Modulation of Cellular Invasion by VEGF-C Expression in Squamous Cell Carcinoma of the Head and Neck

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Objective: To determine how vascular endothelial growth factor C (VEGF-C) affects tumor cell invasion and motility in squamous cell carcinoma of the head and neck (SCCHN).

Design: A molecular biology study. The VEGF-C coding sequence was cloned into an expression vector and stably transfected into the SCCHN cell line SCC116 to create the SCC116-VEGFC line. RNA interference (RNAi) was used to block VEGF-C expression. An adenoviral system for expressing VEGF-C RNAi was developed and tested.

Setting: An academic hospital laboratory.

Main Outcome Measures: Relative VEGF-C RNA levels were determined by real-time quantitative reverse transcriptase–polymerase chain reaction, and protein expression was evaluated by Western blot. Cellular invasion was evaluated by 24-hour semipermeable membrane transit assay.

Results: SCC116-VEGFC cells had markedly increased expression of VEGF-C protein and RNA compared with normal SCC116 controls. SCC116-VEGFC cells produced marked increases in cellular invasion and motility compared with SCC116 cells. Blockade of VEGF-C expression by transfection of a VEGF-C RNAi expression plasmid into both SCC116 and SCC116-VEGFC cells induced a 38% decrease in SCCHN invasion and motility as tested by a semipermeable membrane invasion assay. We developed an adenoviral expression system for VEGF-C RNAi, which also induced a dose-dependent decrease in cellular invasion in the highly invasive DM12 cell line.

Conclusions: These studies demonstrate that intracellular VEGF-C levels modulate in vitro SCCHN motility and invasion. Further work is needed to clarify the specific receptors and signaling pathways that are involved in SCCHN motility. Molecular therapies that inhibit the VEGF-C pathway may have clinical potential in the treatment of lymphatic metastasis in SCCHN.


QUAMOUS CELL CARCINOMA OF the head and neck (SCCHN) metastasizes via lymphatic channels to cervical lymph nodes, and this regional nodal metastasis is the major cause of locoregional recurrence leading to poor prognosis and a high probability of eventual death from disease. Current therapies such as surgery, radiation, and chemotherapy are only marginally effective at inhibiting this biological process. The development of innovative therapies to alter or directly treat cancer metastasis is a critical step in improving therapy for SCCHN.

Neoplastic cells can leave the primary tumor site either by entering established blood and/or lymph vessels or by recruiting new vessels to the peritumoral area. The vascular endothelial growth factor (VEGF) cytokine family is crucial to the development of this new tumor vasculature. Recent research has identified key molecules that regulate the development of new lymphatic vessels, termed lymphangiogenesis. Vascular endothelial growth factor C (VEGF-C) is a member of the VEGF family that has been implicated in driving this process. This growth factor binds to the endothelial-specific receptor tyrosine kinase VEGFR-2 to produce angiogenesis and affects lymphangiogenesis by binding to VEGFR-3, a related yet distinct receptor. The expression of VEGF-C and VEGFR-3 are correlated with increased lymphatic spread and distant metastasis in a number of human tumors, including melanoma and colorectal, gastric, esophageal, thyroid, and breast cancers. Overexpression of VEGF-C has also been demonstrated in SCCHN, and the development of intratumoral and peri-
tumoral lymphatics is known to be critical for the meta-
static spread of head and neck cancers.13,14 Germ-line over-
expression of VEGF-C increases the number of peritumoral lymphatics in mouse models,15 and VEGF-C signaling has been shown to direct cellular motility and invasion through VEGFR-3.16 Therefore, overexpres-
sion of VEGF-C may promote metastasis by increasing the number of peritumoral lymphatics and by increasing the motility of cells into these lymphatics.17 Taken together, these data suggest that VEGF-C plays a critical role in the progression of tumor growth and metastasis in numerous human tumor systems, but the role that VEGF-C expression plays in motility and invasion in SCCHN is still not clear.

RNA interference (RNAi) has emerged as a powerful genetic tool to study knockout phenotypes.18 Intracellu-
lar ribonucleases cleave double-stranded RNA (dsRNA) into 21- to 23-nucleotide (nt) fragments, which are then incorporated into an RNA-induced silencing complex that uses the dsRNA sequence information to destroy the cor-
responding messenger RNA (mRNA).19 This process can be exploited by structuring synthetic 21- to 23-nt dsRNAs that correspond to endogenous genes, called small interfering RNAs (siRNAs), which can efficiently pro-
vide posttranscriptional gene silencing.20 By designing a complementary 21-nt sequence interrupted by a hairpin-
loop sequence, endogenous intracellular ribonucleases can convert a 60-nt short hairpin RNA (shRNA) into a 21-nt double-stranded siRNA. This powerful process has been used to successfully silence genetic expression in mammalian cells, both in vitro and in vivo.21–22 RNAi-
based medical treatments are a realistic and viable alter-
cative to current genetic therapies and are an active area of pharmaceutical research.23,24 Administration of RNAi constructs against known prometastatic genetic targets, like VEGF-C, may theoretically allow for the blockade of lymphatic spread in vivo.25

To our knowledge, no studies to date have evaluated the ability of RNAi to block VEGF-C activity in SCCHN or determined what effect VEGF-C inhibition might have on SCCHN invasion. In the present study, we show that the overexpression of VEGF-C stimulates in vitro cellular invasion in a head and neck cancer model. We also demonstrate that RNAi directed against VEGF-C inhib-
its the motility of SCCHN cells, delivered both by a DNA plasmid and adenoviral techniques. This provides a ba-
sis for the further development of directed molecular therapies against VEGF-C to block lymphatic invasion in patients with head and neck cancer.

METHODS

CELLS AND REAGENTS

All drugs and biochemicals were purchased from Sigma-
Aldrich Fine Chemical Co, St Louis, Missouri, unless other-
wise specified. Tissue culture reagents were purchased from In-
vitrogen Corporation/Gibco Life Sciences, Carlsbad, California. The UM-SCC-1 cell line was generously provided by Thomas Carey, PhD, University of Michigan, Ann Arbor); the UPCI: SCC116 cell line (referred to in this article as SCC116) by Susanne Golfin, PhD, University of Pittsburgh, Pittsburgh, Penn-
sylvania; and the DM12 cell line by Jeffrey Meyers, MD, PhD, MD Andersen Cancer Center, Houston, Texas. The Chinese hamster ovary cells were purchased from American Type Culture Collection, Manassas, Virginia. Cells were grown as monolayer cultures in Dulbecco Modified Eagle Medium supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin, 1% L-glutamine, and 1% nonessential amino acids. Adenoviral VEGF-C RNAi construct, AdshRNA-
VEGF-C, was produced in conjunction with Beverly Davidson, PhD, in the Gene Transfer Vector Core at the University of Iowa, Iowa City.

REAL-TIME QUANTITATIVE REVERSE TRANSCRIPTION–POLYMERASE CHAIN REACTION

Total RNA was prepared by Trizol reagent (Invitrogen/Life Tech-
nologies) and chloroform extraction. Assays were performed using TaqMan polymerase chain reaction (PCR) core re-
agents, and the reactions were recorded and analyzed using an ABI Prism 7700 sequence detector equipped with a 96-well thermal cycler (Applied Biosystems, Foster City, California). RNA samples (100 ng) were then reverse transcribed for 30 min-
utes at 48°C for 30 minutes, followed by heat inactivation at 95°C for 10 minutes. Complementary DNA (cDNA) tem-
plates were then subjected to a 5-minute initial denaturation at 92°C prior to 40 cycles of PCR (92°C for 20 seconds and 62°C for 1 minute, per cycle) in the presence of Taq DNA polymer-
ase and primers spanning exons 2 and 3 of VEGF-C (forward primer, 5′-TCAAGGACAGAAGAGACTATAAAATTTGC-3′; reverse primer, 5′-ACTCCAAACTCCTCTCCCACAT-3′) or glyceraldehyde 3-phosphate dehydrogenase (Applied Bio-
systems).

IMMUNOBLOTTING

Protein lysates were produced in reducing lysis buffer, and protein content was measured by standard Bradford assay. Twenty micrograms of protein was loaded on 12% agarose gels according to standard sodium dodecyl sulfate–polyacrylamide gel electrophoresis protocol, followed by transfer to a nitrocel-
lulose membrane. Equivalent protein loading was confirmed via Ponceau-S staining of nitrocellulose blots. Blots were probed for 1 hour with primary antibody diluted in 5% nonfat dry milk in Tris-buffered saline with 0.1% Tween-20 (TBS-T), followed by three 10-minute washes. Primary antibodies included VEGF-C primary antibody (ZMD.181, 1:250) (Zymed Laboratories, South San Francisco, California) and α-tubulin (1:4000) (Oncogene Research Products, San Diego, California). The blots were then probed with appropriate peroxidase-conjugated secondary antibody (1:10000) (Jackson Immunoresearch Laboratories, West Grove, Pennsyl-
vania) in 5% nonfat dry milk in TBS-T for 1 hour at room temperature. The blots were washed 5 times for 8 minutes in TBS-T and exposed to enhanced chemiluminescence reagents (Amersham Biosciences, Buckinghamshire, England), followed by radiographic exposure and development.

CLONING OF VEGF-C EXPRESSION VECTOR AND SCC116-VEGFC CELL LINE

The VEGF-C coding sequence was cloned into an expression plasmid using standard molecular cloning techniques. Briefly, normal human oral keratinocytes were cultured from human uvula specimens resected from uvulopalatopharyngoplasty procedures under protocols established with the University of Iowa Human Subjects Office. The VEGF-C
DNA was amplified from normal human oral keratinocytes using the primers CCTGCAAAGTTGGGAAGC (forward) and TGGCACAACACTTTTCCATAA (reverse). Reverse transcription–PCR (RT-PCR) was performed using the Qiagen OneStep RT-PCR kit (Qiagen Inc, Valencia, California) with an anneal temperature of 55°C, extension length of 90 seconds, and 40 total cycles of PCR. The PCR product DNA was separated on a 2.5% agarose gel, and the band corresponding to the full-length cDNA of VEGF-C was removed, followed by DNA extraction using the QiAquick Gel Extraction Kit (Qiagen Inc). DNA was then ligated into the pDNA3.1/V5-His-TOPO vector per manufacturer’s instructions (Invitrogen). Vector DNA was transformed into DH5α chemically competent cells (Invitrogen) and plated on ampicillin plates overnight for colony selection. Plasmid DNA was prepared using the QiAprep Spin Miniprep kit (Qiagen). Sample plasmid DNA was digested with BstXI and Xhol, and the digested DNA fragment was gel extracted and sequenced to confirm proper orientation and insertion of VEGF-C prior to transfection into SCC116 cells. pcDNAV5/His-TOPO–VEGF-C plasmid was transfected into SCC116 cells using Effectene transfection reagent (Qiagen Inc). The VEGF-C overexpressing clones of the SCC116 cell line were then obtained through sequential neomycin selection according to standard protocols, and VEGF-C overexpression was confirmed using quantitative real-time RT-PCR and Western blotting.

**RNAi Construct Design, Flow Cytometric Sorting, and Testing**

The pSilencer 1.0-U6 shRNA expression vector was purchased from Ambion Inc, Austin, Texas. Tested sequences were identified using online software available from Ambion. Sequences were derived from National Center for Biotechnology Information sequence for VEGF-C mRNA (VEGFC; GenBank NM_005429). Sequences were numbered according to location within the VEGF-C coding sequence. Test sequences included position 593, GATCGAGGAGCAGTTAC; position 691, AGGAGGGTGACAAACTAC; position 890, CCTACATGTCGTTCCGTCT; position 1153, GACCCTGCCCCCACAATTAC; and position 1640, GAAGTGTGTGTTGTCG. A control shRNA sequence, which did not match any known human gene coding sequence, was obtained from Ambion. Sequences were cloned and DNA was prepared according to manufacturer’s instructions. RNAi vectors were transfected into cells using Effectene transfection reagent at a 3:1 ratio with green fluorescent protein (GFP). Before beginning any RNAi experiments, GFP-positive cells were then sorted on a Becton Dickinson FACs DiVa (Becton Dickinson, San Jose, California). GFP was excited with a laser emitting 150 mW at 488 nm, and emissions were detected after passing through a 560 short-pass dichroic and 530/30 bandpass filter. Debris, dead cells, and aggregates were eliminated by gating on dual-parameter plots of forward scatter pulse area vs side scatter pulse area and forward scatter pulse area vs forward scatter pulse width. This allowed for the identification of a pure population of transfected cells for use in experiments.

**In Vitro Invasion Assay**

Invasion assays were performed using the Membrane Invasion Culture System. A porous polycarbonate membrane (10-µm pore size) was overlaid with a human basement membrane matrix composed of type IV collagen, laminin, and fibronectin and placed in a sandwich apparatus creating 14 wells. For shRNA experiments, cells were transfected with VEGF-C shRNA plasmid and GFP vector (3:1 ratio) for 24 hours using Effectene reagent. Cells were then sorted on a FACS DiVa for GFP-positive fluorescence to select a pure population of transfected cells for experimental purposes as described previously. GFP-shRNA–positive cells (5 × 10⁴) were then plates in the upper well, and incubation continued for 24 additional hours. The medium contained in the lower well was removed, and cells adherent to the underside of the membrane were removed with trypsin-EDTA and combined with the previously removed medium. Cells were collected onto a polylysine-coated 3-µm membrane, stained with hematoxylin, and counted. At least 5 high-powered fields were counted for every single data point, and each specimen was repeated at least 3 times.

**Adenoviral Transduction**

The SCCHN cells (5 × 10⁴) were plated into 60-mm culture dishes in complete medium. Twenty-four hours after plating, plates were washed in serum- and antibiotic-free medium twice, and a final volume of 0.5 mL of medium was added to each dish containing 0 to 200 MOI (multiplicities of infection, based on the plaque-forming units) of adenovirus. Following 6 hours of incubation, cells were allowed to recover to complete medium. Cells were incubated for 24 hours with control LacZ or VEGF-C RNAi adenovirus before beginning any experiments.

**Data Analysis**

All experiments were repeated at least 3 times, and representative data from a single experiment are presented in this article as means, standard deviations, and standard errors. Statistical comparisons between experimental groups included the use of a 2-tailed t test and analysis of variance.

**Results**

**Cloning and Characterization of SCC116-VEGFC Line**

The VEGF-C coding region was isolated and cloned into an expression plasmid using standard molecular cloning techniques. This VEGF-C expression plasmid was transfected into SCC116 cells, and stable clones were selected using serial passage in antibiotic selection medium. Following stable cloning, overexpression of VEGF-C in the SCC116-VEGFC cell line was confirmed by real-time quantitative RT-PCR (Figure 1A). SCC116 cells expressed VEGF-C mRNA at roughly equivalent levels to normal human oral keratinocyte explant cells in repeated experiments (data not shown). The SCC116-VEGFC cell line expressed VEGF-C mRNA at a mean (SD) fold increase of 236.4 (8.3) over baseline expression in repeated experiments. Increased expression was also confirmed by Western blotting (Figure 1B). Minimal mature VEGF-C protein was seen in SCC116 cells at baseline, whereas a large band was observed corresponding to the full-length 58-kDa VEGF-C protein in the SCC116-VEGFC cell line.
SCC116-VEGFC Clones

**Figure 1.** Characterization of the SCC116-VEGFC cell line. A, Real-time quantitative reverse transcription–polymerase chain reaction (RT-PCR) evaluation of vascular endothelial growth factor C (VEGF-C) messenger RNA (mRNA) expression. After stable cloning of the SCC116-VEGFC cell line, RNA was extracted by the Trizol technique, and VEGF-C expression was compared with the parent cell line by real-time quantitative RT-PCR. B, Western blot analysis of VEGF-C protein expression. Total cellular proteins were extracted under reducing conditions, separated using the sodium dodecyl sulfate–polyacrylamide gel electrophoresis protocol, and probed for VEGF-C expression. Error bars indicate SD. *P<.05.

SCREENING AND SELECTION OF VEGF-C shRNA SEQUENCE

We designed 5 different shRNA sequences to target the VEGF-C coding sequence based on the design algorithms available on the Ambion Web site (http://www.ambion.com). These sequences were selected to span the entire length of the coding region of the VEGFC gene. An RNAi sequence that did not correspond to any known human gene was used as a control. RNAi sequences were cloned into the Ambion pSilencer 1.0-U6 shRNA expression vector, and correct sequence insertion was confirmed by DNA sequencing.

All of our SCCHN cell lines have baseline transfection efficiencies of approximately 30% using Effectene (data not shown), making detection of small changes in mRNA or protein expression in the entire population of cells difficult without screening out nontransfected cells. Therefore, we transfected shRNA plasmids into UM-SCC-1 cells together with a GFP expression vector and sorted for GFP fluorescence by flow cytometry 24 hours after transfection. This allowed for isolation of an enriched population of transfected cells. RNA was extracted and VEGF-C levels were assayed by real-time quantitative RT-PCR. A representative experiment is shown in **Figure 2A**. All tested sequences blocked VEGF-C expression by at least 50%. The shRNA sequence targeting the sequence beginning with nt position 1153 in the VEGF-C coding region induced the largest inhibition of VEGF-C mRNA production, decreasing VEGF-C mRNA expression by a mean (SD) of 82.6% (1.4%) compared with controls. On repeated experiments, this construct routinely blocked VEGF-C mRNA expression greater than 80%.

Validation of the VEGF-C shRNA sequence activity was confirmed by Western blotting. The SCCHN cell lines expressed VEGF-C protein at a very low level at baseline, making knockdown difficult to visualize by Western blotting. Therefore, we overexpressed the human VEGF-C protein in a hamster cell background to produce detectable levels of VEGF-C sufficient for knockdown experiments. Short hairpin RNA vectors were transfected into Chinese hamster ovary cells at a 4:1 ratio with the previously described human VEGF-C expression vector. Proteins were extracted and separated by standard sodium dodecyl sulfate–polyacrylamide gel electrophoresis technique and probed for human VEGF-C expression using an antihuman monoclonal antibody. By this technique, only the transfected human VEGF-C protein would be detected by immunoblotting, and flow cytometric sorting to obtain pure populations of transfected cells was obviated. After 24 hours of transfection, a strong human VEGF-C band was observed in Chinese hamster
ovary cells. Cotransfection with the shRNA expression plasmids induced at least a 50% decrease in human VEGF-C expression for all tested constructs. Again, the shRNA plasmid targeting the VEGFC gene at position 1153 had the strongest effect, with no detectable human VEGF-C protein expression 24 hours after transfection. We subsequently used this plasmid targeting the 1153 region of the VEGFC gene for the remainder of these experiments.

MODULATION OF SCCHN CELLULAR INVASION BY VEGF-C EXPRESSION

The shRNA plasmid targeting the VEGFC gene at position 1153 or control plasmid was transfected into the SCC116 and SCC116-VEGFC cell lines together with a GFP expression plasmid for 24 hours, followed by flow cytometric sorting of GFP-positive cells. This allowed for isolation of an enriched population of shRNA transfected cells for experimental use. Sorted cells were plated into the membrane invasion culture assay apparatus and allowed to grow for 24 hours. Cellular invasion was determined in triplicate by counting at least 5 high-power fields for each experimental condition. SCC116-VEGFC cells had a 30% higher baseline rate of invasion compared with SCC116 controls (Figure 3A). Short hairpin RNA plasmid transfection into SCC116 cells reduced invasion by a mean (SD) of 38.1% (4.1%) compared with control transfection. The cell line constitutively overexpressing VEGF-C, SCC116-VEGFC, had cellular invasion reduced by a mean (SD) of 38.3% (5.2%) by shRNA transfection. Representative high-power field images from invasion assays are represented in Figure 3B. These data demonstrate that VEGF-C overexpression in the SCC116-VEGFC cell line leads to increased invasive potential and that blockade of VEGF-C expression with VEGF-C shRNA decreases the invasive potential in both high and low VEGF-C–expressing SCCHN cell lines.

MODULATION OF SCCHN CELLULAR INVASION BY DELIVERY OF VEGF-C shRNA

Adenoviral transduction allows for more efficient shRNA delivery than plasmid delivery and provides a method for possible in vivo shRNA administration. We developed an adenovirus-based shRNA expression system for VEGF-C and tested the ability of adenovirus-delivered shRNA to alter the invasive potential of SCCHN cells. The shRNA sequence targeting the 1153 region of the VEGFC gene was cloned into a U6-promoter–driven adenovirus construct, AdshRNA-VEGFC. We first determined the ability of AdshRNA-VEGFC to decrease VEGF-C mRNA expression in SCCHN cells. UM-SCC-1 cells were transduced with a wide range of MOI doses of VEGF-C shRNA adenovirus for 24 hours, and VEGF-C mRNA levels were assessed by real-time quantitative RT-PCR (Figure 4A). AdshRNA-VEGFC transduction produced a dose-dependent decrease in VEGF-C mRNA expression levels between 0 and 100 MOI. Peak VEGF-C mRNA inhibition was noted at 100 MOI, at which mRNA levels decreased to a mean (SD) of 23% (1.3%) of the baseline levels.

Invasion of SCCHN cells following transduction of AdshRNA-VEGFC was evaluated using the membrane invasion culture assay system as described previously (Figure 4B). We selected the DM12 cell line, a highly invasive SCCHN cell line selected for anoikis resistance, for adenoviral infection assays because of the high level of baseline invasion exhibited by this cell line. DM12 cells were transduced with a dose range of 0 to 50 MOI of AdshRNA-VEGFC, incubated for 24 hours, and subjected to invasion assay. Control cells were transduced with a LacZ–expressing adenovirus to control for adenoviral infection toxic effects. AdshRNA-VEGFC transduction produced a dose-dependent decrease in DM12 invasion. Decreases in invasion were statistically significant at 10, 25, and 50 MOI doses of adenovirus (P < .05). At the 50 MOI dose of AdshRNA-VEGFC, invasion was decreased by a mean (SD) of 71% (8%) compared with the LacZ adenoviral controls, which was a significantly higher decrease (P < .05) than was observed with DNA-plasmid based shRNA delivery. These data demonstrate the ability of adenovirus-delivered VEGF-C shRNA to decrease SCCHN invasion in vitro and corroborate the aforementioned data for DNA plasmid-based shRNA delivery.
Head and neck cancer progression is typified by early lymphatic metastasis to the cervical lymph nodes. 28 Prognosis in advanced disease is significantly decreased by locoregional and distant metastasis. Current treatment options for neck metastasis in advanced SCCHN include cervical lymphadenectomy and/or radiation therapy, and these can have considerable morbidity. Any molecular therapies that could inhibit the invasive and metastatic potential of SCCHN could notably improve overall patient morbidity and mortality in early head and neck cancers and may be able to offer significant improvements toward local disease control in advanced disease. Molecular therapies could also have a role in the treatment of bulky N+ neck disease, since agents that could slow or reverse any progression of lymphatic metastasis would have great clinical utility in conjunction with surgery and/or chemotherapy and radiation. Despite the large clinical need, there are currently no viable adjuvant thera-

pies that could augment standard treatments to inhibit lymphatic metastasis.

Because of its proven association with the progression of metastasis and connection with lymphatic spread, including head and neck cancers, VEGF-C is one of the leading investigational molecular targets for metastatic signaling pathways. 29 Motility of cancer cells by invasion into surrounding stroma and into peritumoral lymphatics is a critical early step in the metastatic pathway, 29 and tumor cell invasion is directly correlated in vivo with metastatic potential in mouse models. 30 We demonstrate for the first time, to our knowledge, that VEGF-C levels correlate directly with invasive potential in 2 SCCHN cell lines, further implicating VEGF-C in the early promotion of metastasis in head and neck cancer. A recent landmark study by Su et al 16 demonstrated similar changes in motility and metastatic potential following VEGF-C overexpression and blockade in several other human tumors. That article 16 showed that increased motility was induced through signaling via VEGFR-3 and the cell adhesion molecule, contactin-1, through the activation of a Src-p38 MAPK-EBP-dependent pathway. Production of a soluble form of VEGF3R that can bind VEGF-C and thereby inhibit endogenous VEGF-3 signaling has been shown to inhibit lymph node metastasis in a melanoma model, corroborating the involvement of VEGF-R-3 signaling in lymphatic cancer spread. 31 We are currently confirming this involvement of VEGF-C signaling through VEGFR-2 and VEGFR-3 in the invasive potential of SCCHN cells in our laboratory. Based on this, therapies directed against the VEGFR-3 receptor and downstream signaling mediators could augment VEGF-C therapies. These therapeutic modalities could lead to clinical therapies to prevent or decrease metastasis in patients with head and neck cancer.

The data presented herein represent relative levels of invasion based on relative levels of intracellular mRNA VEGF-C expression, and it is therefore difficult to directly extrapolate absolute invasive potential between various cell lines using this method. UM-SCC-1 cells express VEGF-C mRNA at baseline, and this invasion appears to increase measurably with the overexpression of VEGF-C in our study. We have noted a general trend toward high baseline invasion of SCCHN cell lines that express VEGF-C at higher levels, and the DM12 cell line appears to generally produce higher VEGF-C mRNA levels than other SCCHN lines used in this study (data not shown). However, we have also noted considerable variance in intracellular VEGF-C mRNA expression based on culture parameters. Because VEGF-C is an excreted cytokine, both intracellular and extracellular VEGF-C protein levels may be important to develop the invasive phenotype. We suspect that varying VEGF-C levels in the culture serum may contribute to this variance. Invasion and motility of cancer cells in vitro and in vivo is most assuredly influenced by multiple cellular and environmental factors, and VEGF-C expression alone is likely not solely sufficient to promote metastasis and invasion in SCCHN. Based on our data, it is not possible to directly extrapolate the effects of VEGF-C expression on in vivo invasion and metastasis in SCCHN. However, based on the established expression of VEGF-C in SCCHN...
tumors and the published relevance of VEGF-C to tumor metastasis in cancer models, our data provide significant confirmatory evidence for the evolving role of VEGF-C expression in SCCHN motility and metastasis.

RNAi-based therapies are evolving as a treatment modality for use in the clinic based on observed antitumor efficacy in mouse models. Standard gene therapy delivery techniques have demonstrated that RNAi against various human oncogenes can inhibit tumor progression in tumor xenografts. To evaluate one possible mechanism of in vivo delivery of VEGF-C RNAi, we produced an adenoassociated viral expression system for VEGF-C shRNA in this study. Compared with DNA plasmid transfection techniques, adenoviral delivery produced much larger decreases in invasion, without an enrichment step for studying only transfected cells. This is most likely due to more efficient production of the shRNA construct by the adenoviral U6-driven promoter and higher SCCHN infection rates with adenovirus than can be achieved with DNA expression plasmid transfection techniques. One group has demonstrated notable in vivo effects from DNA plasmid-based shRNA delivery against VEGF-C, demonstrating decreased lymphangiogenesis, lymph node metastasis, and spontaneous lung metastasis in a mouse mammary tumor model, but the utility of this technique in the operating room and the clinic is questionable. Previous studies have shown that adenovirus is well suited for delivery of shRNA constructs into head and neck cancers owing to high viral titers and the ability to infect broad spectrums of cell types, including epithelial cells. Retroviral expression of shRNAs would allow for maintained baseline expression within a tumor, but integration of the retroviral DNA could induce further oncogenic mutations. Current studies with aden-associated virus have also shown efficacy but are complicated by significant toxic effects due to saturation of intracellular RNAi protein and enzymatic resources. Clinical cancer therapies to inhibit oncogene expression through the delivery of RNAi are evolving from a vague benchtop concept to a potential bedside adjuvant modality, but the exact method for safe and efficient clinical delivery of RNAi remains unclear. Viral techniques for the clinical delivery of gene therapy (adenoviral, retroviral, and adeno-associated viral) currently predominate in the literature, but these need to be highly refined prior to the development of any large-scale clinical applications owing to concerns over toxicity and secondary malignancy. Small-molecule inhibitors are attractive alternatives to viral-based systems, and small-molecule inhibitors of the VEGFR-3 tyrosine kinase are in development. They may be used alone or in combination with RNAi modalities in the future.

In conclusion, we demonstrate for the first time, to our knowledge, that intracellular VEGF-C expression levels modulate SCCHN cellular invasion in vitro. We show that VEGF-C shRNA can successfully inhibit VEGF-C expression in SCCHN and that inhibition of VEGF-C expression by DNA plasmid or adenoviral RNAi delivery techniques induces significant decreases in SCCHN invasion. This directly implicates VEGF-C in the promotion of SCCHN invasive potential and provides an intellectual basis for further investigations into the VEGF-C signaling pathway and its relation to lymphangiogenesis and metastasis in head and neck tumors.

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Author Contributions: Drs Bock, Jackson, Lee, and Trask and Ms Sinclair and Bedford had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

Study concept and design: Bock, Sinclair, Lee, and Trask. Acquisition of data: Bock, Sinclair, Bedford, Jackson, and Trask. Analysis and interpretation of data: Bock, Sinclair, Lee, and Trask. Drafting of the manuscript: Bock, Sinclair, Jackson, and Trask. Critical revision of the manuscript for important intellectual content: Bock, Bedford, Lee, and Trask. Statistical analysis: Jackson, Lee, and Trask. Obtained funding: Trask. Administrative, technical, and material support: Bock and Sinclair. Study supervision: Lee and Trask.

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