Role of VEGF-C in Proliferation and Migration in a Cancer Model

Emily Marie Benke
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ROLE OF VEGF-C IN PROLIFERATION AND MIGRATION
IN A CANCER MODEL

A Thesis submitted in partial fulfillment of the requirements for the degree of Masters of Science in Human Physiology at Virginia Commonwealth University.

by

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University of Mary Washington, B.S. Biology 2005

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Virginia Commonwealth University
Richmond, Virginia
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### Abbreviations

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<tr>
<td>AMD</td>
<td>age-related macular degeneration</td>
</tr>
<tr>
<td>APC</td>
<td>adenomatosis polyposis coli</td>
</tr>
<tr>
<td>BEC</td>
<td>blood endothelial cell</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
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<tr>
<td>CMV</td>
<td>Cytomegalovirus</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modification of Eagle’s Medium</td>
</tr>
<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
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<tr>
<td>ERK</td>
<td>extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>FAK</td>
<td>focal adhesion kinase</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
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<tr>
<td>FGF</td>
<td>fibroblast growth factor</td>
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<tr>
<td>GPCR</td>
<td>G protein-coupled receptor</td>
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<td>HIF-1</td>
<td>hypoxia-inducible factor 1</td>
</tr>
<tr>
<td>HNSCC</td>
<td>head and neck squamous cell carcinoma</td>
</tr>
<tr>
<td>HPV</td>
<td>human papillomavirus</td>
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<tr>
<td>IL</td>
<td>interleukin</td>
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<tr>
<td>LEC</td>
<td>lymphatic endothelial cell</td>
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<tr>
<td>LMD</td>
<td>lymphatic microvessel density</td>
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<tr>
<td>mRNA</td>
<td>messenger RNA</td>
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<tr>
<td>NGF</td>
<td>nerve growth factor</td>
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<td>NTC</td>
<td>non-targeting control</td>
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<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
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<td>PDGF</td>
<td>platelet derived growth factor</td>
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**PI3-K**  phosphoinositide 3-OH kinase
**PKC**  protein kinase C
**PIGF**  placental growth factor
**PMA**  phorbol myristate acetate
**qPCR**  quantitative polymerase chain reaction
**RCC**  renal cell carcinomas
**RISC**  RNA-induced silencing complex
**RNAi**  RNA interference
**RTK**  receptor tyrosine kinase
**shRNA**  short hairpin RNA
**siRNA**  short interfering RNA
**TGF**  transforming growth factor
**TMD**  tumor microvessel density
**TNF**  tumor necrosis factor
**VEGF**  vascular endothelial cell growth factor
**VEGFR**  vascular endothelial cell growth factor receptor
Abstract

ROLE OF VEGF-C IN PROLIFERATION AND MIGRATION IN A CANCER MODEL

By Emily M. Benke, B.S.

A Thesis submitted in partial fulfillment of the requirements for the degree of Masters of Science in Human Physiology at Virginia Commonwealth University.

Virginia Commonwealth University, 2008

Major Director: Dr. Andrew Yeudall
Philips Institute, VCU School of Dentistry

Head and neck cancer ranks high among the most common cancers world wide. In addition, there is a high recurrence rate, as well as a high prevalence of loco-regional tumor spread. Among many factors contributing to metastasis is vascular endothelial cell growth factor C. VEGF-C is primarily an inducer of new lymph vessel formation, typically during embryogenesis; however, some advanced cancers show a significant increase in VEGF-C expression, suggesting a role in metastasis. In the current study, the effects of VEGF-C expression were tested in HN12 cells, which are highly metastatic and known to express high levels of the chemokine CXCL5. A connection between VEGF-C
and CXCL5 expression was made in previous studies. VEGF-C expression was
downregulated or upregulated in appropriate target cells, in order to test its effect on
proliferation and migration. Downregulation of VEGF-C in HN12 cells resulted in a
decrease in proliferation, migration and motility. Conversely, upregulation of VEGF-C in
HN4 cells led to an increase in cell proliferation. In addition, downregulation of VEGF-C
significantly lowered tumorigenicity in athymic mice. All results suggest VEGF-C is
contributing to an increase in proliferation, migration and motility in this HNSCC model
system.
Introduction

1.1 Head and Neck Cancer

Head and neck squamous cell carcinomas (HNSCC) account for a total of 6% of all
cancers, with approximately 40,000 new diagnoses and 12,000 deaths each year (48).
Squamous cell carcinomas, accounting for more than 90% of malignant head and neck
neoplasms, affect various areas including the oral cavity, pharynx, larynx, nasal cavity and
paranasal sinuses, which is due to the prevalence of squamous mucosa covering the
majority of these areas (33). Although great strides have been made in the understanding
of such diseases at the molecular level over the past few decades, mortality and morbidity
still remain high, upwards of 50% (35). In addition, about 50% of patients will return
within 5 years with recurrence of the primary lesion or distant metastasis, suggesting an
inability to gain loco-regional control of the primary lesion (46). As for the establishment
of a mechanism for the invasion, spread and eventual metastasis, there is still some debate,
leading to a difficulty defining possible therapeutic interventions. With this being said,
early diagnosis and treatment still remain the best options for recovery (46).

HNSCC, like all cancers, begin with normal cells that, at some point, attain genetic
abnormalities leading to alterations in cellular processes controlling growth,
differentiation, apoptosis and motility (48). This damage often comes from a combination
of both genetic predisposition as well as environmental cues (50). There are a number of
possible genetic and environmental cues for oncogenesis; the risk factors for HNSCC have grown to include human papillomavirus (HPV), although the primary risk factors for HNSCC are tobacco, including both cigarette smoke and smokeless tobacco, and alcohol use (38). Some other factors that have been suggested included genetic predisposition, weakened immune systems, and narcotic use (marijuana) (12). In spite of these newly highlighted factors, alcohol and tobacco still remain the highest risk factors. As far as genetic predispositions, genetic instability may be acquired or inherited, suggesting a higher risk of developing HNSCC (28). With respect to genetic instability, mutations often occur with several distinct genes. With HNSCC, specific chromosomes have been shown to undergo losses, implicating APC and p53, both tumor suppressor genes, as playing a vital role in the progression of the disease (28). Mutations affecting p53 are found in 50% of oral cancers, while APC mutations are found in 40% of all oral cancers (40, 44).

Mutations in such genes, implicated in the onset of cancer, fall within two categories: proto-oncogenes and tumor suppressor genes, whose normal functions include regulation of cell growth, motility and other processes (50). Damage to either type of gene can lead to alterations in normal physiological processes, such as regulation of cell growth, motility, apoptosis, and differentiation, which are some of the defining characteristics of tumor cells. Proto-oncogenes, when activated by mutation, become oncogenes, which may attribute to an increase in growth (50). Similarly, tumor suppressor genes, which under normal conditions suppress growth, may undergo mutation, which leads to unregulated cell growth (50).
1.2 Metastasis and Lymphangiogenesis

As mentioned previously, the 5 year survival for HNSCC patients is only about 50%. This is thought to be due in part to failure in gaining loco-regional control of the primary tumor. This phenomenon suggests a readily available mechanism for the development of secondary tumor sites. In this process, also referred to as metastasis, tumor cells invade the surrounding tissues, enter the systemic circulation and establish secondary areas of proliferation (50). The defining characteristic of a metastatic tumor is its ability to become invasive and spread to other areas of the body, often via the vascular system. This transformation frequently manifests through the cell’s interactions with its physical boundaries, typically the basal lamina, which lies beneath layers of epithelial cells and the endothelial cells of blood vessels (50). Most cancerous cells employ an ability to degrade the basal lamina in order to penetrate underlying structures.

For HNSCC, the most common mechanism for metastasis is via the lymphatic system, and the presence of metastatic disease is the greatest predictor of disease-specific survival at the time of diagnosis (46). Among determinants of tumor prognosis, tumor microvessel density (TMD) and lymphatic microvessel density (LMD) are key indicators (25). These provide an indication of the tumor’s ability to gain access to the systemic circulation, which often leads to the formation of secondary tumors. The accompanying invasion into the systemic circulation usually occurs via intratumoral lymphatic vessels, preexisting lymphatics or newly formed lymphatics induced by the tumor cells themselves (25). Typically, spread to the lymph nodes through lymphatic vessels is an early event in the systemic dissemination (25).
The lymphatic system consists of a group of inter-connected, thin walled vessels that drain lymph from extracellular spaces into larger collecting tubes that eventually join the circulatory system (14). The system performs a variety of functions including, but not limited to, absorption of lipids from the digestive system, immune homeostasis, and formation of lymphedema. The thin walled vessels are comprised of endothelial cells, commonly referred to as LECs (lymphatic endothelial cells) and lymphangiogenesis refers to the growth of new lymphatic LECs. There are a number of molecular underpinnings that are now just being discovered that control this process. It can be noted that in the first steps of lymphangiogenesis there is the presence of Lyve-1, a marker that identifies the hyaluronan receptor (14). Hyaluronan is one of the chief components of the extracellular matrix (50). In addition to Lyve-1, prox-1 will often be expressed. Prox-1 is a transcription factor responsible for the commitment to lymphangiogenesis and endothelial differentiation (24).

1.3 Vascular Endothelial Growth Factor

Vascular Endothelial Growth Factor (VEGF) plays a fundamental role in the formation of a vascular supply in various physiological processes including differentiation during embryogenesis, organogenesis, wound healing, and reproductive functions (10). While these processes are fundamental to normal physiological functions, VEGF is also implicated in the pathogenesis of some diseases including proliferative retinopathies, age-related macular degeneration (AMD), rheumatoid arthritis, psoriasis and tumor formation
VEGF aids in the growth of the vascular system by functioning as a potent mitogen for micro- and macro- endothelial cells found in arteries, veins and lymphatics (8).

VEGF gene expression is regulated by a variety of factors, most of which are involved in delivering oxygen to tissues. One of the major inducers of gene expression is a hypoxic environment, where cells experience a low oxygen concentration (8). VEGF mRNA expression is rapidly and reversibly induced when cells are subjected to low pO$_2$ (23). This has been established in a variety of tissues and under a number of circumstances inducing a hypoxic environment. For instance, following myocardial infarction, by means of occlusion of the left anterior descending coronary artery, a substantial upregulation of VEGF mRNA was observed, suggesting VEGF is the main factor leading to spontaneous revascularization following ischemia (2).

Furthermore, VEGF mRNA has also been found to increase in various tumor environments. In studies of glioblastoma multiforme, cells adjacent to an area of tumor associated necrosis experience an increase in VEGF transcription (42). Again suggesting the role hypoxia plays in the expression of VEGF. At the transcriptional level, it has been demonstrated that the same transcriptional enhancer involved in the upregulation of erythropoietin, hypoxia-inducible factor 1 (HIF-1), has been implicated in the induction of VEGF transcription (8). With similar mechanisms controlling both erythropoietin and VEGF transcription, there is additional evidence suggesting the role of hypoxia in VEGF regulation.

In addition to hypoxia, it has been shown that several cytokines and/or growth factors induce VEGF transcription. Some of these include epidermal growth factor (EGF),
TGF-β, and keratinocyte growth factor (8, 11), all of which, when added to cultured epithelial cells, induce an increase in VEGF mRNA. From these findings it is possible to suggest a role of VEGF as a paracrine mediator for indirect angiogenic factors, such as TGF-β (36).

While the above circumstances are under normal physiologic control, VEGF has also been shown to take part in several pathological processes, one of which is tumor angiogenesis. In several types of tumors including, but not limited to, lung, thyroid, breast, gastrointestinal, kidney, bladder, ovary, uterine, cervix, angiosarcoma, germ cell tumors, intracranial tumors, and in the current study head and neck cancers, VEGF has been shown to be significantly upregulated. This suggests a connection between the vascularity of the tumor and gene expression. It has also been shown that only the tumor cells themselves undergo upregulation of VEGF mRNA, but not the endothelial cells surrounding the tumors, thus supporting the hypothesis that VEGF may play a role as an autocrine or paracrine factor (37).

1.4 VEGF-C

The VEGF family is comprised of seven secreted glycoproteins designated VEGF-A (also referred to as VEGF), VEGF-B, VEGF-C, VEGF-D, VEGF-E, VEGF-F and placental growth factor (PIGF), with VEGF-C sharing about 30% homology to VEGF. VEGF-C is defined as a sub-group of the family characterized by a unique amino- and carboxy-terminal extension flanking the common VEGF-homology domain (1). Similarly, all members of the VEGF family share parallel mechanisms, which include binding to their
respective receptor tyrosine kinases, and subsequently signaling to downstream targets responsible for angiogenesis or lymphangiogenesis. However, each member of the family has been implicated in different aspects of vascularization. VEGF-C, the focus of the current study, has been characterized as a key modulator of lymphangiogenesis.

In the adult, VEGF-C is found primarily in the heart, placenta, ovary, small intestine and the thyroid gland, while in mid-gestation it is prominently expressed in regions where the lymphatic vessels undergo sprouting from embryonic veins, such as in the perimetaephric, axillary and jugular areas, and in the developing mesenterium (17). These observations suggest a primary role for VEGF-C in lymphangiogenesis, especially during embryogenesis. The role of VEGF-C in lymphangiogenesis is related to its ability to form functional lymphatic vessels. In addition, it was also found that this regulation did not always include angiogenesis (15).

It has been demonstrated that VEGF-C mRNA is upregulated by a number of factors, some similar to VEGF, including proinflammatory cytokines. When epithelial cells are exposed to interleukin-1 beta VEGF-C transcription is induced, suggesting a role in the immune response (39). Other factors, both cytokines and growth factors, demonstrated to increase VEGF-C transcription include PDGF, epidermal growth factor (EGF), transforming growth factor (TGF-α and β) and cytokines TNF-α and IL-1 α, as well as the diacylglycerol analogue phorbol myristate acetate (PMA) (29). While these factors remain similar between the two families, one major regulatory component not present in the transcriptional mechanism of VEGF-C are the hypoxia-inducible factors which, as stated previously, are the main inducers of VEGF transcription (7).
Once VEGF proteins are synthesized within the cell and undergo final processing, they are released and are able to bind to their primary receptors. VEGF-C binds to and activates VEGF receptor 3, and to a lesser extent, VEGF receptor 2. VEGF receptor 3 (VEGFR3) is part of a class of VEGF receptors of which there are three members. VEGFR3 is a receptor tyrosine kinase and, following binding of the VEGF-C ligand, induces a signaling cascade that typically concludes with alterations in cell migration, proliferation and survival (41).

1.5 Receptor Tyrosine Kinases (RTKs)

RTKs belong to a large family of cell surface receptors, all of which are acted upon by extracellular signaling molecules, and at times lead to an activation of transcription factors. All of these receptors bind a number of specific ligands, which trigger activation of downstream signaling pathways. These pathways have a wide variety of functions including regulation of cell survival, proliferation, differentiation, and even cell metabolism. RTKs bind a number of growth factor related ligands including EGF, fibroblast growth factor (FGF), neurotrophins, and VEGFs. Their structure consists of a single hydrophobic transmembrane α helix, an extracellular domain that contains the ligand binding region, and a cytosolic domain that contains the tyrosine kinase activity (50).

For activation of downstream signaling pathways (Figure 1), the first step occurs when the ligand binds to the receptor and induces the formation of receptor dimers, that is two monomeric receptors joining together to form one functional unit. Following
dimerization, the next step, from which the receptor receives is name, the kinase of one receptor unit phosphorylates one or more tyrosine residues near the catalytic site on the other subunit, leading to a conformational change. The conformational change can lead to an enhanced binding of several other factors, for instance ATP or a protein substrate. Subsequent activation of these kinases leads to the phosphorylation of other tyrosine residues within the receptor’s cytosolic domain. This receptor then contains an abundance of phosphotyrosine residues, which can then act as docking sites for signal-transduction proteins.

While the downstream activity for each RTK is specific for the ligand it binds, there exist some commonalities with the first set of signal-transduction proteins. Some RTKs, once activated, can activate Ras, a monomeric GTP-binding switch protein. Here, a cytosolic adaptor protein, GRB2, binds to a specific phosphotyrosine, and with the help of an additional cytosolic protein, Sos, can aid in the activation of Ras.
Figure 1: Receptor Tyrosine Kinases. Upon ligand binding, the receptor undergoes dimerization and phosphorylation of tyrosine residues.

1.6 VEGFR3

Each member of the VEGF family has a high affinity binding receptor. For VEGF-C, this receptor is VEGFR3. The receptors are designated based on the specific VEGF they bind, with VEGFR-1 binding VEGF-A, B and PIGF, VEGFR-2 binding VEGF-A and B, and VEGFR3 binding VEGF-C and D. VEGFR3 is part of the same subfamily of RTKs as are the other VEGFRs, which are characterized by the extracellular region containing seven immunoglobulin-related domains and extracellular domain preserving homology to the
platelet derived growth factor receptor (PDGFR) subfamily (41). The VEGFRs maintain similar mechanisms to other RTKs, with the same dimerization and activation of tyrosine kinases. The downstream targets are also often similar to those other growth factor signaling pathways, such as EGFR and PDGF in their ability to stimulate cell migration, proliferation, and survival. However, VEGFRs are unique in their ability to signal endothelial cells in their formation of three-dimensional tubes and to regulate vascular permeability (30).

**Figure 2:** VEGF receptors. Interaction with each member of the VEGF family with their designated receptor.
VEGFR3, also referred to as fms-like tyrosine kinase 4 (Flt4), binds VEGF-C specifically. VEGFR3 is a member of the endogenous RTKs found in lymphatic endothelial cells, as suggested by the role of VEGF-C in lymphangiogenesis (32).

Expression of VEGFR3 has been shown to be transient and often correlated with lymphangiogenesis in wound healing and within the endothelium of chronic inflammatory wounds (31). When stimulation and regulation of the VEGFR3 gene is needed, the homeobox transcription factor prox-1 is often induced (47). Due to its role in lymphangiogenesis, like its ligand VEGF-C, VEGFR3 plays a critical part in embryogenesis. This has been suggested by Vegf−/− embryos that are unable to form lymphatic vessels and succumb to severe edema and die prenatally (16).

As described previously, the VEGFR3 maintains similar kinase mechanisms to most RTKs, with Tyr1063 and Tyr1068 residues participating in most kinase activity (30). The signal transduction pathway remains elusive however. The downstream pathways for both VEGFR1 and 2 have been previously described, but only a few targets have been identified as participating in the VEGFR3 downstream pathway. These residues include phosphoinositide 3-OH kinase (PI3-K)-Akt pathway, and protein kinase C (PKC)-extracellular signal-regulated kinase 1/2 (ERK) pathway (41). These pathways have been implicated in other systems as being of importance in vasculogenesis, by guiding migration and sprouting of lymphatic endothelial cells (16).

VEGFR3, similar to VEGF-C, has been implicated in a variety of cancers. Given the fact that the environment surrounding tumors contains a rich vascular supply, VEGFR3
is often upregulated at the periphery of such solid tumors as well as within vascular tumors (34).

Figure 3: VEGFR3 Signaling. VEGF-C signals to downstream factors, which eventually lead to transcriptional activation of factors controlling proliferation, migration and survival. Above is a representation of the signaling process.
1.7 VEGF-C and HNSCC

For the current study, the effects of VEGF-C expression in HNSCC will be determined by a variety of assays to measure proliferation, tumorigenicity, migration and signaling. VEGF-C levels will be manipulated via RNA interference or overexpression in two cell lines, HN12 and HN4. VEGF-C will be overexpressed in HN4 cells, derived from a primary squamous cell carcinoma of the tongue, and VEGF-C will be downregulated in HN12 cells, derived from a nodal metastasis from the same patient from which the HN4 cell were derived (49).

These cell lines were chosen based on previous cDNA microarray analysis of their gene expression. It was found that expression of a chemokine ligand, CXCL5, showed increased expression in metastatic HN12 cells (26). CXCL5 belongs to the chemokine superfamily, which comprises a number of small secreted proteins that act as immune modulators and chemoattractants, as well as activators of lymphocytes. However, some chemokines, like CXCL5, can act on non-immune cells, such as endothelial cells involved in the formation of blood vessels (49).

The receptor for CXCL5 is a member of the G protein coupled receptor family (GPCRs), and is designated CXCR2. When CXCL5 binds to CXCR2, downstream pathways are activated. Some mediators suggested in the pathway include Ras, ERK, PI3-K, Rho, Rac and cdc42, all of which are involved in regulating cell migration.

Like many growth factors, not only do chemokines help to maintain normal physiologic function, but they also function in tumor biology. As chemokines have the
ability to control cell motility, tumor cells often take advantage of this characteristic. In the cell lines described above, CXCL5 is upregulated, again suggesting its role in tumor progression. Chemokines have been suggested to play a role in many aspects of tumor cell biology including proliferation, migration, invasion, homing, and survival. For example, CXCL5 expression was inhibited via RNA interference within squamous cell carcinoma cells and as a result tumor growth \textit{in vivo} was completely eliminated (26). The actual mechanism for this abolishment of tumor growth is not known at this point and can only be speculated as having to do with the depletion of the proangiogenic and proliferative effects of CXCL5.

It has been established that VEGF-C expression is high in the HN12 cell line. In previous studies, when CXCL5 expression is downregulated via RNAi, VEGF-C expression is decreased (26). The previous study concluded that decreased CXCL5 expression inhibited the aggressive characteristics of the HN12 cell line. Given the characteristics of VEGF-C and previous studies concerning its role in tumor progression, it is possible that VEGF-C could mediate the function of CXCL5 in HN12 cells. The present study will determine the role of VEGF-C as a downstream mediator of CXCL5 function in proliferation, migration, and motility in HNSCC cells. This will be conducted through two approaches, upregulation of VEGF-C in HN4 cell lines and downregulation of VEGF-C in HN12 cell lines. Following the establishment of appropriate cell lines, a series of proliferation, migration, motility and tumorigenicity assays will be conducted.
Materials and Methods

2.1 Cell Cultures

All cell lines were maintained in a supplemented Dulbecco’s Modified Eagle Medium (DMEM) (4500 mg/L D-Glucose, L-Glutamine and 110 mg/L sodium pyruvate) (Invitrogen, Carlsbad, CA) with 10% fetal bovine serum (FBS) and 0.02% penicillin/streptomycin (10,000 u/mL penicillin, 10,000µg/mL streptomycin sulfate in 0.85% saline) (Invitrogen, Carlsbad, CA). Medium for transfected HN12 cells, in addition to the above supplements, also contained puromycin (1µg/ml), as the pSirenRetroQ plasmid harbors a puromycin resistance gene. HN4 transfected cells were selected in G418 antibiotic (400µg/ml), as the pCMV-SPORT6 plasmid vector contains a neomycin resistance gene. Cells were kept in a humidified 37\(^\circ\) C incubator in 90% air and 10% CO\(_2\).

2.2 Oligonucleotide Synthesis

For the downregulation of VEGF-C expression, the RNA sequence that was targeted for knock down expression was determined using online software (www.clontech.com, 2007). A double stranded DNA oligonucleotide was designed using online software (www.ambion.com) (see Table 1). Oligonucleotides containing 5’ phosphorylations were
synthesized by Sigma-Genosys, (The Woodlands, TX) and contained BamHI and EcoRI compatible ends for ligation into the pSirenRetroQ (see Table 1 for primer sequences). For overexpression of VEGF-C, a pCMV-SPORT6 plasmid vector containing the entire VEGF-C cDNA was purchased from ATCC (Manassas, VA). In addition, a non-targeting control (NTC) was designed for use in the downregulated VEGF-C assays. The non-targeting control (NTC) was designed for insertion into the pSirenRetroQ plasmid vector and contained a scrambled nucleotide sequence that included the same nucleotide composition as the double stranded DNA oligonucleotide sequence for downregulation of VEGF-C. The control used for the VEGF-C upregulation studies was the HN4 cell line transfected with an empty pCMV-SPORT6 plasmid vector (designated HN4/V). (see Table for sequence data).

2.3 Agarose Gel Electrophoresis

To prepare a 1% agarose gel, 0.5g agarose powder (ISC BioExpress, Kaysville, UT) was dissolved in 50 mL 1x TAE buffer (pH 8.0) with the addition of 1 µL ethidium bromide. The samples were combined with a 6x agarose gel loading buffer with Ficoll and run adjacent to a molecular weight marker at 120V for 20-30 min.

2.4 Purification of DNA From Agarose

Following electrophoresis, when contents of the gel were required for future experiments, particularly digested plasmids, the products were extracted from the gel using a QIAquick Gel Extraction Kit (QIAGEN Sciences, MD). The fragment was removed
from the gel using a clean scalp and placed into a microcentrifuge tube along with 300 µL Buffer QG. The tube was incubated for 10 min at 50º C for the gel to completely dissolve. Following incubation, 100 µL of isopropanol was added and then placed in the QIAquick spin column provided within a 2 mL collection tube. The tubes were centrifuged at 13,000 rpm for 1 min. The flow-through was discarded and 750 µL of Buffer PE was added, again the tube was centrifuged on 13,000 rpm for 1 min. The flow through was discarded and the column spun again for 1 min at 13,000 rpm. The column was placed in a clean 1.5 mL microcentrifuge tube and 50 µL of ddH2O was added. The column was incubated at room temperature for 1 min. Following incubation the tube was centrifuged at 13,000 rpm for 1 min. The DNA was eluted from the column and collected.

2.5 Restriction Endonuclease Reaction

To enable insertion of a DNA insert into a plasmid, the plasmid must be linearized by restriction endonuclease digestion. Each plasmid was digested with a combination of two restriction enzymes, BamH1 and EcoR1 (New England Biolabs, Ipswich, MA) for the pSirenRetroQ plasmid and EcoR1 and HindIII (New England Biolabs, Ipswich, MA) for the pCMV-SPORT6 plasmid. To a 1.5 ml reaction tube 20 U/µL of each enzyme was added in addition to 2 µL 10x BSA, 2µL 10x NEBuffer #2 (10mM Tris HCl, 10mM MgCl2, 50mM NaCl, 1mM DTT, pH 7.9) (New England Biolabs, Ipswich, MA), 200 ng pSirenRetroQ or pCMV-SPORT6 plasmid and 4 µL ddH2O, up to a final volume of 20 µL. The final solution was mixed and incubated at 37º C for 1 h.
2.6 DNA Ligation

The ligation was carried out in a sterilized microcentrifuge tube to which was added: 1:3 molar ratio of oligonucleotide:vector, 2 µL of 10x T4 DNA Ligase Buffer (250 mM Tris-HCl pH 7.6, 50 mM MgCl₂, 5 mM ATP, 5 mM DTT and 25% (w/v) polyethylene glycol-8000), 11 µL ddH₂O, and 1 µL T4 DNA ligase (Invitrogen, Carlsbad, CA). Mixture was incubated at room temperature for 1 hour.

2.7 E. coli Transformation

Cells were removed from –80º C and thawed on wet ice. Four microliters of DNA solution, containing a total of 2 µg/ µL DNA, was added to 50 µL of α-Select chemically competent cells (Bioline, Randolph, MA). The microcentrifuge tube was swirled to mix and incubated on ice for 30 minutes. After incubation, the tubes were placed in a 42º C water bath for 45 seconds, followed by an additional incubation on ice for 2 minutes. The transformation reactions were diluted by the addition of 250 µL of SOC medium (2% tryptone, 0.5% yeast extract, 0.4% glucose, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂ and 10 mM MgSO₄) (Bioline, Randolph, MA). Tubes were then incubated at 37º C and shaken at 225 rpm for 30 minutes. Cells were then plated on LB agar plates containing 50 µg/mL ampicillin and incubated overnight at 37º C.
2.7 Small Scale Plasmid DNA Preparation

To prepare miniprep DNA, the Wizard Plus SV Miniprep DNA Purification System (Promega, Madison, WI) was used. Following growth of selected *E. coli* colonies, 1.5 ml of resuspended liquid expansion was centrifuged at 13,200 rpm for 2 min at room temperature. Supernatant was removed and pellet resuspended with 250 µL of cell resuspension solution (50mM Tris-HCl pH 7.5, 10mM EDTA, 100µg/mL RNase A) (Promega, Madison, WI). Following resuspension, 250 µL of Cell Lysis Solution (0.2M NaOH, 1% SDS) (Promega, Madison, WI) was added and inverted by hand 4 times to thoroughly mix the solution. Samples were then incubated at room temperature for 5 min. Next, 10 µL of alkaline protease solution was added to each sample and again inverted 4 times to mix and incubated at room temperature for 5 min. Three hundred fifty microliters of neutralization solution (4.09M guanidine hydrochloride, 0.759M potassium acetate, 2.12M glacial acetic acid) (Promega, Madison, WI) was then added to each sample and inverted 4 times to mix. Samples were then centrifuged at 13,200 rpm for 10 min. At this time the spin column (Promega, Madison, WI) was inserted the collection tube (Promega, Madison, WI). The supernatant was then transferred to the spin column and centrifuged at 13,200 rpm for 1 min, flow through discarded. Seven hundred fifty microliters of wash solution with ethanol (Promega, Madison, WI) was added to the spin column and again centrifuged at 13,200 rpm for 1 min, discarding all flow through. This step was repeated with 250 µL wash solution. At this time, the spin column was transferred to a sterile 1.5
mL microcentrifuge tube and 100 µL of ddH₂O was added to the spin column. Samples were centrifuged at 13,200 rpm for 1 min to elute the DNA. DNA was stored at –20º C.

2.9 RNA Isolation

To ensure high quality RNA, precautions were taken to avoid RNase contamination of all samples and solutions, thus avoiding RNA degradation. Cells grown in monolayer culture were lysed directly in the culture dish by the addition of 1ml of TRIzol (Invitrogen, Carlsbad, CA) per 10 cm² growth area and incubated at room temperature (15 to 30ºC) for 5 minutes. Contents of plates were then removed and transferred to a sterile 1.5mL microcentrifuge tube. Two hundred microliters of chloroform per 1mL TRIzol was added, capped and shaken vigorously by hand for 15 seconds. Samples were incubated at room temperature for 2-3 minutes followed by centrifugation at 12,000 x g for 15 minutes at 4º C. Samples then separated into an upper aqueous phase and lower phenol-chloroform phase, where the upper phase was transferred to a new sterile microcentrifuge tube. Five hundred microliters of isopropyl alcohol per 1mL TRIzol was added to the tube and incubated at room temperature for 10 minutes followed by centrifugation at 12,000 x g for 10 minutes at 4º C. The supernatant was then removed and remaining pellet was washed with at least 1mL of 75% ethanol per 1mL TRIzol used. Samples were then mixed by vortexing and centrifuged at 7,500 x g for 15 minutes at 4º C. The supernatant was again removed and remaining pellet was left to air dry at room temperature for 10-15 minutes. The RNA was redissolved in 20 µL of RNase-free H₂O and stored at –80ºC.
2.10 Reverse Transcription

A solution of 2 µg total RNA, 1 µL oligo(dT)$_{18}$ primer and ddH$_2$O up to 12 µL total was prepared and incubated at 70º C for 5 min. Following incubation, 1 µL 40mM dNTP mix (10mM each), 4 µL 5x reaction buffer (2M Tris HCl pH 8.0, 5M NaCl, 0.5M EDTA) (Bioline, Randolph, MA) and ddH$_2$O up to 19.75 µL were added to the reaction tube. The total solution was thoroughly mixed, followed by the addition of 0.25 µL BioScript reverse transcriptase (200 u/µL; Bioline, Randolph, MA). The mixture was pipetted to mix and left to incubate at 42º C for 60 min. The reaction was then halted by subsequent heating at 70º C for 10 min. Resulting cDNA was stored at –20º C. Alternatively, Superscript II enzyme was substituted (Invitrogen, Carlsbad, CA).

2.11 Cell Transfection

Cells were grown in 10cm plates and subsequently counted to obtain 2 x 10$^6$ cells per transfection. Cells were then centrifuged at 800 rpm for 5 min and supernatant completely discarded. 100 µL Nucleofector Solution (Amaxa, Gaithersburg, MD) was warmed to room temperature and added to the cells in addition to 5 µg desired DNA. Solution was then added to the cuvette provided and placed into the Nuclefector. Depending on cell type, a custom program (T-20) was utilized for transfection. Following transfection, the solution was pipetted to warmed growth medium.
Alternatively, 293-T cells were grown to 60% confluency in 10 cm plates. In a microcentrifuge tube, 10 µL/µg DNA of the TransIT Keratinocyte Transfection Reagent (Mirus, Madison, WI) was added to 200 µL serum-free media and thoroughly mixed by vortexing. The mixture was then incubated for 20 min at room temperature. One microliter green fluorescent protein was then added to the solution along with 3 ng of DNA, pipetting gently to mix. Again the solution was incubated at room temperature for 20 min. The mix was then added in a drop wise fashion to the prepared cells and rocked gently to distribute the DNA mixture.

2.12 Polymerase Chain Reaction

To a 200 µL tube was added 40µM dNTP mix, 10x PCR Buffer (200mM Tris HCl pH 8.4 and 500mM KCl)(Promega, Madison, WI), 10 µM primers, 0.5 µg template DNA, 0.5 µL Taq DNA Polymerase (Promega, Madison, WI) and finally ddH₂O was added up to a total volume of 50 µL. Contents of reaction tubes were thoroughly combined by centrifugation. The tubes were added to the GeneAmp PCR System 9700 (30 cycles 94º C for 30 sec and 68º C for 1 min) (Applied Biosystems, Foster City, CA).

Alternatively, when amplification did not occur, several alterations were made in the above protocol including: use of 1.0 µL Herculase DNA polymerase and 5.0 µL 10x Herculase Reaction Buffer, 0.2 µL high fidelity TAQ enzyme and corresponding 10 x PCR Buffer, addition of MgSO₄.
2.13 Quantitative Polymerase Chain Reaction (qPCR)

To each well of a 96-well optical plate (Applied Biosystems, Foster City, CA) was added 5.0 µL ABsolute SYBR Green ROX Mix (ABgene, Epsom, Surrey, UK), 3.5 µL ddH₂O, and 0.5 µL primer. In addition, 1 µL desired cDNA was added. Tubulin, actin and GAPDH primers were used as housekeeping genes in order to standardize the quantity of cDNA within each sample. A series of standard dilutions and “no template controls” was added to the optical plate. One microliter ddH₂O was added to the no template controls in place of cDNA, 1 µL of a standardized dilution of each target sequence was added to allow for construction of a standard curve for each target. The standard curve allowed for the determination of relative amounts of amplified cDNA in each test sample. One microliter of each test sample was added to triplicate wells. Following addition of all mixes and samples, the plate was covered with an optical cover and then inserted into the 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). The run consisted of 1 cycle at 50º C for 2 min, 2 cycles at 95º C for 10 min, 40 cycles at 95º C for 15 min, and 1 cycle at 6º C for 1 min.
Table 1 Primer Sequences

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>shVEGF-C - sense</td>
<td>5’ GATCCAAGTGTTGCGTTTGTCCCTTTTCAAGAG</td>
</tr>
<tr>
<td></td>
<td>AAAGGGACACAAACGACACACTTTTTTTTACGCGTG 3’</td>
</tr>
<tr>
<td>shVEGF-C – antisense</td>
<td>5’ AATTCACCGGTAACAAAAAGTGCTGTGCTTTGTC</td>
</tr>
<tr>
<td></td>
<td>CTTTCTCTTTGAAAAGGGACACAAACGACACACTTTG 3’</td>
</tr>
<tr>
<td>VEGF-C - sense</td>
<td>5’ GCGGATCAAACCTCACAAG 3’</td>
</tr>
<tr>
<td>VEGF-C - antisense</td>
<td>5’ GCTTTTCGTTTTTGGCCCTTTC 3’</td>
</tr>
<tr>
<td>Tubulin - sense</td>
<td>5’ AGAGGGCTTTGTGCTGTGTC 3’</td>
</tr>
<tr>
<td>Tubulin - antisense</td>
<td>5’ ACCAGCTTTTATAGGATACCTGT 3’</td>
</tr>
<tr>
<td>Prox-1 - sense</td>
<td>5’ CAGCGGTCTCTCTAGTACCGG 3’</td>
</tr>
<tr>
<td>Prox -1 antisense</td>
<td>5’ AGCGATCCCATATCAAACCTGGA 3’</td>
</tr>
<tr>
<td>Actin - sense</td>
<td>5’ CATGTACGTTGCTATCCAGGC 3’</td>
</tr>
<tr>
<td>Actin - antisense</td>
<td>5’ CTCTTAATGTGCAAGCAGAT 3’</td>
</tr>
<tr>
<td>GAPDH - sense</td>
<td>5’ GGAAATCCCATCACCAGTCTCCAG 3’</td>
</tr>
<tr>
<td>GAPDH - antisense</td>
<td>5’ CATCACGCCACAGTTTCCCCGGAG 3’</td>
</tr>
</tbody>
</table>

2.14 Cell Counting Assay

In order to establish cell proliferation rate, cells were first trypsinized using 0.1% trypsin-2.21mM EDTA (Mediatech, Herndon, VA) and counted. A total of $1 \times 10^4$ cells were plated per well, in triplicate, within a 12-well cell culture plate (Greiner Bio-One,
Cells were incubated at 37° C for 5 days, medium was replaced after 3 days. Following incubation, cells were trypsinized and counted using a hemocytometer cell counting chamber under the light microscope using a 10x objective.

Alternatively, 1 x 10^5 cells were plated in triplicate within a 12-well culture plate (Greiner Bio-One, Monroe, NC). Cells were incubated for 24 h at 37° C. Medium was then aspirated from the cells and replaced with DMEM containing 1% FBS. Cells were then incubated for an additional 4 days. Cells were then removed from the incubator and counted as detailed above.

Alternatively, the above methods were repeated, with each cell line plated in triplicate, up to a total for 5 days of counting. Cells were trypsinized and counted daily for 5 days.

2.15 MTT Assay

As an effective assay to determine cell proliferation, cells were trypsinized using 0.1% trypsin-2.21mM EDTA (Mediatech, Herndon, VA) and counted. Cells were then plated at a density of 5 x 10^3 per well, in triplicate, in a 12-well cell culture plate (Greiner Bio-One, Monroe, NC). Cells were incubated at 37° C for 5 days; medium was replaced after 3 days. After incubation, 100 µL MTT solution (3-(4,5-Dimethylthiazolyl-2)-2,5-Diphenyl Tetrazolium Bromide) (MP Biomedicals Inc., Solon, OH) was added to each well and incubated at 37° C for 4 h. At that time, all MTT Solution was aspirated from each well and 1 mL of MTT solubilization solution (10% SDS in 0.01M HCl) was added to each well and incubated 37° C overnight. After incubation and subsequent
solubilization of crystals, absorbance was determined via spectrophotometer using visible light at 570nm.

Alternatively, cells were plated as stated above, following 24 h incubation at 37° C, growth medium was removed and replaced with DMEM containing 1% FBS. Cells were returned to incubation for an additional 4 days and assay continued as described previously.

2.16 Scratch Assay

One common assay to measure cell migration relies on “wounding” or “scratching” a confluent cell monolayer and measuring migration of cells into the space. Thus, cells were trypsinized using 0.1% trypsin-2.21mM EDTA (Mediatech, Herndon, VA) and plated in triplicate in a 12-well cell culture plate (Greiner Bio-One, Monroe, NC) and incubated at 37° C until cells were completely confluent. At this time, a sterilized pipette tip was placed within each well and scratched across the surface of the plate removing the complete layer of cells within the scratch area. Following cell removal, each well was washed once with PBS and then replaced with DMEM. Immediately following, the width of the scratch was measured at a specific point under a 5x objective using a light microscope and AxioVision software. Cells were then returned to a 37° C incubator. Cells were incubated for a total of 6 h, at which time the scratch width was measured again using the same procedure and at the same position as in time 0. Alternatively, cells were plated and allowed to reach full confluency as before. Following initial measurement, cells were incubated for 2 h and the scratch width was measured. Measurements continued every 2 h for a total of 6 h.
2.17 **Conditioned Media**

Cells were washed with PBS and collected from the plate using 0.1% trypsin-2.21mM EDTA (Mediatech, Herndon, VA). Cells were replated at desired concentrations, and returned to incubation for 24 h. At that time, cells were washed twice with PBS and 10 mL DMEM/1% BSA (Invitrogen, Carlsbad, CA) was added to each plate. Cells were incubated for 48 h at 37º C. Following incubation, all medium was collected from the plates, filtered with Acrodisc syringe filters (Pall Corporation, Ann Arbor, MI) and stored at –80º C. Total cell counts from each plate were also determined at this time.

2.18 **Migration Assay**

Cell were first washed twice with PBS and detached from the plate using 1-2 mL Cell Stripper (Mediatech, Inc, Herndon, VA). Next, cells were counted and added in triplicate into the upper chamber of an 8.0 µm pore size Transwell insert (Corning Inc, Corning, NY) at a density of $2 \times 10^4$ per well. One microliter of conditioned media collected from previous cell cultures was added to the lower chamber of each well. The cells were placed in a 37º C incubator for 4 h and allowed to migrate through the porous membrane of the Transwell and attach to the under surface of the membrane. Following incubation, the plates were removed from the incubator and the cells were fixed with 1mL 0.025% glutaraldehyde (Sigma-Aldrich, St. Louis, MO) in PBS. The membranes were then stained for a minimum of 10 min with 1 mL Hematoxylin (Vector Laboratories, Inc.,
Burlingame, CA), and destained by washing with ddH$_2$O a total of 6 times. The nonmigratory cells were removed from the upper surface of the porous membrane by manual cleaning with a Q-tip. Following this, the membranes were mounted on a glass microscope slide with Permount (Fisher Scientific, Fair Lawn NJ) and coverslipped. Cells were counted in 5 random fields per membrane under 20x objective.

**Figure 4**: Migration Assay. Representation of Transwell insert positioned in individual well used in the migration assay. Cells were plated into upper portion of Transwell insert and conditioned media from desired cell lines was inserted into the bottom portion.
2.19 Protein Lysate Isolation

To 1mL of 1x lysis buffer (1M HEPES pH 7.5, 0.5M EGTA pH 8.0, 100mM β-glycerophosphate, 5 mL NP-40 lysis buffer, 2M MgCl₂) was added 1 µL 1M DTT, 10 µL 0.1M PMSF, 1 µL 10 mg/ml aprotinin, 1 µL 10 mg/ml leupeptin, and 2 µL 1M orthovanadate. Selected cells were grown to 70% confluence. Cells were washed once with cold PBS followed by addition of 70-100 µL lysis buffer directly to the plate. Cells were incubated for 5 minutes on ice. Following incubation, plates were scraped and all contents were collected into 1.5 mL microcentrifuge tubes and centrifuged at 12,000 rpm at 4° C for 5 minutes. Next, the supernatant was transferred to a clean microcentrifuge tube and stored at –80° C.

2.20 Western Blot

Western blotting can be used to determine the abundance and molecular size of proteins. In order to determine relative protein expression, protein lysates from selected cell lines were run on a 10% denaturing polyacrylamide gel containing 0.1% SDS (Molecular Cloning: A Laboratory Manual 2001). Proteins were resolved for 2 h at 110V in 1x SDS-PAGE running buffer (20mM Tris-glycine (pH 8.3), 100mM NaCl, 70mM EDTA, 2% (w/v) SDS). Proteins were then transferred to a PVDF membrane (Immobilon-P; Millipore Corp., Bedford, MA) for 16h in 1x transfer buffer (20mM Tris-HCl (pH 7.9), 100mM NaCl, 70mM EDTA, 20% MeOH). Next, the membrane could be dried,
rehydrated and blocked with 5% non-fat dried milk in 0.05% tween-TBS (TBS supplemented with 0.05% polyoxyethylene (20) sorbitan monolaurate) for 1 h at room temperature with constant rocking. Following incubation, the membrane was washed three times for 5 min in T-TBS. The membrane could then be exposed to the primary antibody, (anti-p-ERK, 200 µg/mL (Santa Cruz Biotechnology, Santa Cruz, CA); anti-ERK, 200 µg/mL (Santa Cruz Biotechnology, Santa Cruz, CA); anti-Akt, 500µg/mL (BD Biosciences, San Jose, CA); and anti-p-Akt, 500 µg/mL (Cell Signaling Technologies, Boston, MA)), overnight at 4° C. The membrane was then washed 3 times as stated previously in T-TBS followed by incubation in horseradish peroxidase-conjugated (HRP) goat anti-mouse monoclonal antibody diluted 1:1000 in blocking buffer for 1 h on a shaker at room temperature. The interaction of the primary and secondary antibodies was detected using Western Lightning Chemiluminescence Reagent Plus (PerkinElmer Life Sciences Inc., Boston, MA).

2.2.1 Statistical Analysis

Data were analyzed using an unpaired t-test comparing two means using GraphPad QuickCalcs software (http://www.graphpad.com/quickcalcs/). P-values were compared to an α-value of 0.05.
Results

3.1 Down Regulation of VEGF-C Expression

It has been previously reported that the HN12 cell line, derived from a nodal metastasis in a patient with HNSCC, expressed high levels of VEGF-C as well as CXCL5 endogenously. When down-regulating VEGF-C expression, CXCL5 expression remains high, thus allowing for results reflecting only the function of VEGF-C. Considering the hypothesis that VEGF-C mediates the metastatic characteristics seen in high CXCL5 expressing HN12 cell lines, down-regulation of VEGF-C within HN12 was performed. VEGF-C was targeted for suppression using RNA interference (RNAi). HN12 cell lines were thus transfected with a pSiren-RetroQ plasmid containing specifically engineered shRNA targeting the VEGF-C coding sequence. This particular plasmid maintains the ability to synthesize the shRNA within the cell, so that it may target the synthesis of VEGF-C, allowing for degradation of the newly synthesized RNA. Also contained within the plasmid is a specific gene for the resistance to puromycin, allowing for selection of colonies expressing the plasmid. Following transfection, cells were incubated in puromycin containing medium, where successfully transfected colonies could grow. Individual clones, shVEGF-C (12 total) and shVEGF-C non-targeting control (NTC) pool, were selected and screened for expression of VEGF-C by Quantitative Real Time Polymerase Chain Reaction.
As shown in Figure 5 there was a marked decrease in the expression of VEGF-C of the clone cells as compared to the control cell line as analyzed by qPCR. Each of the clones showed a decrease greater than two fold, with some clones showing a 90% or more down-regulation. Clone 7, 8, 9 and 12 all showed significant decreases in expression of VEGF-C (p-values: 0.0066, 0.0074, 0.0095, and 0.0080, respectively).
Figure 5: RNA was extracted from selected colonies, reverse transcribed and screened for VEGF-C expression by qPCR analysis, as described in Methods. VEGF-C expression is shown normalized to tubulin expression, an internal standard. (* indicates statistical significance (see text), error bars indicate ± S.E.M.)
As a control in some assays, a non-targeting control (NTC) was used. This particular cell line was transfected with a plasmid as described above. A pool of clones was screened for VEGF-C expression. Figure 6 represents VEGF-C expression in HN12 cell line as compared to the NTC cell line.
**Figure 6:** A non-targeting control was constructed as described above. RNA was extracted, reverse transcribed and screened for VEGF-C expression. Similarly, VEGF-C expression was normalized to the internal control tubulin. (Error bars indicate ± S.E.M.)
3.2 Upregulation of VEGF-C Expression

To test the role of VEGF-C in proliferation and migration, VEGF-C expression was also upregulated in cells normally expressing low levels of expression. This was performed similarly to the downregulated VEGF-C clones, by transfection with a plasmid containing the VEGF-C cDNA sequence. Within the pCMV-SPORT6 plasmid vector there is a promoter region that will continuously express VEGF-C cDNA, leading to an increase in VEGF-C expression. HN4 cells were thus transfected with the plasmid and selected in G418 containing medium, due to the G418 resistance coded by the plasmid vector. Following selection, a clone was picked and screened for VEGF-C expression.

Figure 7 shows a greater than 95% increase in VEGF-C expression. This shows a significant upregulation (p-value = 0.045).
Figure 7: RNA was extracted from selected colony, reverse transcribed and screened for VEGF-C expression by qPCR analysis, as described in Methods. VEGF-C expression is shown normalized to GAPDH expression, an internal standard. (* indicates statistical significance (see text), error bars indicate ± S.E.M)
3.3 Down Regulation of VEGF-C Leads to a Decrease in Cell Proliferation

In order to determine if VEGF-C is contributing to an increase in cell proliferation, the VEGF-C downregulated clones were subjected to cell counting proliferation assays in addition to the control HN12 cell lines, as described in Methods. Cells were plated at a density of $1 \times 10^4$ cells per well and allowed to grow in medium containing 10% FBS. Following incubation, cells were counted and a decrease in proliferation was determined.

As seen in Figure 8, the VEGF-C-downregulated clones showed a considerable decrease in cell proliferation. The cell counts showed at least a 2-fold decrease as compared to the control HN12 cells. It was found that all the downregulated clones cell counts were significantly lower than the control HN12 cell line ($p=<0.001$).

Figure 9 shows the results of the assay utilizing the upregulated clones. There is a significant increase in proliferation, $p = <0.001$, between the HN4/V control and the upregulated clone 1.
Figure 8: Cell count assay was conducted, as described in Methods, with knockdown cells and NTC. Both the shVEGF-7 and shVEGF-8 cell lines show significant decrease in proliferation, $p = 0.0028$ and $0.0070$ respectively. Each point represents $n = 3$. (* indicates statistical significance (see text), error bars indicate ± S.E.M)
Figure 9: Cell count assay was conducted as described in Methods. The VEGF-C overexpressing cell line shows a significant increase in proliferation as compared to the empty vector control. Each point represents n = 3. (** represents statistical significance (see text), error bars represent ± S.E.M)
Cells were also subjected to MTT assay to determine the effects of VEGF-C on proliferation. Similar to the cell counting method, all clones and control cells were plated in triplicate at a predetermined cell density, $5 \times 10^3$ cells per well. In addition to the standard DMEM/10% FBS serum, a separate assay was conducted with cells incubated in DMEM/1% FBS medium. The same cell density was used for both assays.

Figure 10 shows the results of the MTT assays, shVEGF-C clones gave a lower absorbance than the control HN12 cell line (Figure 10A). This indicates a significant decrease in cell number as compared to the control. All values were significantly lower than the control HN12. For shVEGF-C clone 12 clone $p=0.0086$; shVEGF-C clone 7 $p=0.0001$; and shVEGF-C clone 8 $p=0.0010$. Figure 10B indicates similar results; however, all cell lines were incubated in 1% FBS supplemented medium. The results show a significant decrease in proliferation among all three clones: for clone 7 $p=0.016$; clone 8 $p=0.0094$; and clone 12 $p=0.0033$.

Figure 11 shows the results from the assay conducted with the upregulated VEGF-C cell lines. The assay was conducted as described above.
**Figure 10:** Equal numbers of each clone were plated in triplicate and incubated under standard conditions for 5 days. (A) 10% FBS (B) 1% FBS. Following incubation, cells were subjected to MTT assay, as described in Methods. Each point represents n = 3. (*) indicates statistical significance (see text), error bars indicate ± S.E.M)
Figure 11: MTT assay was conducted as described above using the upregulated VEGF-C cell lines. The clone shows significant increase in proliferation as compared to the control, p<0.0001. Each point represents n = 3. (** represents statistical significance, error bars represent ± S.E.M)
3.4 Down Regulation of VEGF-C Leads to a Decrease in Cell Motility

An increase in cell motility is often seen in metastatic cell lines, such as the HN12 cells. To test the role of VEGF-C in increased motility, the down regulated clones and HN12 control cells were subjected to the scratch assay. As described above, cells were plated in triplicate and grown to full confluency, the surface of plate scratched, and the width of scratch measured at time 0 and at 6 h. In some experiments, cells were pre-incubated in DMEM/1% BSA medium for 24 h. Again, cells in these assays, the cells would not have the advantage of the supplemented growth factors that are found within the DMEM/10% FBS medium.

The scratch assays were performed with the cells incubated in DMEM/10% FBS supplemented medium (Figure 12A) and in low serum, (1% FBS) (Figure 12B). In Figure 12A all of the clones showed a significant decrease in cell motility: shVEGF-C clone 12 (p = 0.0196), clone 8 (p = 0.0028) and clone 7 (p = 0.0225). In low serum, clone 7 shows a decrease in cell motility compared to the control HN12 cell line (p = 0.0351; Figure 12B).
Figure 12: Cells were plated and grown to confluence in triplicate under standard conditions. (A) 10% FBS (B) 1% FBS. Cells were subjected to scratch assay, as described
3.5 VEGF-C Promotes Cell Migration

It has been demonstrated, not only in metastatic cell lines but also lymphatic endothelial cells, that VEGF-C can increase cell migration. This characteristic is essential in the normal physiological process of neovascularization, but is also implemented in some malignant tumors due to its ability to aid in the metastatic process. HN12 cell lines have been shown to be highly migratory. In order to test if VEGF-C is contributing to an increase in migration, conditioned media from NTC and knock down cells were used as chemoattractants. Following the protocol outlined in Methods, a standard number of shVEGF-C clone 7 cells were plated in triplicate in the upper chamber of a Transwell insert. Conditioned medium from either shVEGF-C clone 7 or shVEGF-C NTC, with VEGF-C acting as a chemoattractant, were placed into the bottom chamber of the Transwell. After 4 h, cells were fixed, stained, and counted to determine if there was an increase in migration.

As shown in Figure 13, the shVEGF-C clone 7 cells migrated significantly less, (p<0.0001), towards the shVEGF-C clone 7 conditioned media as compared to the conditioned media from NTC cell line. This indicates VEGF-C is a strong chemoattractant for HN12 cells, which migrate towards an area of high VEGF-C concentration.
Figure 13: Cells were plated in upper chamber of a Transwell as described in Methods, and allowed to migrate for 4 h. Following migration, cells were fixed, stained, and mounted, and then counted in 5 random fields under 20x objective. Each point represents n = 5 (** represents statistical significance (see text), error bars represents ± S.E.M)
3.6 VEGF-C Promotes Activation of Downstream Targets of VEGFR3

VEGF-C is often released and acts upon VEGFR3 in the same cell, thus forming an autocrine loop. It is suggested VEGF-C is working in an autocrine loop within the HN12, thus when stimulated by VEGF-C, there is subsequent activation of downstream signaling factors from VEGFR3. Several intracellular signaling proteins have been suggested as downstream targets of the VEGFR3, including extracellular signal-regulated kinase (ERK) and Akt. In order to test if these proteins were activated, western blotting was conducted to determine the levels of phosphorylated (active) forms. Protein lysates were collected from the shVEGF-C clone 7, and NTC cell lines. In addition, in some experiments cell lines were incubated in DMEM/1% BSA for 24 h prior to lysate collection.

As shown in Figure 14, there was a slight difference (1.3-fold) between the activated ERK level in the shVEGF-C clone 7 and NTC. This was also seen in cells that were incubated in DMEM/1% BSA medium, containing no exogenous growth factors. For Akt, similar levels of activity were observed in each cell line. These data show activation of ERK and Akt in both the VEGF-C downregulated cell line and the control, suggesting ERK and Akt may not mediate VEGFR3 activity in HN12 cells.
**Figure 14:** Cell lysates from NTC and shVEGF-C cells were collected and subjected to western blot analysis, as described in Methods. Akt and ERK levels were measured. There was a 1.3x increase in ERK activation levels in shVEGF-C clones compared to NTC. The Akt activation was similar within both cell lines.
3.7 Down Regulation of VEGF-C Reduces Tumor Growth *In Vivo*

To study the effects of VEGF-C on tumorigenicity, an *in vivo* experiment was conducted to test the effect of downregulating VEGF-C in HN12 cells. Two million HN12 cells were injected simultaneously in one flank and the same number of shVEGF-C clone 7 in the opposite flank of athymic mice and allowed to grow. This was repeated for a total of 10 mice. The sizes of the tumors were determined weekly and the assay was continued for 3 weeks.

Figure 15 shows the tumor volume following a 3-week incubation period. There is a significant decrease (p <0.0001) in tumor size in the shVEGF-C clone. This shows a significant decrease in tumorigenicity in the downregulated VEGF-C cell lines compared to the HN12 control. This suggests VEGF-C is contributing to an increase in tumorigenicity.
Figure 15: $2 \times 10^6$ shVEGF-C clone 7 or HN12 control cells were injected into a mouse flank and allowed to grow 3 weeks. Measurements of tumor size were taken every week. Each point is a measurement of n = 10. (** represents statistical significance (see text), error bars represents $\pm$ S.E.M)
3.8 Prox-1 Expression is Regulated by VEGF-C Expression

Prox-1, a homeobox transcription factor, has been shown in previous studies to regulate expression of VEGFR3 (47). To test if VEGF-C may contribute to VEGFR3 expression through prox-1, the level of prox-1 was determined by qPCR analysis. Levels of expression were tested in both the HN12 cell line and the VEGF-C downregulated cell lines. Figure 16 shows the qPCR results. There is a significant decrease in prox-1 expression in the shVEGF-C cells compared to the HN12, p <0.0001.

Figure 16 shows the results of qPCR analysis of prox-1 expression in the VEGF-C upregulated cells. There is not a significant increase in prox-1 expression when comparing the upregulated cell line to the control.
Figure 16: RNA was extracted from selected cell lines, reverse transcribed and screened for prox-1 expression by qPCR analysis, as described in Methods. Prox-1 expression is shown normalized to GAPDH expression, an internal standard. (** indicates statistical significance (see text), error bars indicate ± S.E.M)
Figure 17: Prox-1 expression was measured using qPCR analysis, as described above, in the upregulated VEGF-C cell lines. Expression is normalized to GAPDH, an internal standard. There is no significant difference in expression between the control and the clone. (Error bars represent ± S.E.M)
Discussion

4.1 VEGF-C Expression in HNSCC

It has been shown in previous studies that expression of both CXCL5 and VEGF-C is high in HN12 cells (26). In these studies, a number of proliferation, migration and \textit{in vivo} tumorigenicity experiments were performed to determine the extent to which CXCL5 controls these metastatic characteristics. It was found that CXCL5 contributed to an increase in proliferation, migration, and when downregulated, expression led to the complete inhibition of tumor growth \textit{in vivo} (26). In addition to testing these factors, CXCL5 expression was tested in regards to other growth factor expression levels, one of which was VEGF-C. When CXCL5 expression was inhibited; it was found that VEGF-C was also reduced (26). This connection in expression between the two factors has led to the formation of the current hypothesis, that VEGF-C is mediating the effects of CXCL5 such as an increase in proliferation, migration, and tumorigenicity. Considering the lymphangiogenic properties of VEGF-C, its propensity to increase proliferation and migration in other cancer models, and connection with CXCL5 expression, VEGF-C was hypothesized to mediate the effects of CXCL5 in HN12 cells (4). To test this hypothesis, the expression of VEGF-C was downregulated in HN12 cells and a number of assays were conducted to test VEGF-C expression and its role in proliferation, migration, VEGFR3 signaling and possible regulatory factors mediating its effects.
To modify the HN12 cell lines, RNA interference was used to degrade transcribed mRNA of the selected gene, in this case VEGF-C. A pSirenRetroQ plasmid vector was designed with an oligonucleotide insert encoding a short hairpin RNA complementary to VEGF-C.

Due to the fact pSirenRetroQ contains an antibiotic resistance gene, the transfected HN12 cells could be selected for using puromycin. Following selection, colonies grown from single cells were picked, expanded and screened for VEGF-C expression using collected RNA. Here there was a large decrease in the expression of VEGF-C as compared to the parental HN12 cell line, or cells transfected with a non-targeting control plasmid. On average, there was a greater than 90% downregulation of VEGF-C expression. This indicates the vector was transfected successfully and is providing excellent RNA inhibition. Considering such high percentages of downregulation, the three best VEGF-C downregulated clones were selected for use in proliferation and migration assays.

VEGF-C expression was also upregulated to aid in determination of possible pro-tumorigenic effects. This was carried out in HN4 cells, which express low levels of CXCL5 and VEGF-C (27). Since the HN4 cells were derived from the same patient as the HN12 cells, the question of varying genetic backgrounds is minimized (27). HN4 cells were transfected using the same method as the HN12 cells; however, the vector introduced into the cell carried the entire coding region for VEGF-C under control of a CMV promoter, which provided constitutive expression of the inserted coding region. As with the HN12 cell line, the transfected cells could be selected for using an antibiotic resistance gene encoded by the vector. Following selection, the clones were screened for VEGF-C
expression. The results showed a significant increase in expression of VEGF-C as compared to the control HN4 cell line transfected with empty vector. This suggests the vector is successfully expressing the VEGF-C coding region, essentially producing an increase in VEGF-C product. The successful clone was also subjected to proliferation and migration assays, as with the HN12 cells, to determine the effect of increased VEGF-C expression.

While using HNSCC cell lines in the current study has proven to be an efficient model for the study of VEGF-C in cancer biology, some restrictions remain. For one, this is an in vitro model that carries many limitations. With this come a variety of factors that the cells are not exposed to, as they would be under normal body conditions. Despite all efforts to maintain the best in vitro growth environment for the cells, i.e. temperature, humidity, etc, the conditions remains very selective. While the cells are supplemented with fetal bovine serum, which contains a myriad of growth factors and nutrients needed for cell growth and maintenance, this serum may not accurately represent the endogenous factors the cells would be exposed to in vivo. With these factors in mind, it was imperative to expand the current study to include in vivo assays.

### 4.2 VEGF-C Expression Induces Increased Proliferation

The primary function of VEGF-C is a stimulator and regulator of lymphangiogenesis, and as a ligand to VEGFR3 located primarily on the surface of lymphatic endothelial cells, has been clearly established (8). VEGF-C maintains this function by acting as a potent mitogen for endothelial cells, allowing for the growth of new lymph vessels towards areas
of high VEGF-C concentration (8). For continued formation of new lymphatic vessels, it is essential to increase proliferation of the targeted endothelial cells; this enables the cells to continuously grow towards the desired area. Increase in proliferation occurs following binding of the VEGF-C ligand to the VEGFR3 and activation of downstream signaling pathways that induce proliferation. Considering increased proliferation is often a characteristic of cancers, studies have linked VEGF-C to tumor biology, with several tumor types exhibiting an increase in VEGF-C and VEGFR3 expression (9). HN12 cells have been characterized as maintaining high levels of proliferation (26). To determine if VEGF-C contributes to this increase, both shVEGF-C and VEGF-C upregulated clones were subjected to proliferation assays.

The data for proliferation assays clearly support the hypothesis that VEGF-C expression contributes to an increase in proliferation. Each clonal cell line, showing a considerable decrease in VEGF-C expression, showed a significant decrease in proliferation as compared to the control cell line (26). Also when the assay was performed using the VEGF-C upregulated cell lines, there was an increase in proliferation as compared to the control cell line. In addition, MTT assays provide data to support the above hypothesis. When cultured in low serum conditions, there was also a significant decrease in proliferation in VEGF-C knockdown cells compared to control. A reduced serum environment eliminates the presence of additional growth factors, which allows for more focused analysis of the effects of VEGF-C.

Clearly, VEGF-C expression contributes to increased proliferation. This result can be seen in other cancer models as well. In similar studies, where the expression of VEGF-C
has been downregulated in high expressing cell lines via RNAi methods, similar results are seen. He and colleagues examined a similar system in colorectal cancer. LoVo, a human colorectal adenocarcinoma cell line, which was used in this particular study, shows an increase in the expression of VEGF-C (13). The approach used to establish a connection between VEGF-C expression and lymphangiogenesis within this particular model was very similar to the current study. By utilizing RNAi, the authors performed a variety of proliferation and migration assays to test the hypothesis. Similarly, they found a decrease in cell proliferation \textit{in vitro} following down regulation of VEGF-C. In addition, there was also an inhibition of tumor growth \textit{in vivo} (13). These findings provided additional support for the results of the current study. Thus, it is clear VEGF-C increases the rate of proliferation in tumor cells.

While the cell counting and MTT assays proved to be effective models in determining the difference in cell proliferation, as with all assays some limitations exist. With the cell counting assay, it may be possible to misinterpret the data based on the presence of non-viable cells while counting. This limitation may be avoided by using a trypan blue stain. When cells are exposed to this staining procedure, viable cells with intact membranes exclude the dye, and those with damaged membranes take up the dye. Another limitation that may exist is the accurate plating of the specified cell number. Due to the nature of VEGF-C autocrine / paracrine signaling, the density of the cells may have an effect on the cell growth. If there are an unequal number of cells at the start of the assay, there may be a difference in cell growth as the assay progresses. One way to overcome this limitation is
count the number of individual cells within each well to determine if a growth advantage exists.

### 4.3 VEGF-C Induces Increased Migration

In addition to testing VEGF-C and its ability to regulate cell proliferation, a role in cell migration was also investigated. Previously it was discovered that not only did the HN12 cells show increased proliferation, there was also an increase in cell migration as compared to controls (26). Again, considering the innate expression of CXCL5 and VEGF-C within the cell, and in the absence of CXCL5 a complete lack of tumorigenic qualities, the role of VEGF-C and migration was tested. Within normal physiologic function, VEGF-C acts to induce a pro-degradative environment suitable for the sprouting and migration of endothelial cells towards areas of new vascular growth (6). In this pro-degradative environment there is an increase in expression of serine proteases urokinase-type and tissue-type plasminogen activators and metalloproteinase interstitial collagenase (8). These factors act to degrade the surrounding connective tissue allowing for the sprouting and migration of endothelial cells from existing vasculature (8). Along with creating an environment suitable for the migration of endothelial cells, VEGF-C also acts to stimulate the actual migratory properties of the cell (9). Often for an increase in cell motility, there is an alteration in the genes controlling the actin cytoskeleton (45). In metastatic cancers, there is often an increase in actin remodeling which suggests an increase in cell migration and ability to gain access to the systemic circulation (45). For many cancers, including head and neck cancers, VEGF-C signaling and ability to induce an increase in cell
migration, has been suggested to control metastatic migration (3).

The current study included two types of migration assay, Transwell and scratch assay. Both tested the effect of reduced VEGF-C expression on migratory ability over a specific time period. The Transwell assay tested the ability of VEGF-C to act as a chemoattractant to cell expressing VEGFR3. We found that the shVEGF-C clone migrated significantly more towards the conditioned media from control cells in the Transwell assays. The results show at least a 6-fold difference in the number of cells that have migrated. This increase in migration towards a medium containing higher levels of VEGF-C supports the theory that VEGF-C signaling increases cell motility.

Similarly, the scratch assay was also used to test the migratory ability of the HN12 cell line as compared to the shVEGF-C cell lines. If VEGF-C were contributing to an increase in migration, the cells expressing higher levels of VEGF-C, the HN12 cells, would be expected to migrate at a higher rate than cells expressing lower levels of VEGF-C. The data show that the HN12 cell lines have a higher rate of migration when compared to the shVEGF-C clones. When considering possible limitations to the scratch assay, one possibility is examining the extent of cell migration versus cell proliferation within the assay. To address this consideration, we examine the actual distance the cells migrated within 6 hours compared to average proliferation. Typically, the doubling time for the HN12 cells is 24 hours. For the average cell, with a width of about 5 μm, within a 24-hour period, there would be an increase in movement totaling 10 μm. When examining the migration within the scratch assay, if a rate of 20 μm/hr or higher is seen, this suggests the motility is due to an increase in migration rather than cell proliferation. In addition, another
way to determine if the decrease in scratch width is due to proliferation or migration is to employ time-lapse photography or video microscopy during the scratch assay to visualize the migration of the cells.

In further examination of increased migratory properties of high expressing VEGF-C cell lines, Chen and colleagues examined a similar system in mammary tumors. Mammary tumors have also been found to metastasize via the lymphatic system, which is hypothesized to be attributed to an increase in VEGF-C expression. Chen et al. utilized similar methods for downregulation of VEGF-C in the murine mammary tumor cell line Cl66. Following successful downregulation, the authors performed a variety of assays to test the hypothesis that the downregulation of VEGF-C may reduce lymphangiogenesis and lymph node metastasis. One of the characteristics of lymphangiogenesis as well as metastasis is an increase in cell migration. The authors performed *in vivo* migration assays, and found a significant decrease in cell migration from the original injection site to the lung tissue in cells lacking VEGF-C. In addition to supporting this particular hypothesis, the data also complement the results of the current study. As in the current study, VEGF-C expression shows a relationship to increased cell migration (5).

Some limitations do exist with the migration assays. For instance in the scratch assay, the width of the scratch made within each well varied. If there is a VEGF-C concentration gradient established within the scratch the difference in the gradient would correspond with the variance in scratch width. To overcome this limitation in the future it may be possible to use the exact same width instrument in all wells, by determining the proper speed and angle for an appropriate size scratch and repeating exactly with each well.
4.4 Inhibition of VEGF-C Expression Decreases Tumorigenicity In Vivo

Given the results seen in the in vitro assays, namely a decrease in VEGF-C expression levels contributing to a decrease in proliferation and migration, the shVEGF-C clones were then tested in vivo. This in vivo assay provides a more stringent representation of the effects of decreased VEGF-C on migration and proliferation due to the fact that cells are subjected to factors not found while in culture. In previous studies examining the effects of CXCL5 expression in HN12 cells, downregulation of expression led to a complete inhibition of tumor growth in vivo (26). This result provided evidence for the role of CXCL5 in tumor progression. If VEGF-C is modulating the effects of CXCL5, tumor growth would be expected to decrease in vivo with downregulation of VEGF-C.

The results showed a significant decrease in tumor size arising from VEGF-C knockdown cells. These findings suggest a role for VEGF-C in tumorigenicity downstream of CXCL5. It is significant that the results seen in the in vitro assays have been replicated by in vivo assays, providing strong evidence to support the hypothesis that VEGF-C expression leads to an increase in proliferation and migration in HNSCC. The results also help to connect VEGF-C and CXCL5. Considering the previous study connecting CXCL5 expression and inhibition of tumor growth, it was hypothesized the loss of tumorigenic potential was due, at least in part, to a loss of VEGF-C expression. There was a significant decrease in tumor size, but not a complete loss of tumorigenic potential as seen with CXCL5 knockdown. This suggests VEGF-C does play a vital role in the tumorigenicity of the HN12 cells, but that additional factors regulate tumor growth.
It is possible that another VEGF family member, such as VEGF-A, also mediates the effects of CXCL5 in tumorigenicity. In the HN12 cell line, an increase in VEGF-A expression has also been shown (27). Again, a similar connection may exist between CXCL5 and VEGF-A. VEGF-A and C are both members of the same growth factor family, and both factors mediate related functions through similar pathways. VEGF-A is mainly responsible for angiogenesis, the formation of new blood vessels (9). The mechanism for VEGF-A stimulation and signaling is similar to VEGF-C. As with VEGF-C, the main inducers of VEGF-A gene expression are hypoxia, oncogenes, and transcription factors (9). The receptor for VEGF-A is VEGFR2, another receptor tyrosine kinase. Similar to VEGF-C, VEGF-A ligand binds to its receptor, initiating a series of downstream signaling cascades, leading to an increase in migration and proliferation (9).

VEGF-A has also been reported to be upregulated in several metastatic cancers, including melanoma, breast, colorectal, gastric, breast, hepatic, lung and squamous cell tumors (9). VEGF-A signaling within these cells increases proliferation, migration, cell survival and angiogenesis (9). In other metastatic cell models, a connection between VEGF-A and CXCL5 expression and metastasis has been suggested. Mestas and colleagues (22) have tested the presence of VEGF-A and CXCL5 within renal cell carcinomas (RCC). High concentrations of both VEGF-A and CXCL5 were found to be present in the serum of RCC patients. When the receptor for CXCL5, CXCR2, was downregulated in RCC tumor cells, a decrease in tumor growth was found. Upon further investigation into the mechanism for inhibition of tumor growth, a decrease in VEGF-A expression was found. This suggests the decrease in tumor growth was due to a decrease
in VEGF-A induced angiogenesis and associated proliferation and migration. This
connection between VEGF-A and CXCL5 expression in RCC may account for some of the
properties of HN12 cells.
Figure 18: VEGFR Signaling Pathways. Possible downstream signaling targets for VEGFR2 and 3.

4.5 VEGFR3 Signaling

The lymphangiogenic effects of VEGF-C are modulated via VEGFR3 signaling. As mentioned earlier, VEGFR3 receptors are found on the surface of endothelial cells and provide the signaling cascade for the induction of increased proliferation and migration (6). In tumor biology, VEGFR3 is often expressed on the surface of tumor cells expressing high levels of VEGF-C (6). This suggests VEGF-C is signaling through an autocrine or
paracrine loop in order to increase cell proliferation and migration within the tumor cell. Considering this process, downstream mediators of VEGFR3 were tested for activation in the current study. Unfortunately, the proposed mechanism for the VEGFR3 signaling pathway remains incompletely characterized. There are, however, some signaling proteins that have been proposed in the VEGFR3 signaling pathway, including ERK and Akt (41). Both proteins, when activated via phosphorylation, are reported to increase proliferation, migration and survival in this pathway (41).

In the current study, the levels of activated ERK and Akt were measured by western blot analysis. Upon examination of the results, the level of activated Akt were similar between the NTC cell line and shVEGF-C. However, the level of activated ERK increased by a total of 1.3 fold in the shVEGF-C cell lines when compared to the NTC. With ERK activation, the results were not as predicted: if the activation occurred as suggested in the literature, ERK activation would have been significantly higher in the NTC cell lines compared to the shVEGF-C.

If the pathways regulating proliferation and migration do not involve Akt or ERK, perhaps signaling is occurring through other mediators. Bock and colleagues have shown an increase in the cell adhesion molecule contactin-1 through activation of Src-p38 MAPK-C/EBP-dependent pathway following VEGF-C binding to VEGFR3 in HNSCC (3). The increase in contactin-1 correlates with an increase in migratory ability in other cancer models, such as lung carcinomas (43). Besides ERK, another possibility to explain the increase in proliferation is the involvement of signaling factors known to act downstream of other VEGF receptors. Considering that many of the pathways for
VEGFR3 have yet to be fully characterized, there are a large number of possibilities for downstream pathways. One possible mediator is focal adhesion kinase (FAK), which is known to increase proliferation following VEGF-A binding to VEGFR2 (5). One study has indicated the presence of VEGFR3 on the surface of bone marrow endothelial cells and following VEGF-C binding, there was an increase in FAK activation (20).

From the western blot analysis, ERK activation is seen in the shVEGF-C clones. ERK typically is associated with an increase in proliferation; however, both the in vivo and in vitro assays show a significant decrease in proliferation with the shVEGF-C clones. Thus, activation may be induced by another mechanism. It has been shown that ERK activation may vary in strength and duration, and as a result there is a difference in signaling specificity (21). When studied in PC-12 cells, used primarily as a model of neuronal differentiation, epidermal growth factor (EGF) induced a strong but transient activation in the ERK cascade, but when the cells were exposed to nerve growth factor (NGF), there was a weaker but prolonged activation of the cascade (21). When the cells were exposed to EGF there was an increase in proliferation, but when exposed to NGF there was an increase in differentiation. This suggests a transient activation of ERK leads to an increase in proliferation, but a longer activation increases differentiation. In HNSCC and other tumors, a significant de-differentiation is observed with increased tumor progression (19). Thus, it could be that the increase in ERK activation is indicating a re-differentiation of the shVEGF-C cells, or the reversal of the mesenchymal phenotype of HN12 cells.
As mentioned previously, the transcription factor prox-1 has been suggested as a regulator of VEGFR3 expression. In the current study, the expression of prox-1 was determined with qPCR. It was found there is a significant decrease in prox-1 expression in the shVEGF-C cell lines. Therefore, if there is a decrease in prox-1 expression there may be a decrease in VEGFR3 expression, leading to a decrease in receptor numbers at the cell surface. This decrease in expression also indicates a connection between VEGF-C expression and VEGFR3 expression via prox-1 signaling. When this was tested with the upregulated VEGF-C expressing cells, there was not a significant difference between the control and clone. Perhaps VEGF-C participates in expression of VEGFR3 expression through prox-1 signaling, but also requires other factors for this increase.

4.6 RNA Interference Limitations

RNA interference (RNAi) provides a well-established and excellent means to down regulate the expression of a target gene. Many studies have employed this technique with great success in order to modify the expression of the gene of interest. For these reasons the current study utilized this particular method for the down regulation of the VEGF-C gene. With this technique however, come many challenges in creating a stable and reliable knock down.

For instance, several designs of interfering RNA can be used for use in RNAi. With each design are several limitations including stability and off-target binding (18). In the current study, short hairpin RNA (shRNA) was utilized in order to address some of these limitations. shRNA has been shown to overcome the transient expression often
experienced with short interfering RNA. The main difference between siRNA and shRNA lies with its endogenous structure; shRNA is able to form a stable hairpin loop that is analogous to naturally occurring microRNA, on which the technique is based. With this advantage, the shRNA is not easily degraded, allowing for continual activation of the RISC complex and down regulation of the target gene.

Designing the coding sequence of the shRNA is one of the most crucial steps to a successful RNA interference. Within the processes of designing the particular coding sequence, it is important to determine if the sequence shows any homology to other proteins. This allows for a degree of specificity. However, despite these measures, it is not possible to completely eliminate any chance of a non-specific binding, or off target binding, which results in the down regulation of an unknown gene (18). To avoid this problem, the targeting sequence designed showed little to no sequence homology to other human genes. While it may be possible to test expression of possible homologous genes, this would be very costly and time consuming. Despite this possibility, rigorous design remains the greatest method for specific gene targeting.

4.6 Future Studies

In the current study, it has been shown that VEGF-C affects the proliferation, migration and motility of HN12 cells, in addition to tumorigenicity \textit{in vivo}. While these findings are significant and important in furthering the study of HNSCC development, many uncertainties still remain. For instance, the downstream targets of the VEGFR3 have
not been well established in previous tumor studies, and in the current study the findings suggest HNSCC may not use the downstream mediators postulated in the current literature. One way to determine possible mediators is to extend the western blot analysis. Once established, it could be possible to specifically target these mediators for down regulation or inhibition and determine their effect on cell proliferation, migration and motility. This could lead to a determination of the exact mechanism of VEGFR3.

In addition it might also be an option to down regulate VEGFR3 via RNA interference in both HN12 cell and the shVEGF-C clones. It would be possible to use a similar approach as was used for shVEGF-C transfections, but using an alternative antibiotic resistance in order to properly establish shVEGFR3 clones. Following successful transfection and screening of established clones, similar assays could be conducted. It would be possible to determine if VEGFR3 expression is the limiting factor in the metastatic characteristics or if the cells would even be viable. It could even be possible to determine if the cells began to use one of the other VEGFRs in order to avoid apoptosis. All of these are vital questions in determining the biology and molecular mechanisms underlying HNSCC development and progression.

As mentioned earlier, to avoid some of the limitations in the assays performed and the shRNA limitations, several steps could be taken in future experiments. For the determination of a concentration gradient, it would be possible to manipulate the some of the factors within the migration assay in order to address this limitation. The migration assay could be conducted as previously established, but in the presence of varying concentrations of VEGF-C. From this it would be possible to establish at which
concentration, if any the cells migrated more effectively. To overcome the limitations in the utilization of shRNA, it is possible to use a blocking antibody specific for VEGF-C and/or its receptor.

In summary, the results of the current study indicate a role of VEGF-C in proliferation, migration and tumorigenicity. These results support the hypothesis that increased VEGF-C expression leads to an increase in migration, proliferation and tumorigenicity. VEGF-C has also been shown to mediate some of the effects of CXCL5 in tumorigenicity, which supports the proposed hypothesis. In addition, further research can be conducted to determine what other factors mediate the role of CXCL5 in tumorigenicity.
Literature Cited


VITA

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