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Small Interfering RNA Decreases VEGF mRNA Expression and Proliferation of Colorectal Cancer Cells

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Abstract:

SMALL INTERFERING RNA DECREASES VEGF mRNA EXPRESSION AND PROLIFERATION OF COLORECTAL CANCER CELLS. Stephen M. Ward, Abby L. Mulkeen, Teresa Silva, Peter S. Yoo, John C. Schmitz, Edward Uchio, Edward Chu, Charles Cha. Department of Surgery, Yale University, School of Medicine, New Haven, CT.

Vascular endothelial growth factor (VEGF-A) was first described in 1989 for its angiogenic and mitogenic properties. Early studies indicated that VEGF-A acts primarily in a paracrine pathway which is limited to vascular endothelium. Further investigation showed that VEGF-A and VEGF receptor-2 (VEGFR-2) are expressed by many solid tumors and improve cell growth and survival. Therefore, VEGF-A may act via an autocrine pathway that effects tumor cellular proliferation by binding VEGFR-2 at the cell surface. This study utilizes small interfering RNA (siRNA) technology to investigate the presence of an autocrine loop in human RKO colorectal cancer cells. RT-PCR demonstrated the expression of VEGF-A, VEGF-B, VEGF-D, placental growth factor (PlGF), VEGFR-2, neuropilin-1 (NP-1) and neuropilin-2 (NP-2) *in vitro* by RKO cells. Transfection with siRNA against VEGF-A resulted in a 94% knockdown of VEGF-A expression by ELISA. Northern blot, quantitative real time PCR and semiquantitative RT-PCR confirmed the knockdown data. In addition, transfected RKO cells showed a 67% decrease in cellular proliferation by WST-1 assay. This data correlated to the ELISA results. In summary, the presence of VEGF-A and VEGFR-2 argues in favor of an autocrine loop in human colorectal cancer cells. siRNA targeting of VEGF-A remains a promising anti-tumor therapeutic strategy.

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I would like to thank the many faculty and students at Yale University School of Medicine who have inspired me to ask questions, solve problems, think, reason and learn.

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Introduction:

Cancer claims over half a million lives per year in the United States alone (1). Bronchogenic carcinoma remains the leading killer in both men and women. However, colorectal cancers account for 9.5% and 10.5% of total cancer deaths in men and women, respectively (1). The pathogenesis of colorectal cancer, therefore, warrants further investigation.

The last several decades has produced an overwhelming body of scientific literature regarding the pathogenesis of cancer. There is clearly great interplay between cellular damage, genetics and environmental factors in tumor heterogeneity. But regardless, all neoplastic cells are ultimately dependent on their stromal microenvironment for growth, survival and metastatic potential.

Neoplastic cells, like all cells, require the efficient exchange of metabolic waste products for oxygen and nutrients. Host circulation provides normal physiologic exchange and can support the initial burden of transformed cells. However, a neoplasm is essentially diffusion limited at approximately 2-3 mm in diameter. As neoplastic cells undergo rapid cell division, host circulation can no longer meet tumor metabolic needs. Mouse mammary gland carcinoma cells, for example, divide more slowly as distance from vascular endothelium increases (2, 3).

To the dismay of cancer patients, tumors often overcome diffusion limitations. Tumor angiogenesis makes this possible. Angiogenesis is a process which occurs in conjunction with or in addition to an underlying neoplastic transformation. As expected, cells capable of initiating angiogenesis possess a distinct survival advantage over those which can not (4).

The study of angiogenesis and its related mechanisms has far reaching implications for cancer therapy. The "angiogenic switch" refers to the trigger, or pathophysiologic mechanism, by which a cell induces angiogenesis. If it's possible to understand the angiogenic switch, it may be possible to design novel anti-angiogenic strategies to suppress tumor growth and survival. In fact, limiting tumor growth potential may directly impair metastatic spread and greatly improve host survival.

Although the precise molecular mechanisms are incompletely understood, the past two decades have been instructive. It appears that many factors govern the initiation and regulation of angiogenesis. There is consensus in the scientific community that many pro-angiogenic and anti-angiogenic cytokines are at work simultaneously. In a given microcellular environment, whether physiologic or pathological, the balance between pro-angiogenic and anti-angiogenic factors determines whether the angiogenic switch occurs.

Neoplastic cells are, by definition, dysregulated. In addition to defects in apoptosis, for example, tumor cells suffer from an imbalanced expression of angiogenic cytokines. Several such cytokines have been identified. Pro-angiogenic cytokines include platelet derived growth factor (PDGF-*B*), fibroblast growth factor (FGF), epidermal growth factor (EGF) and vascular endothelial growth factor (VEGF). Thrombospondin-1, angiostatin and others down regulate angiogenesis. Each cytokine contributes uniquely towards the angiogenic switch via receptor tyrosine kinases (RTKs) and complex molecular pathways (4).

In 1989, a landmark paper established that VEGF-A increases vascular permeability and has strong mitogenic properties. The mitogenic action is specific to

vascular endothelial cells (5). Since then, VEGF-A mRNA has been detected by in situ hybridization in lung, breast, GI, kidney, bladder, pituitary, thyroid, ovary, intracranial cancers and hemangioblastomas (5).

VEGF-A abundant primary breast cancers have a worse prognosis and a higher postoperative recurrence rate than breast cancers with low levels of VEGF-A. This suggests that tumor angiogenesis influences survival rates (6). VEGF-A has also been shown to impair dendritic cell development which may block host immune surveillance and promote tumor cell survival (7).

VEGF-A was subsequently cloned and shown to exist in three major isoforms, $VEGF_{189}$, $VEGF_{165}$ and $VEGF_{121}$. These represent the amino acid lengths resulting from alternative splicing (2, 8). They have unique but overlapping activities. $VEGF_{189}$, for example, is secreted via exocytosis and binds extracellular matrix proteins (8, 9). Essentially, it is inactive until released by heparinase or another proteoglycan. This implies a role for matrix degradation in angiogenesis (5).

$VEGF_{165}$ is secreted but some remains bound to the cell surface and some adheres to the extracellular matrix. $VEGF_{121}$, in contrast, is freely soluble and readily diffuses into the intracellular environment. Therefore, matrix degradation and direct secretion are both important as we consider how cytokines access neighboring vascular endothelium in the stroma (2, 9).

In 1992, the Max Planck Institute provided the first evidence that VEGF-A induces angiogenesis *in vivo*. Mouse embryologic studies detected the expression of VEGF-A mRNA in regions of blood vessel growth and maturation (10). The precise mechanism of action remained unknown until deVries described a high affinity receptor

tyrosine kinase (RTK), which he named Flt-1 (11). The name was later changed to VEGFR-1. A second high affinity RTK, VEGFR-2, is highly homologous (12).

VEGF-A binds both VEGFR-1 and VEGFR-2. To determine the clinical significance of the receptors, researchers disrupted each gene individually and observed that deficiency in either resulted in mouse fatality (13). Both receptors are expressed predominantly in vascular endothelium, further supporting the role of VEGF-A in angiogenesis (14, 15). This discovery led to the postulation that VEGF-A may act in paracrine fashion. However, VEGF-A receptors are now known to exist on the cell surface of many tumors, indicating a possible autocrine loop as well (16, 17).

VEGF-A receptor activity is highly complex. VEGFR-2 is the predominate receptor which mediates VEGF-A action. It is expressed embryologically in the yolk sac and vascular endothelium as well as the endocardial and mesodermal layers. All of these sites undergo extensive angiogenic activity.

Adults do not normally experience widespread vascular proliferation. As anticipated, adults express much lower levels of VEGFR-2 mRNA (18). This implies that VEGFR-2 is essential during embryologic development. In fact, VEGFR-2 deficient mice fail to form embryologic blood islands (19). VEGFR-2 may be activated in adults under pathologic conditions or on an as needed basis.

Interestingly, VEGFR-2 is coupled with cell surface adhesive proteins such as integrin $\alpha_v\beta_3$ in the extracellular matrix. This integrin is exclusive to angiogenic endothelium. VEGF-A and VEGFR-2, therefore, appear to be associated with endothelial cell survival (20).

A second class of receptors, the neuropilins, also potentiates VEGF-A activity. Neuropilins are generally known to preserve axonal function. However, neuropilin-1 (NP-1) serves two important roles in angiogenesis. It's a cell surface glycoprotein which acts as a cofactor, presenting $VEGF_{165}$ to VEGFR-2 and thereby enhancing its effect (19). NP-1 also competes with $VEGF_{165}$ for a binding site at VEGFR-1 (21). Less is known about neuropilin-2 (NP-2), although it may be involved in lymphatic vessel development through $VEGF_{165}$ activity (22).

In order to devise novel anti tumor therapies, it is important to understand how cells are initially stimulated to release VEGF-A. Cellular hypoxia is a primary stimulus. VEGF-A, VEGFR-1 and VEGFR-2 are, in fact, upregulated in hypoxic regions of mouse lung vasculature *in vivo* (23, 24). One study demonstrated that glial tumors, which are known to be extremely sensitive to hypoxia, express very high levels of VEGF-A (25). These findings are consistent with an angiogenic model for tumor progression, since vessel proliferation undoubtedly improves tissue oxygenation and cell survival.

VEGF-A mRNA is upregulated in the left anterior descending (LAD) artery distribution of pig myocardium following occlusion. Myocardial ischemia, then, is another setting which may induce local angiogenesis in response to hypoxic injury (26). Interestingly, hypoxia seems to serve a dual function in angiogenesis. In addition to enhancing VEGF-A mRNA transcription, it also stabilizes the mRNA itself (25).

Hypoxia is not the only stimuli effecting VEGF-A expression. VEGF-A can be found ubiquitously, secreted by kidney, heart, lung and brain cells. Secretion often occurs in the absence of overt hypoxia. This suggests that VEGF-A may be involved not only in angiogenesis, but also in the maintenance of existing vascular endothelium as a

type of survival factor (15, 27). Recently, VEGF-A has been shown to upregulate *bcl-2*, an anti-apoptotic protein, yielding further evidence for cell survival signaling (28, 29).

Many factors stimulate angiogenesis, but the precise mechanisms of VEGF action are incompletely understood. Researchers have identified three additional proteins that share structural homology with VEGF-A. They are VEGF-B, VEGF-C and VEGF-D. A new receptor, VEGFR-3, has also been recently described. A more thorough understanding of these proteins and their interactions is necessary in order to devise new therapeutic strategies.

Northern blot analysis shows abundant VEGF-B in the skeletal muscle and cardiac muscle of developing mice. This again indicates possible angiogenic function for this protein (30). VEGF-B is also present in adult mice and human muscle. VEGF-B exists in two known isoforms produced by alternative splicing. VEGF-B_{167} remains cell surface associated until released by heparinase. VEGF-B_{186} is freely secreted (31).

A unique feature of VEGF-B_{167} is its ability to form heterodimers with $VEGF_{165}$ at the cell surface. Since $VEGF_{165}$ is otherwise freely secreted, this association may influence $VEGF_{165}$ bioavailability and may also limit VEGF-B action to a paracrine pathway (30, 31). Upon release, VEGF-B binds to VEGFR-1 using NP-1 as a cofactor (32).

VEGF-C was originally cloned from human prostatic carcinoma. It is proteolytically cleaved in the ECM and binds to both VEGFR-2 and VEGFR-3 (32). It is less potent than VEGF-A, but will successfully induce vascular permeability and angiogenesis in large enough concentrations. VEGF-C stimulates the migration of bovine capillary endothelial cells *in vitro* in a collagen gel matrix model (33).

Unlike VEGF-A, VEGF-C responds to inflammatory cytokines more than to hypoxia. VEGF-C also induces lymphangiogenesis through VEGFR-2 and VEGFR-3 activity (34, 35). In situ hybridization reveals mRNA expression at day 8 in the mouse embryo, for example, particularly in regions of lymphatic vessel proliferation (36). VEGF-C overexpression in the pancreatic β cells of transgenic mice stimulates lymphatic proliferation around the islets of langerhans. However, this does not appear to influence rate of tumor formation (35). Lymphatic hyperplasia in dermal keratinocytes is also associated with VEGF-C overexpression (37).

VEGFR-3 is found at the lymphatic endothelial cell surface during development and is normally expressed exclusively there (35, 38). It is present pathologically on lymphatic endothelial cells in metastatic lymph nodes, lymphangiomas and vascular skin tumors (33).

Regional lymph nodes are often the first to develop metastases. It's not clear whether lymphatics are accessed by way of new lymph vessels, or by invasion of preexisting lymph vessels from within the tumor. Peritumor lymphatics are clearly associated with cancer metastasis (39). However, it was less certain if the density of intratumor lymphatic vessels is related to metastasis until VEGF-D was described (40).

Like VEGF-C, VEGF-D binds to VEGFR-2 and VEGFR-3. It is proteolytically cleaved and is therefore dependent on the extracellular matrix for its bioavailability (8). VEGF-D has mitogenic and angiogenic properties and promotes tumor metastasis via lymphatic spread in a mouse model. It is also upregulated in human melanomas compared to baseline melanocytes (41).

VEGF-D was recently investigated in the setting of papillary thyroid carcinoma. The study statistically correlated metastasis with VEGF-D mRNA and protein levels, correlating lymphatic vessel density with metastases (42). Another study confirmed this finding, and determined that VEGF-D is an independent marker for overall survival in colorectal cancer with lymphatic involvement (43).

To further understand cancer pathophysiology, one study assessed VEGF-A, VEGF-C, VEGF-D, VEGFR2 and VEGFR-3 levels as adenomatous polyps progressed to colorectal carcinoma. Interestingly, VEGF-D mRNA expression was significantly lower in both polyps and carcinoma cells compared with normal mucosa. VEGF-A_{121} and VEGF-A_{165} levels were significantly higher in cells undergoing lymph node metastasis. Ironically, there was no relationship between VEGF-C or VEGF-D levels and lymphatic invasion. This may indicate that lower levels of these factors make more receptor binding sites available for VEGF-A (44). Another report confirms that VEGF-A levels are, in fact, related to advanced disease (45). VEGFR-2 and VEGFR-3 levels were similar in tumor cells and normal mucosa.

Clearly, VEGF-C, VEGF-D and VEGFR-3 are potential therapeutic targets against tumor metastasis (8). One study inhibiting VEGF-D successfully impaired angiogenesis, lymphangiogenesis and metastasis in a mouse tumor model (42).

Until recently, a lack of genetic tools hindered the study of mammalian cancer cells. Today we are fortunate to have a wealth of resources that help elucidate molecular mechanisms. Plasmids, for example, are used to transfect target cells with exogenous DNA that confer a desired phenotype. This maneuver is fairly efficient since plasmids self replicate independently from the host genome and therefore do not interact with the

cell's molecular machinery. Viral vectors are also useful but transfection tends to be less efficient (46).

Plasmid or virus transfection is considered a gain of function technique. Target cells transcribe exogenous DNA. Other promising techniques are the exact opposite. It may be desirable to shut down gene expression in order to deduce the downstream function of individual proteins. This can be done at the level of transcription, translation, exocytosis or protein action at receptors.

Small interfering RNAs (siRNAs) are very useful in such gene silencing studies. siRNAs are small double stranded RNAs approximately 20 nucleotides in length. They can be introduced to target cells where they bind to an RNA induced silencing complex (RISC). The RISC then facilitates binding of the siRNA to a complementary mRNA sequence. This results in mRNA cleavage and gene silencing.

Many preliminary gene knockdown studies are encouraging. However, cancer mortality rates remain unacceptably high despite 20 years of remarkable advances in the scientific understanding of neoplastic transformation and a virtual explosion of molecular biology research tools.

Little is known about the angiogenic switch and how it is regulated. Angiogenesis and lymphangiogenesis are relatively recent complements to our understanding of cancer pathophysiology. A more detailed understanding of angiogenic mechanisms is necessary. This field presents a marvelous opportunity to contribute to the future of cancer therapy.

Statement of Purpose:

VEGF-A and VEGFR-2 are highly active mitogenic signals and angiogenesis factors. The presence of both of these proteins implies an autocrine loop that may be important to colorectal cancer growth and survival.

This study hypothesizes the existence of an autocrine loop in the human RKO colorectal cancer cell line. Evidence for this pathway requires VEGF-A and VEGFR-2 mRNA expression, and correlation between expression level and cell proliferation. Preliminary data from ELISA and northern blot assays of wild type and siRNA knockdown cells support this hypothesis. Data is confirmed by RT-PCR followed by semi-quantitative analysis.

This study further hypothesizes that RKO colorectal cancer cells may possess complex molecular pathways involving VEGF-B, VEGF-C and VEGF-D. In addition, RKO cells may express VEGFR-1, VEGFR-3, NP-1 and NP-2 indicating broad angiogenic activity. Greater understanding of cellular mechanisms in colorectal cancer may lead to novel anti-tumor strategies.

Methods:

Cell Culture

Human RKO colorectal carcinoma cells were cultured in RPMI 1640 media supplemented by 9% fetal bovine serum (Invitrogen, Carlsbad, CA). Cell were cultured and incubated in T-75 vented flasks at 37^{o} C using 5% CO_2 humidified air. Every 3 days, cells were passed with trypsin-EDTA (Invitrogen) upon reaching 70% confluency.

siRNA Design

Elbashir and Tuschel published helpful guidelines for selecting siRNA targets (47). To maximize transfection efficiency, these guidelines suggest that sequences contain a total GC ratio of 40-60%, lack three successive guanine or cytosines and begin with two adenosines.

Several VEGF gene specific siRNA sequences were selected accordingly. A BLAST search of the human gene database confirmed gene specificity (ID 7422). A previously published siRNA sequence, VP, was chemically synthesized by Dharmacon (Lafayette, CO) and used in our study design (3). VP (5' atgtgaatgcagaccaaagaa 3') targets nucleotides 1082-1102 in exon 4 of the VEGF-A coding region.

siRNA Transfection

RKO cells were cultured in a T-75 flask until reaching 70% confluency, as described. Cells were then harvested by trypsinization and plated for transfection at a density of 100,000 cells. Plating occurred in T-25 flasks in RPMI 1640 media containing 9% fetal bovine serum (FBS).

The VP siRNA was diluted to 50 nM in OPTIMEM I (Invitrogen) and plated. After 24 hours, RKO cells were transfected with the VP siRNA using oligofectamine (Invitrogen). Media was changed at 24 hours and supernatants were collected at 24, 48, and 72 hours post-transfection. All supernatants were stored at -20^{o} C and cell debris was centrifuged out prior to sandwich ELISA.

Controls

A GL2 siRNA against luciferase served as control. The sequence is given in table 1 and it has no other known human genetic homology. RKO wild type cells were treated with either oligofectamine alone (UT) or oligofectamine plus GL2 siRNA (50nM) for negative control.

Transient Transfection with VEGFSP Hairpin siRNA

This study required an anti-VEGF hairpin siRNA (pVP). It was designed, chemically synthesized and cloned into a psiRNA-hH1neo G2 plasmid by Invivogen. The hairpin sequence is shown in table 1. 500,000 cells were plated to T-25 flasks. After 24 hours, they were transfected with 3μg of the hairpin containing plasmid.

Untransfected cells received lipid only and were plated as negative controls. Lipofectamine 2000 (Invitrogen) served as the lipid vehicle. 24 hours post-transfection, media was changed and replaced with 5ml of fresh complete medium. 48 hours post-transfection, supernatant was collected from each flask for sandwich ELISA analysis.

Northern Blot Analysis

RKO cells were plated at 100,000 cells per flask and transfected with 50 nM of VP or GL2 siRNA. 48 hours after transfection, cells were trypsinized and prepared for analysis using the NorthernMax-Gly kit (Ambion, Austin, TX). Quiagen's RNeasy kit (Valencia, CA) was used to extract total RNA from cultured cells. 20 ug of the extracted total RNA was then electrophoresed on a glyoxal gel and transferred to a BrightStar-Plus positively charged nylon membrane (Ambion).

A riboprobe was produced using a MEGAshortscript T7 kit and labeled using the BrightStar labeling reagent (Ambion). The probe was amplified from cDNA using the primer sequences shown in table 1. Membranes with the VEGF riboprobe were hybridized overnight at 65^{o}C.

RT-PCR Primer Design

The VEGF-A gene consists of 8 exons which are alternatively spliced into three major isoforms. VEGF-A_{189} is complete. VEGF-A_{165} lacks exon 6 and VEGF-A_{121} lacks exons 6 and 7 (48). The sense stand was placed in exon 3 and the anti-sense strand in exon 8 to target regions common to all isoforms. VEGF-A_{165} and VEGF-A_{121} amplify to different nucleotide lengths and separate upon electrophoresis.

VEGF-B consists of 7 exons. Alternative splicing in exon 6 yields 2 isoforms (31). The sense primer in exon 3 and the antisense primer in exon 4 capture both isoforms. VEGF-C has 7 exons that undergo alternative splicing in exon 4, 6 and 7 (36). The sense primer is in exon 1 and the antisense primer in exon 4. VEGF-D has 8 exons

and 7 introns (49). The sense primer binds to exon 5 and the anti-sense primer binds to exon 6.

VEGFR-1 consists of 7 exons. Interestingly, alternative splicing results in a soluble form lacking a transmembrane region coded by exon 6. Primer sites span this transmembrane region in order to amplify the cell surface receptor (50, 51). VEGFR-2, the most biologically active receptor, is a type-III receptor tyrosine kinase (52, 53). It has a highly regulated promoter region (54). Primers bind in exons 5 and 10.

NP-1 has 17 exons and undergoes alternative splicing. There are several membrane bound and soluble isoforms (55). The sense primer in exon 14 and antisense primer in exon 15 bind to conserved regions. NP-2 also undergoes extensive alternative splicing resulting in cytoplasmic and transmembrane domain variations (55). The primer sequences are both within exon 17, which is conserved.

All accession numbers are given in tables 1 and 2. Each mRNA sequence is available at www.ncbi.nih.gov/entrez under the appropriate accession number. Primer specificity and binding locations in the literature were confirmed with BLAST searches and ALIGN searches at www2.igh.cnrs.fr.

Primer Resuspension

VEGF-R1, VEGF-R2, NP-1 and NP-2 primers were resuspended in 400 µl of H_2O then diluted 200 times for spectrophotometry.

Single stranded DNA concentrations were calculated using the formula:

$$[\ ] \text{ in ng/µl} = (\text{absorbance in AU}) * 33 \text{ ng/AU/µl} * 200$$

(absorbance in AU): from spectrophotometer

33 ng/AU/µl: ssDNA conversion

200: dilution correction

Concentrations in ng/µl were converted algebraically to µM:

$$[\ ] * (1g / 1x10^9 \text{ ng}) * (1x10^6 \text{ µl} / 1) * (1 \text{ mol} / 330g) * (1x10^6 \text{ µmol} / \text{mol}).$$

330g: average molecular weight of nucleotide

VEGF-B, VEGF-C and VEGF-D primer concentrations were calculated by the same method. VP, GL2 and UT mRNA concentrations were confirmed with an absorbance conversion of 40 ng/AU/µl and a 25 times dilution.

RT-PCR

An RNeasy Kit (Qiagen) isolated total RNA from cultured RKO cells. A Superscript III First Strand Synthesis Kit and Platinum Taq DNA Polymerase (Invitrogen) was used for cDNA synthesis. RT-PCR primer sequences are given in tables 2 and 3. The RT-PCR program for VEGF, VEGFR-1 and VEGFR-2 consisted of an initial denaturation step at 95°C for 2 minutes, followed by 35 amplification cycles (denatured at 95°C for 1 minute, annealed at 59°C for 1 minute and extended at 68°C for 2 minutes) with a final extension step at 68°C for 10 minutes.

Slightly different conditions were used to separate the VEGF-A isoforms and to identify VEGF-B, VEGF-C, VEGF-D and PlGF. 40 amplification cycles (denatured at

95°C for 30 seconds, annealed at 55°C for 30 seconds and extended at 68°C for one minute) optimized primer binding. These conditions were also used to amplify VP, GL2 and UT cDNA following Superscript III first strand synthesis.

Agarose Gel Electrophoresis

1.5 g of Ultrapure agar (Invitrogen) was added to 100 ml tris-borate-EDTA (TBE) and 2 μg ethidium bromide. The gel ran at 100V. All lanes contained 15 μl of cDNA plus 15 μl of loading buffer at 1:3 dilution. They ran against 30 μl of a 1 kb DNA ladder (Invitrogen).

Quantitative Real Time PCR

Cells were transfected to a 50 nM final concentration as described above. At 48 hours post transfection, they were harvested and total RNA was extracted using the RNeasy Kit (Qiagen). A Superscript III First Strand cDNA Synthesis Kit (Invitrogen) was used for cDNA synthesis. Each cDNA was amplified using TaqMan Universal PCR master mix (Applied Biosystems). The VEGF TaqMan Expression Assay was used with the B-actin TaqMan Expression Assay (Applied Biosystems) as positive control. These kits contained predesigned sense and antisense primers and a FAM dye-labeled probe.

PCR was carried out on an ABI Prism 7900 HT sequence detection system for 40 cycles. The system used SDS 2.2 software for relative quantification of mRNA expression levels. VEGF mRNA expression levels from siRNA knockdown cells were compared to levels from wild type RKO cells. B-actin served as control.

Semiquantitative Analysis

To add further support for RKO knockdown, mRNA from the quantitative analysis of VP, GL2 and UT transfected cells was used for semiquantitative analysis. This involved repeating the RT-PCR and optimizing the cell cycle number to avoid the amplification plateau. At 30 cycles, densitometry analysis measured the relative concentration of cDNA present in each sample. Gels were photographed using an Eagle Eye II by Stratagene and scanned using NIH Image version 1.62.

Sandwich ELISA Analysis

RKO cells were cultured and transfected as described above. Cells were collected at 24, 48, and 72 hours post transfection. A DuoSet ELISA Kit for Human VEGF measured protein levels (R&D Systems, Minneapolis, MN). Plates were read with a Powerwave 340 microplate spectrophotometer (Bio-Tek, Winooski, VT).

To calculate the effectiveness of siRNA knockdown using protein levels, absorbance at 450 nm was plotted against VEGF protein standards in the 0-1000 pg/ml concentration range. This calibration allowed comparison of protein levels in VP siRNA transfected supernatant with protein levels in untransfected (UT) wild type supernatant.

WST-1 Proliferation Assay

This assay measured cellular proliferation using WST-1 tetrazolium salt (Roche, Indianapolis, IN) in accordance with manufacturer's instructions. RKO cells were placed into a 24 well place at a density of 2,500 cells per well. These cells were cultured in the RPMI 1640 media containing 9% FBS. 24 hours after plating, the cells were transfected

with VP or GL2 siRNA in 0.1 nM to 100 nM concentration ranges. Negative controls were wells with media alone, and wells with media plus wild type RKO cells transfected with lipid only. All cells were plated in triplicate.

Following transfection, plates were incubated at 37ºC with 5% CO_2 for 72 hours, and then retransfected exactly as previous. Cellular proliferation was determined 48 hours post-retransfection (5 days post original transfection) using the WST proliferation assay. Plates were then incubated for another 90 minutes after the addition of 45 µl of WST reagent.

A 60µl aliquot of each supernatant was transferred to a 96-well plate for spectrophotometry. A Powerwave 340 microplate spectrophotometer measured absorbance at 460 nM. These WST-1 proliferation experiments were performed in duplicate and each experimental condition was plated in triplicate.

To investigate whether siRNA knockdown of the VEGF gene alone results in decreased cell proliferation, reduced serum media (2% FBS) was used for additional proliferation experiments. In these experiments, either conditioned media (taken from cultured wild type RKO cells) or 10 ng of recombinant human VEGF-A_{165} (Calbiochem) were added back to the wells of transfected cells in an attempt to reverse the observed decrease in proliferation. This was done at 48 hours post-transfection in accordance with above procedures.

Flow Cytometry

VP siRNA transfected RKO cells were further assessed by flow cytometry. Cells were treated with either 50 nM siRNA or lipid control and harvested at 48 hours. They were washed with 1x PBS and fixed with 70% ethanol. Then, cells were treated with RNase A and stained with propidium iodide (Sigma-Aldrich, St. Louis, MO). A FACSCalibur cytometer (BD Biosciences) with FlowJo software (Tree Star Inc, Ashland OR) performed cell cycle analysis.

Western Blot Analysis

Cultured RKO cells were assayed for VEGFR-2 and VEGFR-3 using Western blot analysis. Cells were grown under WST assay conditions, transfected with 25 nM siRNA and lysed at 48 hours post transfection. All cells were lysed in RIPA buffer consisting of 10mM tris-HCl at pH 7.4, 15mM NaCl, 0.1% SDS, 1% NP-40, 0.5% deoxycholic acid and protease inhibitor. Lysates were centrifuged at 13,000 rpm for 10 minutes.

PARP and caspase-3 protein levels were determined by a DC protein assay kit (Bio-Rad Laboratories, Hercules, CA). Equal amounts of protein were loaded onto a 12% SDS-PAGE gel (Bio-Rad) for evaluation. VEGFR-2 and VEGFR-3 were evaluated with a 4-20% tris-HCl precast ready-gel (Bio-Rad). Both gels were transferred to a polyvinylidene membrane (Millipore Corporation, Bedford, MA.). Membranes were blocked for one hour with 5% non-fat dry milk, 0.05% tween and 20/1x PBS at pH 7.4. They were incubated for one hour with the appropriate primary antibodies.

The primary antibodies were 1:1000 anti-caspase-3 (Upstate, Lake Placid, NY), 1:1000 anti-PARP (Upstate), 1:1000 anti-VEGFR-2 (Santa Cruz Biotechnology, Santa Cruz, CA), and 1:1000 anti-VEGFR-3 (Zymed Laboratories, San Francisco, CA). All antibodies were rabbit anti-human.

The secondary antibody was a 1:200 dilution of goat-anti rabbit IgG (Zymed) conjugated with horseradish peroxidase. Proteins were detected by a SuperSignal West Pico chemiluminescent substrate (Pierce, Rockford, IL).

Table 1: Primer Sequences

GL2 siRNA	5' cgtacgcggaatacttcga 3’
Hairpin	
Sense:	5’ tcccaatgtgaatgcagaccaaagaattcctgtcattctttggtctgcattcacattt 3’
Antisense:	5’ caaaaaatgtgaatgcagaccaaagaatgacaggaagttctttggtctgcattcacatt 3’
Riboprobe	
Sense:	5’ ctgctgtcttgggtgcatt 3’
Antisense:	5’ taatacgactcactataggtgatgttgga ctcctca 3’

Table 2: VEGF Primer Sequences With Accession Numbers and References

VEGF-A			
Sense:	5’ cgaagtggtgaagttcatggatg 3’		
Antisense:	5’ ttctgtatcagtctttcctggtga 3’	M_63971	
VEGF-B			
Sense:	5’ cagaggaaagtggtgtcatgga 3’		
Antisense:	5’ accggatcatgaggatctgca 3’	U_52819	
VEGF-C			
Sense:	5’ ctctcaaggccccaaacca 3’		
Antisense:	5’ aggtcttgttcgctgcctga 3’	NM_005429	
VEGF-D			
Sense:	5’ gatcgctgttcccattcca 3’		
Antisense:	5’ atcatgtgtggcccacagaga 3’	NM_004469	
PlGF			
Sense:	5’ ggcgatgagaatctgcactgt 3’		
Antisense:	5’ caccttttccggcttcatcttc 3’	X_54936	
B actin			
Sense:	5' gtggggcgccccaggcacca 3'		
Antisense:	5' ctccttaatgtcacgcacgatttc 3’	N/A	

Table 3: Receptor Primer Sequences With Accession Numbers and References

VEGFR-1			
Sense:	5' gtcacagaagaggatgaaggtgtcta 3’		
Antisense:	5' cacagtccggcacgtaggtgatt 3'	X51602	52
VEGFR-2			
Sense:	5' ctggcatggtcttctgtgaagca 3'		
Antisense:	5' aataccagtggatgtgatgcgg 3'	AF035121	52
NP-1			
Sense:	5’ ctggtgagccctgtggtttattcc 3’		
Antisense:	5’ actaatgtcatccacagcaatccc 3’	NM_003873	56
NP-2			
Sense:	5’ ccgaagctgcaccacactgg 3’		
Antisense:	5’ caaaatagaactgtgtgacccc 3’	AF281074	55

Statement of Student Involvement:

In the course of this study, I conducted an extensive literature search of journals pertaining to vascular endothelial growth factor (VEGF). This included review of past and current angiogenesis research, especially in cancer models. I also researched articles describing VEGF and its various forms, receptors, and pathophysiologic pathways. Dr. Cha, Dr. Mulkeen and Terry Silva of the VA Medical Center, West Haven, CT ensured that my background reading included the most relevant landmark studies.

Cell culture, siRNA design and RKO cell transfection was performed by other members of the lab team. Concurrently, I performed the RT-PCR work. The lab had already ordered pre-published primers for the VEGF-A isoforms, VEGF-B, VEGF-C, VEGF-D, PlGF, VEGFR-1, VEGFR-2, NP-1 and NP-2. However, the binding sites and primer specificity were not verified. Therefore, I underwent the process of original primer design to verify all primers.

This required a search of the NIH human gene database for the appropriate mRNA/cDNA sequences and confirmation of primer originality with BLAST and ALIGN. I also searched the literature for articles describing the precise location of introns and exon boundaries, and compared all primer binding sites to those junctions. This ensured amplification of the cDNA of interest. I accounted for alternative splicing to ensure amplification of appropriate isoforms for each protein. I also calculated all primer melting temperatures and verified that CG content meets accepted PCR guidelines.

I resuspended all primers, with the exception of VEGF-A, and titrated them to the appropriate concentration for RT-PCR. In addition, I conducted my own mRNA

extractions and first strand synthesis in order to use my own cDNA consistently throughout the study. Later, I used mRNA previously extracted by other members of the lab team to verify my RT-PCR results.

I prepared all of the necessary reagents for RT-PCR and performed my own agarose gel electrophoresis. I also optimized RT-PCR conditions independently and ran all semiquantitative densitometry studies.

Other team members performed the Northern blots, Westerns, ELISA and cell proliferation studies concomitantly. The quantitative analysis was performed by the Keck lab at Yale.

Results:

RKO colon cancer cells express VEGF-A, specifically the 165 and 121 isoforms. They also express VEGF-B, VEGF-D and PlGF. In addition, RKO cells express VEGFR-2, NP-1 and NP-2. The duel expression of VEGF and its receptors implies that RKO cells may be activated exogenously or by an autocrine loop. Cells transfected with anti-VEGF siRNA show a 94% knockdown in VEGF expression and a 67% decrease in cellular proliferation.

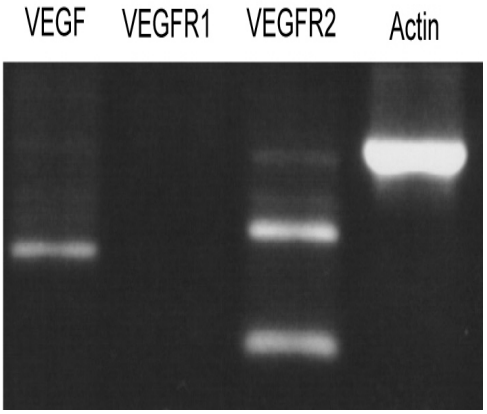


Figure 1: RT-PCR showing RKO expression of VEGF-A and VEGFR-2. Note the absence of VEGFR-1. B-actin is positive control.

First, VEGF-A, VEGFR-1 and VEGFR-2 were assessed by RT-PCR. Separate primers were used for each and B-actin served as control (figure 1). Repeat RT-PCR looked specifically for VEGFR-2 (figure 2). Northern blot analysis also confirmed the RT-PCR results and showed expression of VEGF-A and VEGFR-2 but not VEGFR-1. Western blot corroborated the presence of VEGFR-2 expression.

RT-PCR analysis using isoform specific primers showed RKO expression of VEGF-A_{165} and VEGF-A_{121} but not VEGF-A_{189} (figure 3).

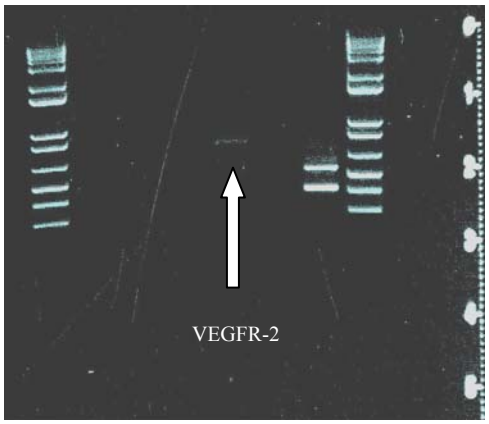


Figure 2: RT-PCR showing RKO expression of VEGFR-2 at 790 bp. Lane 6 is a –cDNA water control. Lane 7 is a VEGF-A positive control. Note the absence of VEGFR-1.

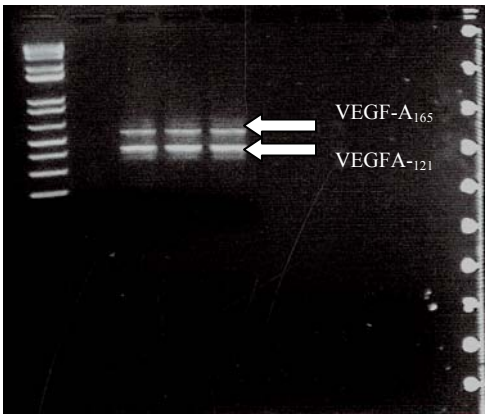


Figure 3: RT-PCR showing RKO expression of VEGF-A isoforms. Run in triplicate. Top bands correspond to VEGF-A_{165} at 535 bp. Bottom bands correspond to VEGF-A_{121} at 403 bp. Note that VEGF-A_{189} is not present (expected size 607 bp).

Neuropilin-1 and neuropilin-2 are both expressed by RKO cells (figure 4). In addition, RKO cells strongly express VEGF-B and PlGF (figure 5).

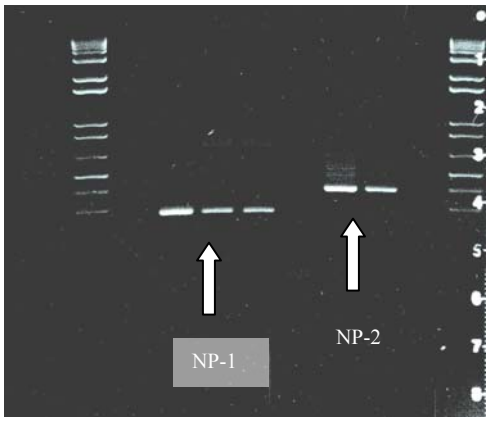


Figure 4: RT-PCR showing RKO expression of NP-1 (lanes 3-5) at 275 bp and NP-2 (lanes 7,8) at 390 bp. Lanes 1 and 10 are 1 kb DNA ladders. Lanes 2 and 6 are –cDNA water controls. Amplified for 40 cycles and run on 1.5% agarose.

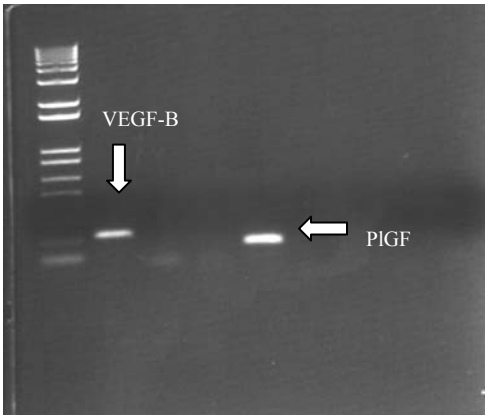


Figure 5: RT-PCR showing expression of VEGF-B, VEGF-C, VEGF-D and PlGF by RKO cells. Amplification was not optimized for VEGF-C and VEGF-D (lanes 3 and 4).

siRNA Knockdown

RKO cells transfected with VP siRNA decrease VEGF-A protein secretion by 94% on ELISA. mRNA from cells transfected with VP, GL2 and untransfected controls show VEGF-A knockdown at the mRNA level on Northern blot (figure 6).

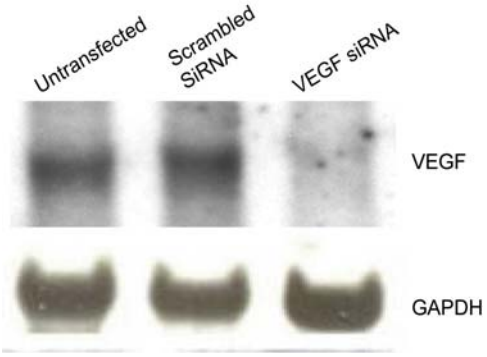


Figure 6: Northern blot of VEGF mRNA. RKO cells transfected with VP siRNA express less VEGF mRNA than UT (left) and GL2 (middle) controls. Courtesy of A. Mulkeen, MD.

To characterize VEGF-A knockdown conditions, RKO cells were transfected with several different concentrations (1nM, 12nM, 24nM, 36nM, 48nM, and 50nM) of VP or GL2 siRNA. Untransfected cells were used as controls. Collection of supernatant followed by sandwich ELISA at 24, 48, and 72 hours showed

greatest knockdown at 48 hours using 12nM VP siRNA. Analysis of supernatant revealed that knockdown ranged from 67.5 to 94% and remained as high as 59% at 5 days post transfection (figure 7).

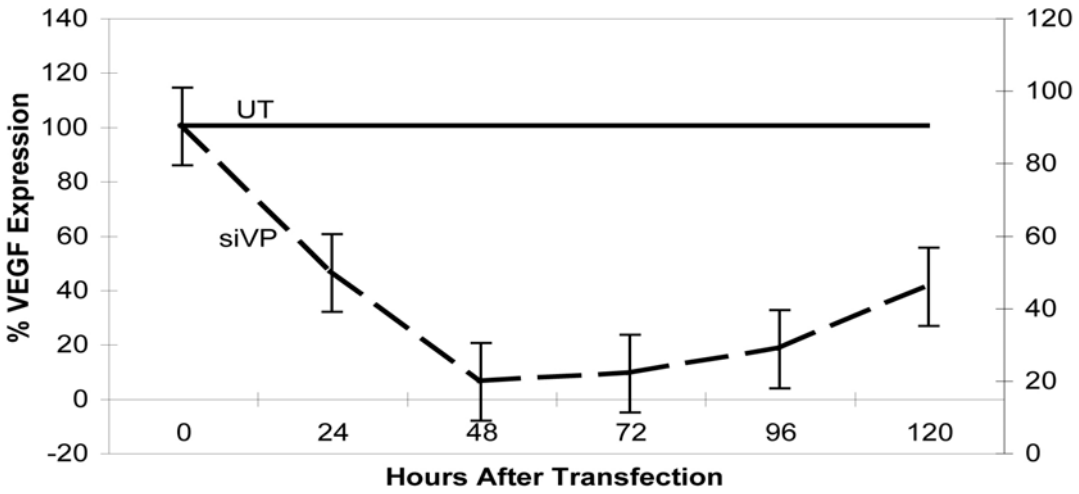


Figure 7: This graph shows % VEGF expression against time by ELISA. RKO cells transfected with 12 nM of VP siRNA were followed at various time points. Maximum VEGF knockdown occurred at 48 hours. Although the results were transient, cells remained 59% knocked down after 5 days. Courtesy of A. Mulkeen, MD.

Following ELISA and Northern blot analyses, RT-PCR was used to further strengthen the evidence for effective siRNA interference against VEGF-A. Using isoform specific primers, VP and GL2 transfected RKO cells were compared to untransfected controls by densitometry. VEGF-A_{165} knockdown ranged between 70-81% and VEGF-A_{121} knockdown ranged between 71-74% using this method. This experiment was run in triplicate. Gels and densitometry plots are given in figures 8-13.

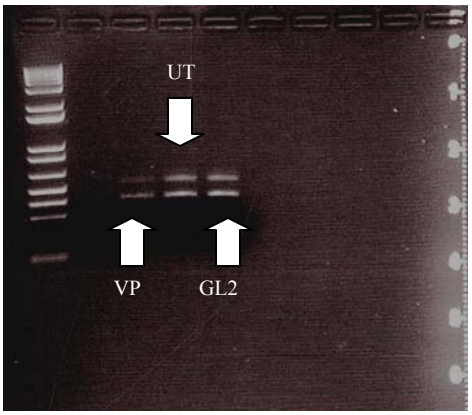


Figure 8: RT-PCR showing VEGF-A expression by RKO cells. Lane 1 is a 1 kb DNA ladder. Lane 2 is a –cDNA negative control.

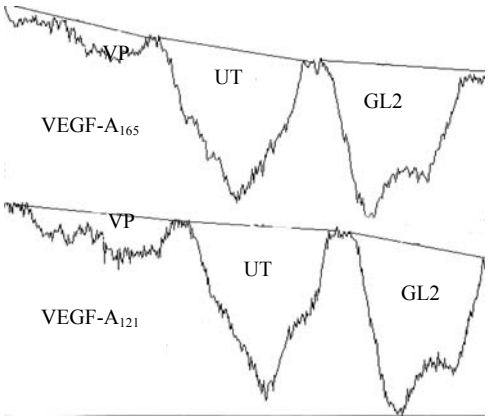


Figure 9: Densitometry analysis of VP, UT and GL2 transfected RKO cells. VEGF-A_{165} is knocked down by 81%, VEGF-A_{121} by 71%.

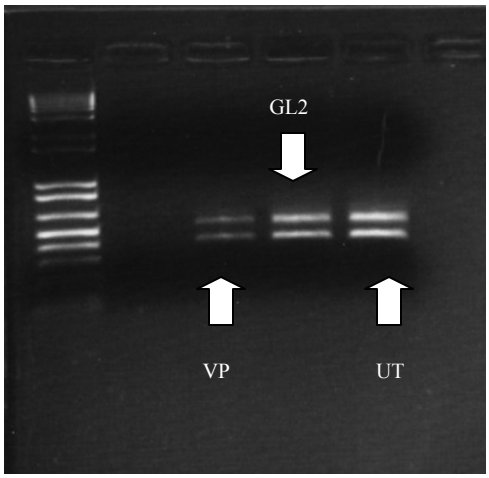


Figure 10: Repeat RT-PCR of RKO cDNA transfected with VP, GL2 and UT at 30 cycles. Lane 1 is a 1 kb DNA ladder. Lane 2 is a –cDNA water control.

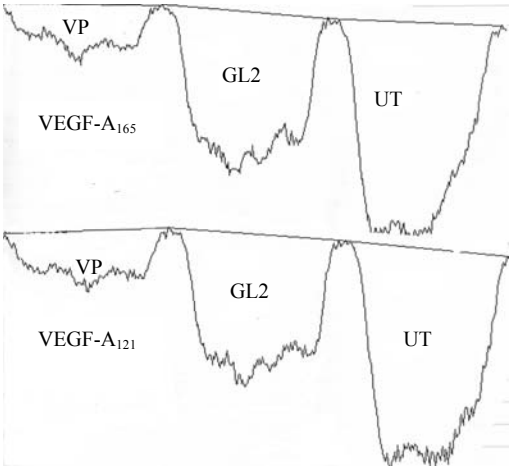


Figure 11: Repeat densitometry analysis of VP, GL2 and UT transfected RKO cells. VEGF-A_{165} is knocked down by 78%, VEGF-A_{121} by 74%.

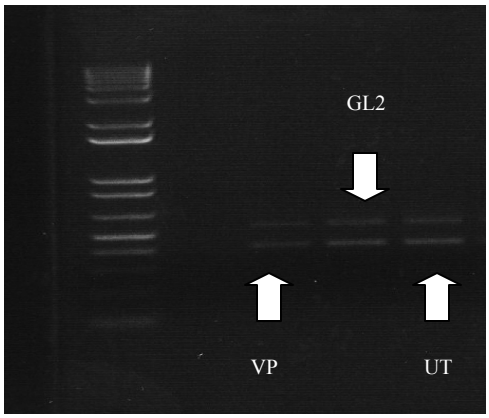


Figure 12: Repeat RT-PCR of RKO cDNA transfected with VP, GL2 and UT at 30 cycles. Lane 1 is a 1 kb DNA ladder. Lane 2 is a –cDNA water control.

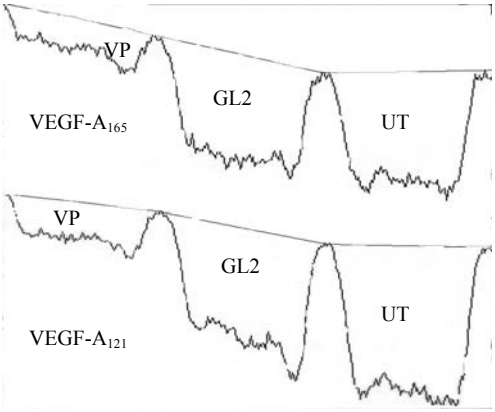


Figure 13: Repeat densitometry analysis of VP, GL2 and UT transfected RKO cells. VEGF-A_{165} is knocked down by 70%, VEGF-A_{121} by 73%.

Quantitative real time PCR confirmed the ELISA and RT-PCR data. Knockdown ranged from 61-67% in cells that were transfected with VP siRNA and harvested 48 hours post transfection (figure 14).

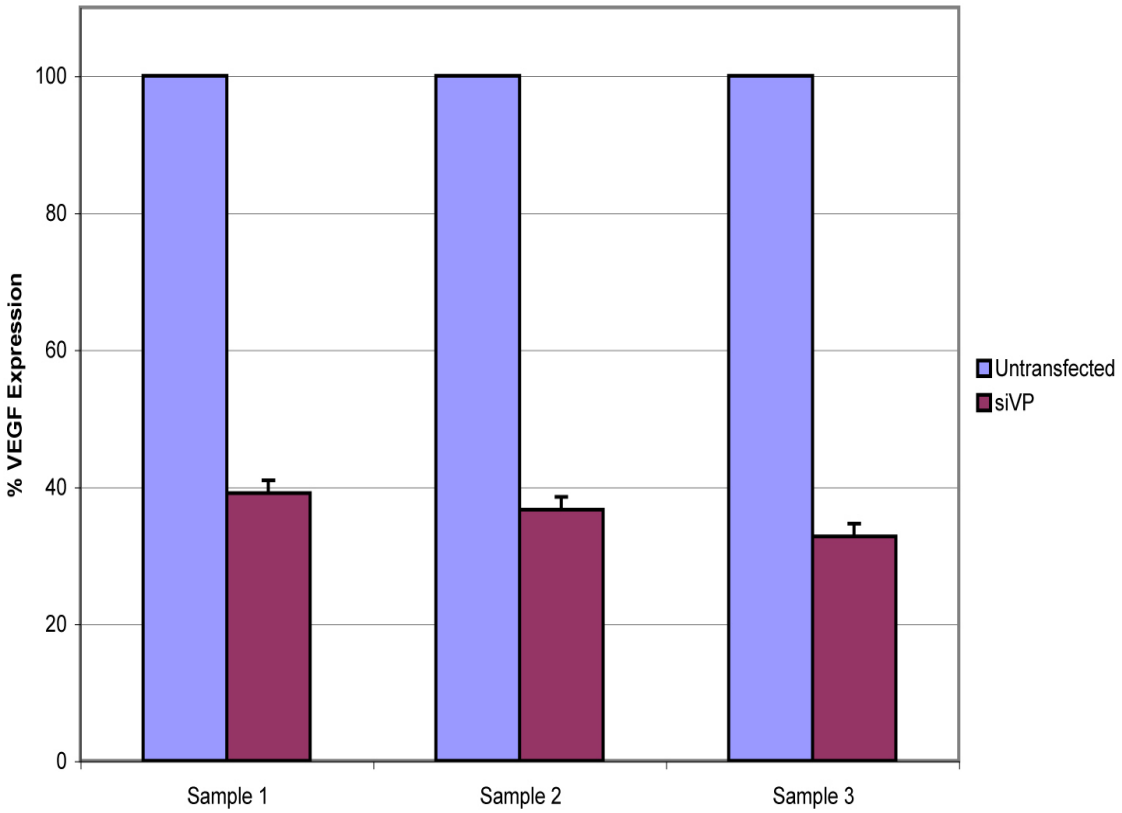


Figure 14: Quantitative real time PCR measuring VEGF expression by RKO cells transfected with 12 nM VP siRNA. Cells were harvested at 48 hours post transfection. Knockdown ranged from 61-67% compared to untransfected controls. Courtesy of A. Mulkeen, MD.

RT-PCR using primers previously described showed that RKO cells express VEGF-B and VEGF-D (figures 15, 16). Densitometry analysis demonstrated a 48% knockdown of VEGF-B in cells treated with VP siRNA. There was no significant knockdown of VEGF-D (figures 17, 18).

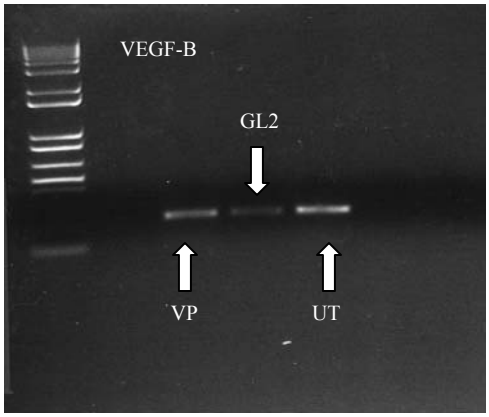


Figure 15: RT-PCR showing VEGF-B expression by RKO cells. Lane 1 is a 1 kb DNA ladder. Lane 2 is a –cDNA water control.

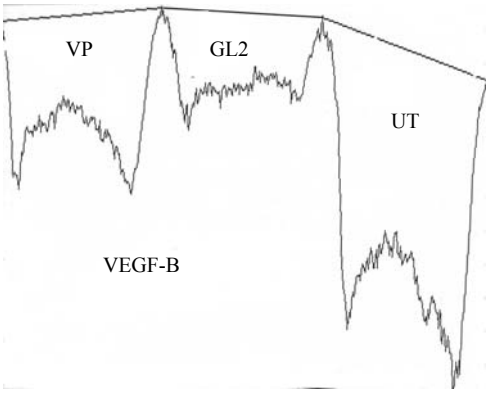


Figure 16: Densitometry analysis of VEGF-B expression by RKO cells. VP transfection results in 48% knockdown compared to untransfected (UT) cells.

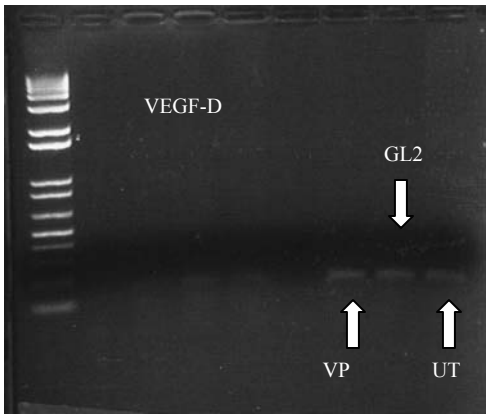


Figure 17: RT-PCR showing VEGF-D expression by RKO cells. 40 amplification cycles. Lane 1 is a 1 kb DNA ladder. Lane 2 is a –cDNA water control.

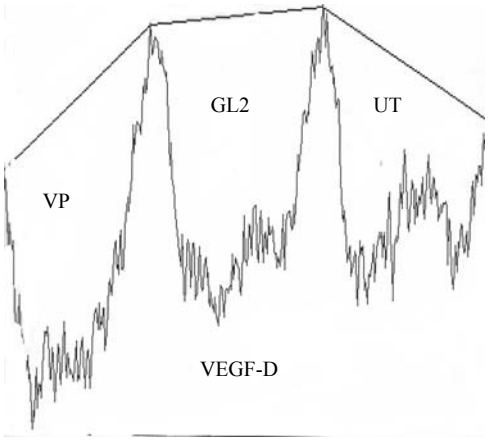


Figure 18: Densitometry analysis of RKO VEGF-D expression. There is no significant knockdown between VP, GL2 and UT cells.

RKO Cell Proliferation and Apoptosis

WST-1 reagent determined the effect of VEGF-A on RKO cell proliferation. RKO cells were transfected with different concentrations (0.1nM, 1nM, 10nM, and 100nM) of either VP siRNA or scrambled control siRNA. Untransfected wild type cells served as controls. Cells were transfected twice over a 5 day period. Proliferation

decreased by 67% in VP treated cells compared to control (figure 19). ELISA correlated this result with decreased VEGF-A knockdown of over 90%.

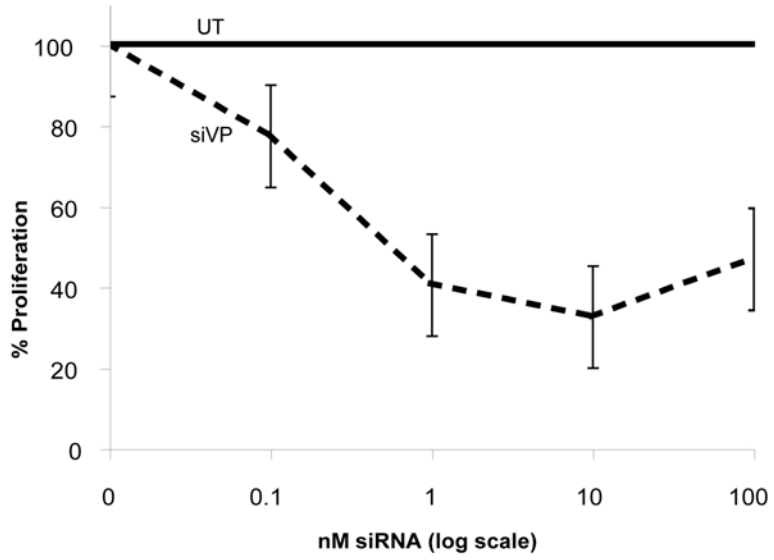
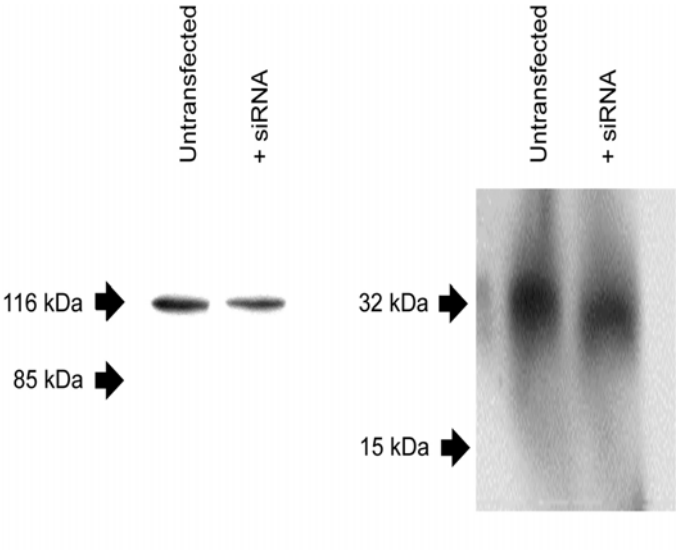


Figure 19: This graph shows percent proliferation compared to baseline as RKO cells are treated with increasing concentrations of VP siRNA. After transfection with 10nM, RKO cell proliferation decreased by approximately 67%. Interestingly, cell proliferation increased with greater siRNA concentration beyond this point. Courtesy of A. Mulkeen, MD.

Proliferation rates did not return to normal after adding back recombinant human VEGF (rhVEGF) or conditioned media to VP transfected cells. Cells treated with rhVEGF and plated in complete media showed a 53% decrease in proliferation, and the cells plated in 2% FBS decreased by 52%. Cells treated with conditioned media did not recover proliferation either.

Cells were assessed for apoptosis to investigate a possible basis for decreased cell proliferation. In accordance with the WST assay, transfected cells and controls were harvested and lysed 5 days after transfection and plating. Western blot analysis did not show evidence of apoptosis using antibodies against PARP and caspase-3 in either

transfected or control cells (figure 20). PARP and caspase 3 are normally cleaved during apoptosis and yield 85 kDa and 15 kDa fragments, respectively.



Transfected and untransfected cells differ in the number of cells in G1. 40% of transfected cells were in G1 compared to 25 % of untransfected. This is a 64% increase (figure 21).

Figure 20: Western blot assessment of RKO cell apoptosis. No apoptosis is observed in VP siRNA transfected RKO cells compared to control. PARP and caspase 3 are normally cleaved during apoptosis and yield 85 kDa and 15 kDa fragments, respectively. Courtesy of A. Mulkeen, MD.

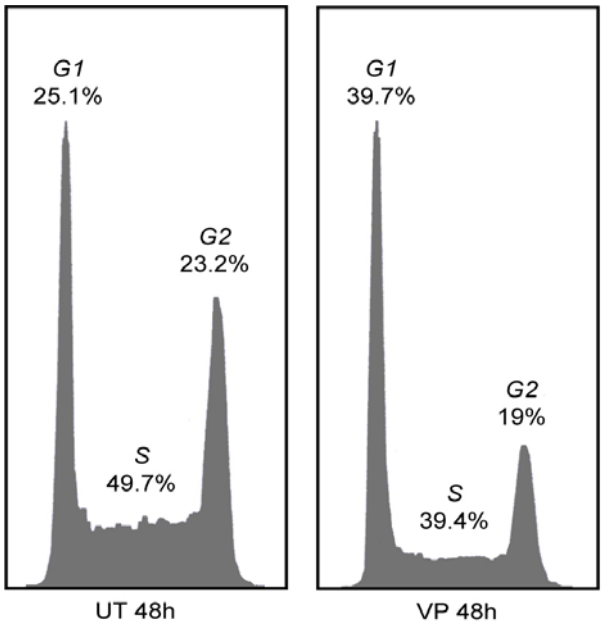


Figure 21: Comparison of RKO cells in G1 and S phase of the cell cycle. 40% of VP siRNA transfected cells are in G1 compared to 25% of untransfected controls. Courtesy of A. Mulkeen, MD.

Discussion:

Despite recent advances, cancer continues to claim over half a million lives per year in the United States alone (1). Colorectal cancers account for 9.5% and 10.5% of total cancer deaths in men and women, respectively (1). With an appropriate sense of urgency, researchers continue to investigate angiogenic mechanisms for their great therapeutic potential. Clearly, cells which trigger the "angiogenic switch" possess a distinct survival advantage over those which can not.

Warren Lewis from John Hopkins University was the first to systematically implicate blood vessels in tumor pathogenesis. In 1927, he observed that rat tumors displayed aberrant vasculature. Further, he observed phenotypic variations in the vasculature of different tumor types. He correctly concluded that tumor microenvironment is an important determinant in vessel density, tortuosity and morphology (2, 8).

VEGF-A was first recognized in 1989 for its strong mitogenic properties and ability to increase vascular permeability (5). In 1992, an in vivo mouse model demonstrated that VEGF-A effects survival rates in solid tumors by directly inducing angiogenesis (10). The mechanism of action was unclear until high affinity binding to VEGFR-2 resulted in increased endothelial cell survival (4, 32). This interaction is important in normal physiology. It is also the basis for the VEGF model for tumor angiogenesis and cell survival.

Originally, VEGF-A and VEGFR-2 were thought to be expressed exclusively on vascular endothelial cells. Further investigation showed that these important proteins are expressed by solid tumors as well (6, 57, 58, 59, 60). Therefore, a tumor cell which

secretes VEGF-A may allow VEGFR-2 activation at its own cell surface. This led to the hypothesis that an autocrine loop may confer a survival advantage to tumor cells by promoting growth and survival independent of angiogenesis (17, 23, 61).

This study shows that human RKO colorectal cancer cells express VEGF-A and VEGFR-2. Additionally, RKO cells express the VEGF-A isoforms, VEGF-B, VEGF-D, PlGF, NP-1 and NP-2. The presence of VEGF-A and VEGFR-2, with NP potentiation, implies that an autocrine loop may be present in colorectal cancer.

With fresh evidence of an autocrine pathway in colorectal cancer, this study reemphasizes the dynamic activity of VEGF and its receptors in the pathogenesis of cancer. The autocrine loop is further understood as a potential target for novel anti-tumor therapies which are independent of the stroma.

VEGF-A receptor activity is highly complex. However, it appears that VEGFR-2 is the predominate receptor which mediates VEGF-A action. It is expressed embryologically in the yolk sac and vascular endothelium as well as the endocardial and mesodermal layers. All of these sites undergo extensive angiogenic activity. However, VEGFR-1 is also significant. Deficiency in either VEGFR-1 or VEGFR-2 results in embryonic mouse fatality (13).

Placental growth factor (PlGF) binds VEGFR-1 and potentiates the same effects as VEGF-A, although more weakly (62). PlGF may compete with VEGF-A for a binding site. If this occurs, then VEGFR-1 can be thought of as a decoy receptor, which by competitive inhibition, makes more VEGF-A available for binding at VEGFR-2 (32). Of note, VEGFR-1 is not expressed by RKO cells in this study. Such a conspicuous absence warrants further investigation.

The autocrine pathway may not be limited to VEGF-A. VEGF-D is known to bind to VEGFR-1 and VEGFR-2. Since VEGF-D and VEGFR-2 are both present in RKO cells, VEGF-D may have an autocrine effect in colorectal cancer as well. This is significant because VEGF-D has been previously shown to promote tumor proliferation and metastasis (1).

VEGF-B also has a known angiogenic function (33). It acts on VEGFR-1 with NP-1 as a cofactor. Since VEGFR-1 is not expressed by RKO cells, VEGF-B action may be restricted to a paracrine pathway in colorectal cancer.

Despite further understanding of pro-angiogenic factors, cytokine secretion alone does not adequately explain tumor progression. Genetic mutations, infection and hormonal influences must also be considered. Neoplastic transformation alone may lead to the angiogenic switch independent of any other influences. For example, VEGF-A overexpression is related to *ras* mutations in colorectal cancers (44). Similarly, p53 mutations are pervasive in human cancers and result in the loss of hypoxia inducible factor (HIF-1α) degradation. Loss of degradation leads to greater HIF-1α activity, which induces transcription at the VEGF promoter site (26). The VEGFR-1 promoter sequence and the erythropoietin gene contain a HIF-1α binding site as well, linking angiogenesis to increased red cell mass (25).

Another study demonstrated that p53 mutations result in the upregulation of matrix metalloproteinase-9 (MMP-9) transcription. This enzyme acts in the extracellular space. Since $VEGF_{189}$ and $VEGF_{165}$ are at least partially bound there, MMP-9 frees these isoforms and facilitates their access to VEGF-A receptors (63). Increased VEGFR-1 activity is also linked to MMP-9 expression (64).

Angiogenesis can be stimulated by infectious and hormonal influences, too. For example, human herpesvirus 8 (HHV-8) partially codes for a VEGF-A receptor. This may be a critical step in the pathogenesis of kaposi sarcoma (13). Thyroid stimulating hormone (TSH) upregulates VEGF-A in thyroid carcinoma, and adrenocorticotrophic hormone (ACTH) acts similarly in human fetal adrenal cortical cells (59). Human chorionic gonadotropin (hCG) and estradiol are pro-angiogenic factors, and androgens stimulate VEGF-A expression in prostate cancer (60, 65).

It is important to note that angiogenesis is a normal physiologic function often required for cellular survival. Although relatively uncommon in adults, angiogenesis does normally occur in tissues with high metabolic demand, such as in the female reproductive tract, or during episodes of tissue repair. These observations gave rise to the hypothesis that angiogenesis is initiated by hypoxia and also occurs in response to inflammatory mediators. Evidence for this hypothesis is observed in many non-neoplastic diseases, such as diabetic retinopathy and HSV-1 keratitis (66).

Although angiogenesis may result from several related and seemingly unrelated pathways described above, this study emphasizes the utility of gene knockdown as a potential therapeutic modality. VEGF-A expression was knocked down by 94% on ELISA following siRNA interference. Since siRNA transfection is often inefficient and yields varying results, data was confirmed by RT-PCR, quantitative real time PCR, Northern and Western blot analysis. All techniques showed significant inhibition of VEGF-A.

VP siRNA is directed specifically against VEGF-A mRNA. There is approximately 45% homology between VEGF-A and VEGF-B, which is located on

chromosome 11q13 (31). This homology may account for the 48% partial knockdown of VEGF-B observed. VEGF-D, located at chromosome Xp22.31, is less homologous and does not show significant siRNA interference (49). This is expected.

This study determined that 12nM of siRNA optimizes VEGF-A knockdown. Cells transfected with this concentration of siRNA were followed over time and were maximally knocked down at 48 hours. Although siRNA technology has transient effects, significant knockdown (59%) remained at 5 days post transfection.

RKO cell proliferation and apoptosis were investigated using siRNA technology. Transfection with 10nM of siRNA targeting VEGF-A decreased RKO cellular proliferation by 67%. This data corresponds to the 94% VEGF-A knockdown at a similar concentration (12nM). Apoptosis was not detected. In fact, 40% of siRNA treated cells were observed in G1 compared to 25% of control cells. This is an increase of 64%. The cell proliferation and apoptosis data clearly demonstrates a role for VEGF in tumor growth and survival pathways.

VEGF-A expression and tumor cell proliferation were both significantly impaired as a result of siRNA interference. However, the effects were incomplete. The VEGF-A protein was still synthesized at low levels and tumor cells continued to grow slowly. Administration of recombinant VEGF or controlled conditioned media did not reverse these effects. Therefore, alternative proliferation pathways besides the VEGF-A and VEGFR-2 mechanism may be active and are interesting future therapeutic targets.

VEGF-A inhibition impairs tumor growth (67). However, we have seen that VEGF-A may act via both paracrine effects on surrounding stroma or autocrine pathways. The interplay between stromal effects and autocrine effects on cellular

proliferation remains unclear. By plating transfected RKO cells in vitro and following cellular proliferation, this study lacked stromal tissue and therefore isolated an autocrine pathway that likely involves VEGFR-2 ligand binding. Another study supports this hypothesis, where treatment of colon cancer cells with anti-VEGFR-2 antibody in vitro resulted in decreased proliferation (68).

siRNA interference is a useful technique with observable effects on VEGF mRNA expression and cellular proliferation. However, siRNA targets VEGF-A expression at the mRNA level. This post transcriptional effect is one approach of many. VEGF-A expression may also be blocked by either downregulating genetic transcription itself, impeding translation, protein exocytosis, or receptor action.

Receptors can be similarly targeted. Neutralizing antibodies and receptor blockade both ultimately impair ligand binding. Cells may upregulate VEGF and receptor expression as a compensatory response, negating the therapeutic effect of receptor blockade. Therefore, novel combination therapies which include siRNA technology are particularly promising (69).

Although exciting, siRNA studies are not easily done. Cell types tend to differ in their propensity to undergo transfection. This is a problem for standardized technique. Signal sequences are also limited by sequence length. If an siRNA is over 30 nucleotides long, for example, it triggers an interferon response which globally degrades mRNA translation via RNAse activity (70). This effect can usually be overcome by RNAse cleavage of long stranded siRNAs into shorter ones in vitro prior to transfection (71).

siRNA interference is also transient. Once inside the cell, siRNAs tend to be highly unstable (46). Since the gene of interest is still active, continuous interference is

necessary to effect long-term knockdown. Furthermore, not all siRNAs are mRNA specific. This has two important consequences. First, studies may be confounded by non-specific results. Secondly, large scale siRNA libraries are not practical, requiring researchers to design siRNAs individually (72).

VEGF-A expression results in numerous effects which appear to be rate limiting in pathologic angiogenesis. Therefore, blocking VEGF-A activity is a natural goal for anti-angiogenic therapies. Several studies have investigated the potential utility of this type of therapy. VEGF-A may be blocked by either downregulating genetic transcription, impeding translation, protein exocytosis, or action at the receptors. Similar techniques can be used against the receptors. Novel combination therapies aimed at multiple mechanisms are particularly exciting.

Linomide, for example, prevents the progression of prostate and mammary cancers in rats by 50% by downregulating VEGF-A and FGF mRNA expression (65). SU5416, which inhibits VEGFR-2, has been shown to block neovascularization in mouse pancreatic islet cell tumors (73). Platelet derived growth factor receptor (PDGFR) inhibition by SU6668 slows tumor regression in the same model by stimulating pericyte detachment from tumor vessels (74).

One exciting study in 1993 showed that anti-VEGF-A antibody reduces the growth rate of mouse glioblastoma cells by 80%. The antibody did not have any observable effect on the tumor cells themselves, suggesting that tumor growth is angiogenesis dependent (58).

Low dose chemotherapy with vinblastine preferentially damages endothelial cells. When combined with anti-VEGFR2 antibody, vinblastine reduces VEGF-A activity and

has an endothelial cell survival benefit (68). Bevacizumab, an anti-VEGF-A antibody, decreases tumor perfusion, vascular density and the number of vascular endothelial cells involved in colorectal cancer (75). Phase II clinical data shows that bevacizumab, when combined with 5-FU and leucovorin, increases survival time in metastatic colorectal cancer (64).

Despite progress in anti-tumor strategies, some tumor types compensate for drug induced VEGF-A knockdown by upregulating gene expression. This speaks to the complexity of molecular interactions involved in the angiogenic switch and the need to overcome confounding variables in therapeutic approaches (27, 71).

Gene knockdown remains promising despite these obstacles. To test gene silencing *in vivo*, researchers engineered *Drosophila* species to express luciferase and then transfected them with siRNA against luciferase. This resulted in the loss of luciferase mRNA expression and subsequent loss of luminescence (76). In a separate study, siRNAs were used to treat HSV-1 keratitis. Infection normally results in recruitment of inflammatory cells which release VEGF into the avascular cornea (66). Female BALB/c mice were inoculated with HSV-1 and allowed to develop keratitis. siRNA against VEGF, VEGFR-1 and VEGFR-2 were administered topically or systemically via tail vein injection. mRNA levels and protein secretion were measured by RT-PCR and ELISA. Angiogenesis was significantly reduced in mice that received siRNA topically alone and also in those that received systemic injection alone (77).

Future studies will require siRNA delivery *in vivo*. Difficulties abound but may be addressed by using creative transport techniques such as ligand directed nanoparticles. Nanoparticles containing siRNA have the potential to overcome uptake and stability

limitations (78). Some animal studies show potential utility for other approaches such as systemic delivery of naked siRNA or plasmid and viral vectors (71). Despite various limitations, siRNA technology remains an important therapeutic tool and a fruitful field for further investigation.

This study demonstrates that human RKO colorectal cancer cells do indeed express many pro-angiogenic cytokines, including VEGF-A and VEGFR-2. These factors are particularly important in angiogenesis. The presence of VEGFR-2 in the absence of stroma implies an autocrine loop at the cell surface that is important to tumor growth and survival.

The autocrine hypothesis is supported by siRNA knockdown data which correlates decreased VEGF-A mRNA expression with decreased cellular proliferation. Accordingly, it is appropriate for novel anti-tumor strategies to target both the paracrine and autocrine pathways involved in tumor pathogenesis.

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