masuda et al., 2008) reported a novel function of the 5′-UTR of *vegf* mRNA in growth and survival of CRC cells. Lichtneberger et al. recently showed that epidermis-specific Flt1 deletion also impairs tumorigenesis and proliferation of skin tumors (Lichtenberger et al., 2010). These reports and the presence of a functional VEGF receptor in CRC cells (demonstrated previously by our laboratory (Fan et al., 2005)) led us to hypothesize that autocrine/intracrine VEGF has an essential growth/survival function in CRC cells.

In this study, using two VEGF-deficient human CRC cell lines generated by genetic deletion, we demonstrate that VEGF is a survival factor for human CRC cells. *In vitro,* VEGF-deficient cells displayed decreased cell viability and increased rates of spontaneous apoptosis when grown under low-serum conditions, a form of stress. We further demonstrate that loss of VEGF increased the susceptibility of CRC cells to apoptosis caused by 5FU treatment. Consistent with these findings, western blot analysis demonstrated upregulation

of several pro-apoptotic mediators. Treatment of CRC cells with bevacizumab, an anti-VEGF antibody (that acts exclusively on secreted/extracellular VEGF), did not affect cell survival, suggesting an intracrine survival function of VEGF in these cells. Additionally, treatment of the CRC cells with an intracellular suppressor of VEGF tyrosine kinase receptor activation, SU5416, did not affect cell survival, suggesting that the VEGF-mediated survival function in these cells is independent of kinase activity. It is possible that VEGF may bind to the non-kinase receptors neuropilin-1 or neuropilin-2 to mediate it's intracellular survival effects. Although PlGF was elevated in the VEGF−/− cells, it was unable to compensate for the loss of VEGF in these cells, suggesting again that the kinase activity of VEGFR-1 does not mediate survival signals in CRC cells.

While our studies have focused on function of intracrine VEGF signaling in mediating survival of CRC cells, the precise mechanisms by which VEGF mediates survival of CRC cells remain to be elucidated. In the case of endothelial cells, there is evidence for several mechanisms, including the induction of Bcl-2 (Nor et al., 2001) and interactions with integrins (Hutchings et al., 2003). Evaluation of the receptor status of the CRC cells revealed the expression of mRNA corresponding to both VEGFR-1 and VEGFR-2; however, only VEGFR-1 protein was detectable by western blot analysis, indicating that VEGFR-1 is the predominant VEGF tyrosine kinase receptor in these cells.

VEGF is one of the few genes where heterozygous knockout is embryonic lethal due to vascular abnormalities (Carmeliet et al., 1996; Ferrara et al., 1996), and thus held great promise as a therapeutic target. Although VEGF targeting has been shown to be a very effective therapeutic strategy in certain tumor types, such as renal cell carcinoma (Escudier et al., 2009; Rini et al., 2008), the overall benefit of blocking VEGF activity in other solid malignancies is marginal and has led to some skepticism in the field. However, understanding the ubiquitous role of VEGF intracrine signaling may provide new opportunities for targeting this protein, which is not only an angiogenic factor, but also an intracrine survival factor for both tumor cells (our study) and endothelial cells (Lee et al., 2007a). Newer technologies, such as siRNA therapeutics, may provide new opportunities to improve upon current VEGF targeted therapies.

Our study reveals a novel role of autocrine VEGF in mediating survival of CRC cells. These studies suggest that strategies to block VEGF signaling based on agents that neutralize VEGF (bevacizumab) or inhibit its receptors (kinase inhibitors) may not have a direct effect on tumor cells, but other methods that decrease or ablate intracellular VEGF may provide new therapeutic opportunities to exploit VEGF-dependent survival of these cells.

## Materials and Methods

### Cell lines and cell culture

The human CRC cell lines HCT116 and LS174T with homologous recombination–mediated deletion of VEGF alleles (HCT116/VEGF−/− and LS174T/VEGF−/−) were developed as previously described (Dang et al., 2006). The cell lines were maintained in minimal essential media (MEM) supplemented with 10% FBS, 2 mM L-glutamine, penicillin and streptomycin at 37°C with 5% $CO_2$.

### Reagents and antibodies

Bevacizumab (Genentech BioOncology, San Francisco, CA, USA) was obtained from the pharmacy at M. D. Anderson Cancer Center. SU5416 was obtained from Sigma-Aldrich (St. Louis, MO, USA). Antibodies and suppliers for western blot analysis were as follows: anti-VEGF-A (R&D Systems, Minneapolis, MN, USA); anti-VEGFR-1 (Calbiochem, San Diego, CA, USA); anti-NRP-1 and anti-NRP-2 (Santa Cruz, CA, USA); anti-vinculin and anti-actin (Sigma-Aldrich); and anti-caspase-3, anti–cleaved caspase-3, anti-PARP, anti–cleaved PARP, anti-Bax and anti-survivin (Cell Signaling Technology, Danvers, MA, USA).

### Cell proliferation assay

Cell growth was examined using an MTT assay (Sigma). Cells were plated in 96-well plates at 2,000 cells/well with MEM plus 10% FBS and allowed to attach for 24h. The medium was then replaced with MEM plus 1% FBS, and cells were incubated for 24, 48 or 72h. The MTT assay was done according to the manufacturer's protocol. Absorption was read at 570 nm.

**Isolation of mRNA and RT-PCR—**Total RNA was extracted from 60% to 70% confluent tumor cells growing in culture using TRIzol reagent according to the manufacturer's protocol (Life Technologies, Grand Island, NY, USA). RT-PCR was performed as described previously (Fan et al., 2005). Briefly, PCR amplification of VEGFR-1 was done under the following conditions: 40 cycles of 30 sec denaturation at 95°C, 30 sec annealing at 50°C and 1 min extension at 72°C. PCR amplification of VEGFR-2, VEGFR-3, NRP-1 and NRP-2 was done under the following conditions: 35 cycles of 30 sec denaturation at 95°C, 30 sec annealing at 57°C and 1 min extension at 72°C. PCR products were analyzed by electrophoresis of 20 μL of each PCR reaction mixture in a 1% agarose gel, and bands were visualized by ethidium bromide staining. The following primers were used: VEGFR-1 (516 bp): sense primer (5′-GACCTGGAGTTACCCTGA-3′) and antisense primer (5′-GACACGGCCTTTTCGTA-3′); VEGFR-2 (402 bp): sense primer (5′-CATCACATCCACTGGTATTGG-3′) and antisense primer (5′-GCCAAGCTTGTACCATGTGAG-3′); VEGFR-3 (381 bp): sense primer (5′-CCCACGCAGACATCAAGACG-3′) and anti sense primer (5′-TGCAGAACTCCACGATCACC-3′); NRP-1 (452 bp): sense primer (5′-ACGATGAAATGTGGCGATACT-3′) and anti sense primer (5′-AGTGCATTCAAGGCTGTTGG-3′) and NRP-2 (407 bp): sense primer (5′-ACCGTGCGGAGGTCGTTT-3′ and anti sense primer (5′-ATTCGATGGTCCCGTTGG-3′).

### Western blot analysis

Cells were solubilized in 20 mM Tris-Cl (pH 8.0), 137 mM NaCl, 1% Triton X-100, 1 mM $Na_3VO_4$ and 2 mM ethylenediaminetetraacetic acid with protease inhibitor. Extracts were cleared by centrifugation. The protein content was determined using the Bradford assay. Fifty micrograms of protein per sample was separated on 8% sodium dodecyl sulfate polyacrylamide gels and transferred to polyvinylidene difluoride membranes. Membranes were probed with primary antibodies overnight at 4°C, and the next day, they were washed

thrice for 10 min with Tris-buffered saline and 0.1% Tween-20 and reprobed with the appropriate secondary antibody for 1h at room temperature. After incubation and three washes, immunostained proteins were detected by chemiluminescence (New England Biolabs, Boston, MA, USA).

### Cell culture supernatants

VEGF secretion into culture medium was examined using western blot analysis. Cell lines were plated with MEM plus 10% FBS and allowed to attach for 24h. The medium was then replaced with MEM plus 1% FBS, and cells were incubated for an additional 48h. Conditioned medium was collected and spun down at 1800 rpm for 5 min. Supernatant was concentrated using Amicon Ultra-4 centrifugal filter tubes with a 10,000 dalton molecular weight cut-off. Protein concentration was determined using the Bradford assay, and volumes corresponding to equal amounts of total protein were used for loading the gel.

### Propidium iodide staining and flow cytometry

Cell lines were plated with MEM plus 10% FBS and allowed to attach for 24h. The medium was then replaced with MEM plus 1% FBS, and cells were further incubated for 24h. For 5FU treatment, 2 μg/ml 5FU was added to the medium. Cells were trypsinized, washed in phosphate-buffered saline (PBS) and fixed in 70% ethanol at 4°C overnight. DNA was stained with PI (10 mg/ml of PBS) and DNase-free RNase (2.5 g/ml of PBS) for at least 30 min before cells were subjected to flow cytometry in a Coulter EPICS XL-MCL fluorescence-activated cell analyzer (Beckman Coulter, Fullerton, CA, USA).

### Annexin V staining

The percentage of apoptotic cells was assessed using the FITC Annexin V Apoptosis Detection Kit I (BD Pharmingen, San Diego, CA, USA) according to the manufacturer's instructions. Annexin V quantitation was performed using a Coulter EPICS XL-MCL fluorescence-activated cell analyzer equipped with System II software (Beckman Coulter). For apoptotic studies following exogenous VEGF-A treatment, HCT116 VEGF$^{-/-}$ cells growing in reduced-serum medium were switched to medium supplemented with recombinant human VEGF-A (10 ng/ml, R&D Systems, Minneapolis, MN) for 24 hours. Cells were then stained with Annexin V/FITC and analyzed by flow cytometry.

### Placental growth factor enzyme-linked immunosorbent assay

PlGF production in culture supernatants from HCT-116 VEGF +/+ and VEGF –/– cells was examined using a human PlGF-specific enzyme linked immunosorbent assay (ELISA) according to the manufacturer's instructions (Quantikine; R&D Systems). Cells were plated at 80% cell density in a 100-mm cell culture dish in minimal essential medium supplemented with 1% fetal bovine serum, 2 mM l-glutamine, and 200 μg/mL streptomycin. After 24hours, medium was removed for PlGF analysis, and cells were solubilized in lysis buffer. PlGF concentration was normalized to the total protein content of each culture dish, as measured by the Bradford assay.

### siRNA mediated transient knockdown of VEGF expression

Human VEGFA siRNA (siVEGF-A, ON-TARGET plus SMART pool L-003550-00-0005) and human non-targeting scrambled siRNA duplex (siCntr) were purchased from Dharmacon (Lafayette, CO). Transient transfection of HCT116 cells was performed using X-tremeGENE transfection reagent (Cat. 04476093 001; Roche, Palo Alto, CA) according to manufacturer's instructions. The efficiency of VEGF knockdown in culture supernatant was assessed after 48 hr of incubation by western blot analysis. To test chemosensitivity, the cells were further incubated without or with 5FU for additional 48 hours and expression of apoptotic and antiapoptotic markers were assessed by western blot analysis.

### Immunohistochemical staining of in vivo tumor tissue

Male athymic nude mice (6 – 8 weeks old) were purchased from the National Cancer Institute – Frederick Cancer Research Facility (Frederick, MD) and maintained under specific pathogen-free conditions. All animal experiments met the requirements of the MDACC Animal Care Facility, IACUC, and the National Institutes of Health (NIH) guidelines on animal care. $1.0 \times 10^6$ HCT-116 VEGF$^{+/+}$ and VEGF$^{-/-}$ were injected in 0.1 mL of Hanks balanced salt solution per mouse subcutaneously (right rear flank). Tumor growth was monitored and mice were killed by $CO_2$ asphyxia when tumors were approximately 1 cm in diameter. The VEGF$^{-/-}$ tumors were harvested about 30 days after the VEGF$^{+/+}$ tumors so that they were of similar size at the time of harvest. The excised tumors were fixed in formalin. Paraffin embedded tissue specimens of VEGF$^{+/+}$ and VEGF$^{-/-}$ tumors were IHC stained for cleaved caspase 3.

### Statistical analyses

All data are expressed as mean ± standard error. Statistical analyses were performed with InStat 2.01 statistical software (GraphPad Software, San Diego, CA, USA). The differences between groups were determined by Student's *t*-test. A *p*-value of less than 0.05 was considered significant.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

## References

Bachelder RE, Crago A, Chung J, Wendt MA, Shaw LM, Robinson G, et al. Vascular endothelial growth factor is an autocrine survival factor for neuropilin-expressing breast carcinoma cells. Cancer Res. 2001; 61:5736–5740. [PubMed: 11479209]

Barr MP, Bouchier-Hayes DJ, Harmey JJ. Vascular endothelial growth factor is an autocrine survival factor for breast tumour cells under hypoxia. Int J Oncol. 2008; 32:41–48. [PubMed: 18097541]

Brown LF, Detmar M, Claffey K, Nagy JA, Feng D, Dvorak AM, et al. Vascular permeability factor/vascular endothelial growth factor: a multifunctional angiogenic cytokine. Exs. 1997; 79:233–269. [PubMed: 9002222]

Calvani M, Trisciuoglio D, Bergamaschi C, Shoemaker RH, Melillo G. Differential involvement of vascular endothelial growth factor in the survival of hypoxic colon cancer cells. Cancer Res. 2008; 68:285–291. [PubMed: 18172321]

Carmeliet P, Ferreira V, Breier G, Pollefeyt S, Kieckens L, Gertsenstein M, et al. Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. Nature. 1996; 380:435–439. [PubMed: 8602241]

Cohen GM. Caspases: the executioners of apoptosis. Biochem J. 1997; 326 (Pt 1):1–16. [PubMed: 9337844]

Connolly DT, Heuvelman DM, Nelson R, Olander JV, Eppley BL, Delfino JJ, et al. Tumor vascular permeability factor stimulates endothelial cell growth and angiogenesis. J Clin Invest. 1989; 84:1470–1478. [PubMed: 2478587]

Cryns V, Yuan J. Proteases to die for. Genes Dev. 1998; 12:1551–1570. [PubMed: 9620844]

Dang DT, Chen F, Gardner LB, Cummins JM, Rago C, Bunz F, et al. Hypoxia-inducible factor-1alpha promotes nonhypoxia-mediated proliferation in colon cancer cells and xenografts. Cancer Res. 2006; 66:1684–1936. [PubMed: 16452228]

Dias S, Shmelkov SV, Lam G, Rafii S. VEGF(165) promotes survival of leukemic cells by Hsp90-mediated induction of Bcl-2 expression and apoptosis inhibition. Blood. 2002; 99:2532–2540. [PubMed: 11895790]

Escudier B, Eisen T, Stadler WM, Szczylik C, Oudard S, Staehler M, et al. Sorafenib for treatment of renal cell carcinoma: Final efficacy and safety results of the phase III treatment approaches in renal cancer global evaluation trial. J Clin Oncol. 2009; 27:3312–3318. [PubMed: 19451442]

Fan F, Wey JS, McCarty MF, Belcheva A, Liu W, Bauer TW, et al. Expression and function of vascular endothelial growth factor receptor-1 on human colorectal cancer cells. Oncogene. 2005; 24:2647–2653. [PubMed: 15735759]

Ferrara N, Carver-Moore K, Chen H, Dowd M, Lu L, O'Shea KS, et al. Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. Nature. 1996; 380:439–442. [PubMed: 8602242]

Ferrara N, Gerber HP, LeCouter J. The biology of VEGF and its receptors. Nat Med. 2003; 9:669–676. [PubMed: 12778165]

Gerber HP, Malik AK, Solar GP, Sherman D, Liang XH, Meng G, et al. VEGF regulates haematopoietic stem cell survival by an internal autocrine loop mechanism. Nature. 2002; 417:954–958. [PubMed: 12087404]

Gerber HP, McMurtrey A, Kowalski J, Yan M, Keyt BA, Dixit V, et al. Vascular endothelial growth factor regulates endothelial cell survival through the phosphatidylinositol 3′-kinase/Akt signal transduction pathway. Requirement for Flk-1/KDR activation. J Biol Chem. 1998; 273:30336–30343. [PubMed: 9804796]

Houck KA, Ferrara N, Winer J, Cachianes G, Li B, Leung DW. The vascular endothelial growth factor family: identification of a fourth molecular species and characterization of alternative splicing of RNA. Mol Endocrinol. 1991; 5:1806–1814. [PubMed: 1791831]

Hutchings H, Ortega N, Plouet J. Extracellular matrix-bound vascular endothelial growth factor promotes endothelial cell adhesion, migration, and survival through integrin ligation. Faseb J. 2003; 17:1520–1522. [PubMed: 12709411]

Lee S, Chen TT, Barber CL, Jordan MC, Murdock J, Desai S, et al. Autocrine VEGF Signaling Is Required for Vascular Homeostasis. Cell. 2007a; 130:691–703. [PubMed: 17719546]

Lee TH, Seng S, Sekine M, Hinton C, Fu Y, Avraham HK, et al. Vascular endothelial growth factor mediates intracrine survival in human breast carcinoma cells through internally expressed VEGFR1/FLT1. PLoS Med. 2007b; 4:e186. [PubMed: 17550303]

Leung DW, Cachianes G, Kuang WJ, Goeddel DV, Ferrara N. Vascular endothelial growth factor is a secreted angiogenic mitogen. Science. 1989; 246:1306–1309. [PubMed: 2479986]

Lichtenberger BM, Tan PK, Niederleithner H, Ferrara N, Petzelbauer P, Sibilia M. Autocrine VEGF signaling synergizes with EGFR in tumor cells to promote epithelial cancer development. Cell. 2010; 140:268–279. [PubMed: 20141840]

Masuda K, Teshima-Kondo S, Mukaijo M, Yamagishi N, Nishikawa Y, Nishida K, et al. A novel tumor-promoting function residing in the 5′ non-coding region of vascular endothelial growth factor mRNA. PLoS Med. 2008; 5:e94. [PubMed: 18494554]

Nor JE, Christensen J, Liu J, Peters M, Mooney DJ, Strieter RM, et al. Up-Regulation of Bcl-2 in microvascular endothelial cells enhances intratumoral angiogenesis and accelerates tumor growth. Cancer Res. 2001; 61:2183–2188. [PubMed: 11280784]

Pidgeon GP, Barr MP, Harmey JH, Foley DA, Bouchier-Hayes DJ. Vascular endothelial growth factor (VEGF) upregulates BCL-2 and inhibits apoptosis in human and murine mammary adenocarcinoma cells. Br J Cancer. 2001; 85:273–278. [PubMed: 11461089]

Rini BI, Halabi S, Rosenberg JE, Stadler WM, Vaena DA, Ou S-S, et al. 2008; 26:5422–5428.

Santos SC, Dias S. Internal and external autocrine VEGF/KDR loops regulate survival of subsets of acute leukemia through distinct signaling pathways. Blood. 2004; 103:3883–3889. [PubMed: 14726393]

Senger DR, Galli SJ, Dvorak AM, Perruzzi CA, Harvey VS, Dvorak HF. Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. Science. 1983; 219:983–985. [PubMed: 6823562]

Senger DR, Perruzzi CA, Feder J, Dvorak HF. A highly conserved vascular permeability factor secreted by a variety of human and rodent tumor cell lines. Cancer Res. 1986; 46:5629–5632. [PubMed: 3756910]

Tischer E, Mitchell R, Hartman T, Silva M, Gospodarowicz D, Fiddes JC, et al. The human gene for vascular endothelial growth factor. Multiple protein forms are encoded through alternative exon splicing. J Biol Chem. 1991; 266:11947–11954. [PubMed: 1711045]

Vincent L, Jin DK, Karajannis MA, Shido K, Hooper AT, Rashbaum WK, et al. Fetal stromal-dependent paracrine and intracrine vascular endothelial growth factor-a/vascular endothelial growth factor receptor-1 signaling promotes proliferation and motility of human primary myeloma cells. Cancer Res. 2005; 65:3185–3192. [PubMed: 15833849]
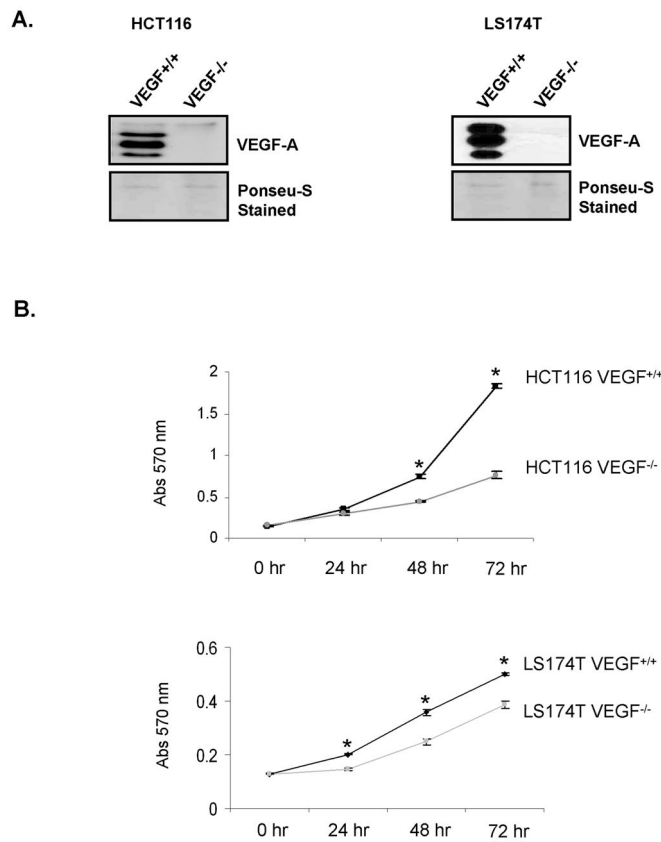
**A.**



**B.**



**Figure 1. Effect of loss of VEGF expression on proliferation of CRC cells**
**A.** Loss of VEGF expression in CRC cells with deletion of VEGF alleles. VEGF-A levels in conditioned medium from HCT116 and LS174T VEGF$^{+/+}$ and VEGF$^{-/-}$ cells were determined by western blot analysis. VEGF-A expression was undetectable in the VEGF$^{-/-}$ cells. Equalproteinloading of the gels was verified by Ponseu-S staining of the membranes.
**B.** The growth rates of HCT116 and LS174T VEGF$^{+/+}$ and VEGF$^{-/-}$ cells were assessed in terms of absorbance at 570 nm in an MTT assay. VEGF$^{-/-}$ cells showed significantly reduced proliferative activity compared with VEGF$^{+/+}$ cells.
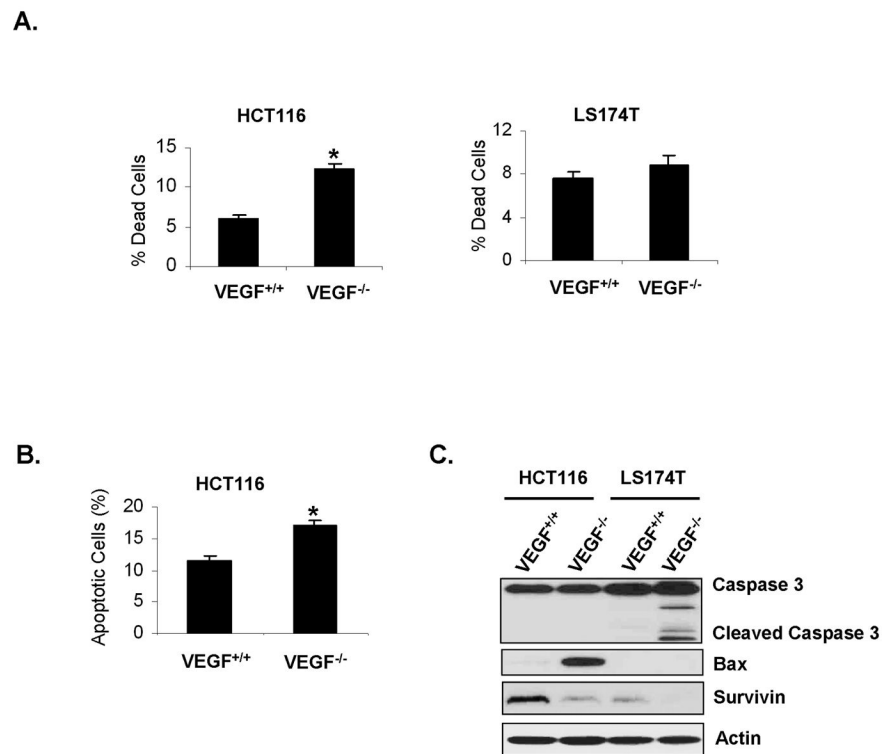
**A.**



**B.**

**C.**



**Figure 2. Effect of loss of VEGF expression on viability of CRC cells *in-vitro***
**A.** Loss of VEGF expression led to increased spontaneous cell death. Cells were grown in 1% FBS medium for 48h, fixed and stained with PI. Cell death was assessed by flow cytometry. **B.** Increased spontaneous apoptosis in HCT116 VEGF$^{-/-}$ cells. Cells were grown in 1% FBS medium for 48h, and apoptosis was assessed by flow cytometry following Annexin V staining. **C.** Altered expression of apoptotic mediators in VEGF$^{-/-}$ cells. Whole-cell lysates of cells growing in 1% FBS medium for 48h were collected and analyzed for expression of caspase 3, cleaved caspase-3, bax and survivin by western blot analysis. Actin served as a loading control.
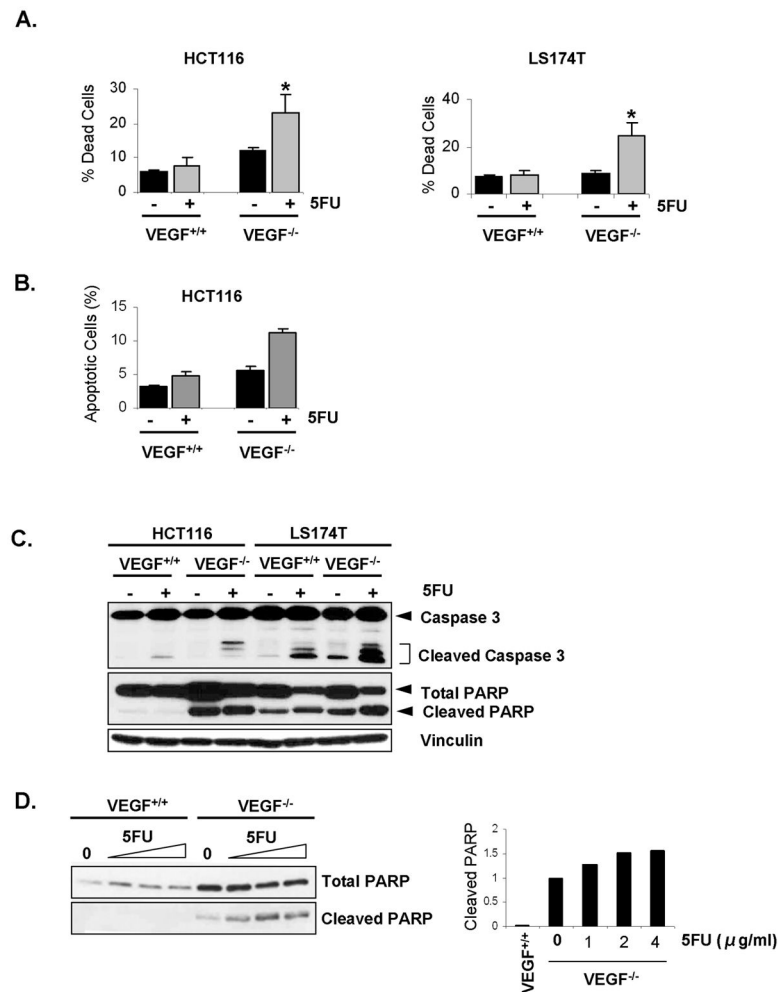
**Figure 3. Effect of loss of VEGF expression on chemosensitivity and signaling in CRC cells**
**A.** Increased chemosensitivity of VEGF$^{-/-}$ cells to 5FU treatment. HCT116 and LS174T VEGF$^{+/+}$ and VEGF$^{-/-}$ cells growing in 1% FBS medium were treated without or with 5FU for 48h, fixed and stained with PI; cell death was then assessed by flow cytometry. **B.** 5FU treatment led to increased apoptosis in HCT116 VEGF$^{-/-}$ cells. HCT116 VEGF$^{+/+}$ and VEGF$^{-/-}$ cells growing in 1% FBS medium were treated without or with 5FU for 48h, then stained with Annexin V; apoptosis was then assessed by flow cytometry. **C.** 5FU treatment led to increased expression of proapoptotic mediators in HCT116 and LS174T VEGF$^{-/-}$ cells. Whole-cell lysates were collected from cells treated without or with 5FU for 48h and were analyzed for expression of caspase-3, cleaved caspase-3, PARP and cleaved PARP by western blot analysis. Vinculin served as a loading control. **D.** 5FU treatment led to increased PARP cleavage in HCT116 VEGF$^{-/-}$ cells in a dose-dependent manner. Whole-cell lysates were collected from HCT116 VEGF$^{+/+}$ and VEGF$^{-/-}$ cells treated without or with increasing concentrations of 5FU for 48h; the lysates were analyzed for expression of PARP and cleaved PARP by western blot analysis. The bands for cleaved PARP were scanned, densitometrically quantitated using NIH Image J software and the resulting data were plotted (right panel.)
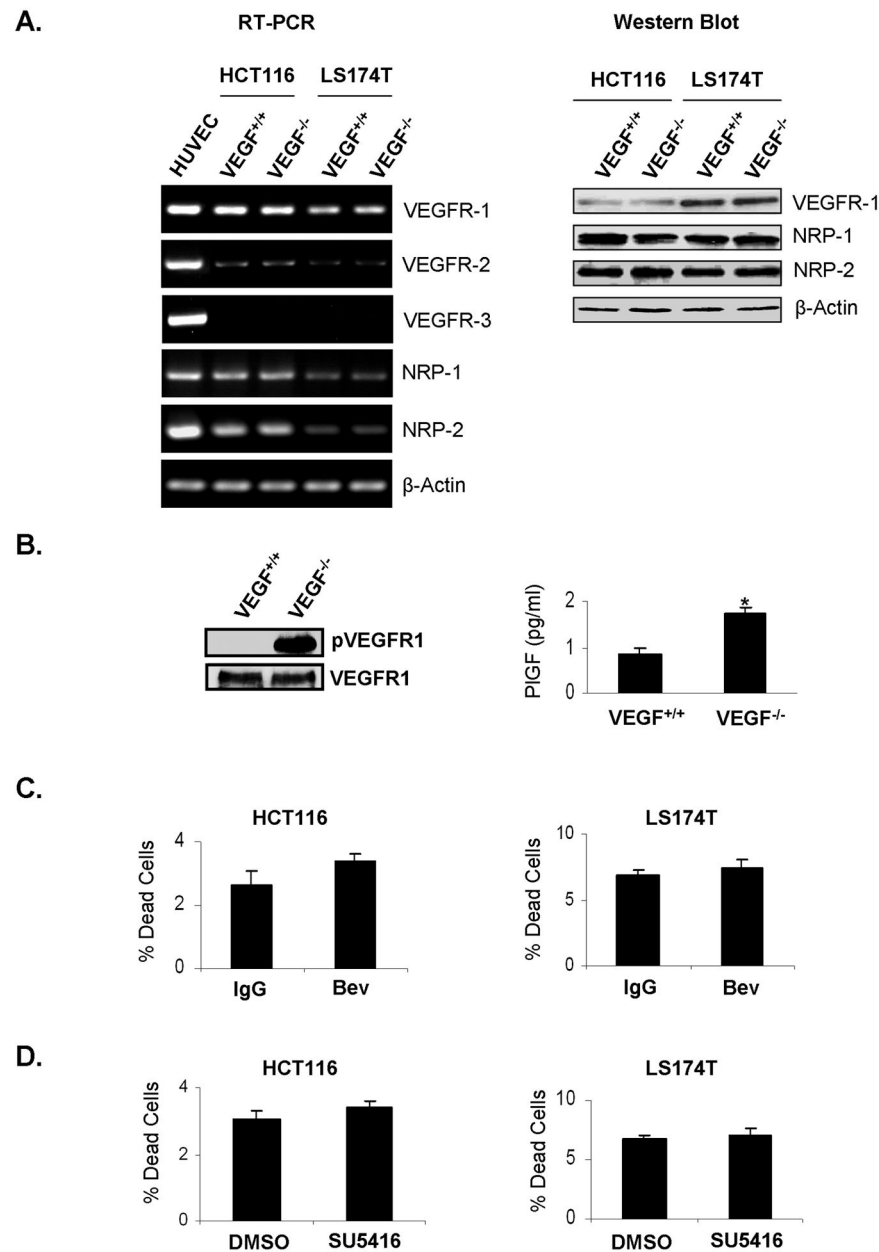
**Figure 4. Status of VEGF receptor expression in the VEGF$^{+/+}$ and VEGF$^{-/-}$ CRC cells**
**A.** VEGF receptor expression and levels in the VEGF$^{+/+}$ and VEGF$^{-/-}$ cells. The expression of the VEGF receptors was assessed by RT-PCR (left panel) and western blot analysis (right panel). HUVEC (Human umbilical vein endothelial cell) cells served as a positive control for VEGF receptor expression. **B.** Increased VEGFR1 activation and PlGF expression in VEGF$^{-/-}$ cells. The phosphorylation of VEGFR1 in the HCT116 VEGF$^{+/+}$ and VEGF$^{-/-}$ cells was assessed by western blot analysis (left panel). Total VEGFR1 served as a loading control. Conditioned medium was collected from HCT116 VEGF$^{+/+}$ and VEGF$^{-/-}$ cells and PlGF level was assesses by ELISA (right panel). **C.** Anti-VEGF antibody treatment did not affect spontaneous cell death in CRC cells. HCT116 and LS174T cells growing in 1% FBS

medium were treated with IgG (as control) or bevacizumab (250 μg/ml) for 48h, fixed and stained with PI; cell death was then assessed by flow cytometry. **D.** Treatment with VEGFR tyrosine kinase inhibitor did not affect spontaneous cell death in CRC cells. HCT116 and LS174T cells growing in 1% FBS medium were treated with DMSO (as solvent control) or SU5416 (1 μM) for 48h, fixed and stained with PI, cell death was assessed by flow cytometry.