2007

Effects of CXCL8 Overexpression on Tumor Cell Proliferation and Migration in an HNSCC Cell Model

Emil Paul Christofakis
Virginia Commonwealth University

EFFECTS OF CXCL8 OVEREXPRESSION ON TUMOR CELL PROLIFERATION AND MIGRATION IN AN HNSCC CELL MODEL

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

by

EMIL P.CHRISTOFAKIS
University of Mary Washington, B.S. Biology, 2005

Director: DR. ANDREW YEUDALL
PHILIPS INSTITUTE, VCU SCHOOL OF DENTISTRY

Virginia Commonwealth University
Richmond, Virginia
May 2007

Acknowledgement

First and foremost I would like to thank my PI, Dr. Andrew Yeudall. If it were not for his vast knowledge in the field of cancer biology, willingness to take a chance on a new student, and most importantly, his unending patience, I would have never had such a great opportunity to learn so much. I would also like to thank Dr. Hiroshi Miyazaki. His explanation of biochemistry, unforgettable analogies, and knowledge of laboratory technique have helped me progress in my understanding of cancer biology. I would also like to thank Huixin Wang, who was always there to help me with any question I had and most importantly, to catch me before I made any critical mistakes. Finally, I would like to thank my lab partner, Anisha Patel. She was always there to listen to my frustrations and celebrate my accomplishments. I do not think I could have made it through the year with out her.

Table of Contents

Page

List of Tables

Page

List of Figures

Page

Abstract

EFFECTS OF CXCL8 OVEREXPRESSION ON TUMOR CELL PROLIFERATION AND MIGRATION IN AN HNSCC CELL MODEL

By Emil P. Christofakis, B.S

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

Virginia Commonwealth University, 2007

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

Head and neck squamous cell carcinoma is the 6th most common malignancy worldwide. Recently, a link between cancer and inflammation has been found. Mediating this relationship are the chemotactic cytokines known as chemokines. CXCL8 (Interleukin-8), a CXC ELR^{+} Chemokine mainly responsible for neutrophil chemoattraction, has been implicated in increased tumor proliferation, migration and angiogenesis. The current study tests the effects of CXCL8 on the tumor proliferation and metastasis. By genetically

modifying cells to knockdown or overexpress the CXCL8 gene we tested its biological role in head and neck cancer progression. Overexpression of CXCL8 in HN4 tumor cells with low endogenous CXCL8 levels was found to increase tumor growth, as judged by cell counting and MTT assays. Conversely, RNAi-mediated knockdown of CXCL8 expression in HN12 cells, which express high levels of this chemokine, resulted in a decrease in proliferation. Similarly, overexpression of CXCL8 enhanced migration of HN4 cells in vitro, while knockdown inhibited HN12 cell migration and invasion through a basement membrane substitute. Taken together, these findings support the hypothesis that CXCL8 affects multiple processes involved in head and neck cancer tumor progression. The data suggest that CXCL8 is a potential therapeutic target for head and neck, and other, cancers.

Introduction and Background

1.1 Cancer

Worldwide, out of every 100,000 people, anywhere from 100 to 300 will die of cancer and approximately one fifth of the deaths in the United States each year are cancer related (Lodish, *et al.,* 2004). Of these vast numbers, head and neck cancer accounts for about six to seven percent of new cancers (McMahon and Chen, 2003). 40,000 people in the United States and 500,000 people worldwide are diagnosed with head and neck cancer each year (Kim and Califano, 2004). Diagnosis and treatment of head and neck cancer has had many advances in the past 20 years, unfortunately survival rates have not improved very significantly (McMahon and Chen, 2003). The 5-year survival rate has remained at approximately 50% with some sites and more advanced lesions having considerably poorer outcomes (Kim and Califano, 2004). Head and neck cancers involve four anatomical areas of the body: the oral cavity, oropharynx, hypopharynx, and larynx. Since all of these areas are covered by squamous mucosa, the most common type of malignancy (accounting for approximately 90%) is squamous cell carcinomas (McMahon and Chen, 2003).

Cancer is the result of genetic mutations occurring in normal cells, which leads to alterations in their normal physiological processes including proliferation, differentiation,

apoptosis and motility (Yeudall and Miyazaki, 2007). These mutations occur in two large classes of genes: proto-oncogenes and tumor suppressor genes (Kim and Califano, 2004; Lodish, *et al.,* 2004). Proto-oncogenes normally have a proliferative effect on cells. When a gain-of-function mutation occurs in these genes they may become constitutively active leading to the cancer phenotype (Kim and Califano, 2004; Lodish, *et al.,* 2004). On the other hand, tumor suppressor genes are normally regulatory and anti-proliferative. A loss-of-function mutation in these genes causes them to lose the ability to regulate proliferation, and similarly to the proto-oncogenes, they too may develop a cancer phenotype (Kim and Califano, 2004; Lodish, *et al.,* 2004).

These mutations can occur via environmental influences or genetic factors (McMahon and Chen, 2003). Environmental factors are typically carcinogens, such as certain chemicals or ultraviolet radiation that humans are exposed to. Certain viruses, such as human papillomavirus (HPV) can also cause cancer. The most preventable environmental risk factor for HNSCC is tobacco use, which includes cigarette smoke and smokeless tobacco. (McMahon and Chen, 2003; La Vecchia, *et al.,* 1997). Also included in this preventable sub-grouping is alcohol. It has been found that the risk for cancer by alcohol and tobacco together is greater than multiplicative (La Vecchia, *et al.,* 1997). It was also reported that the relative risk for HNSCC among tobacco and alcohol abusers is approximately 20 times great than those who use neither (McMahon and Chen, 2003; La Vecchia, *et al.,*1997). Several theories have been developed as to the role that alcohol plays in this duo, which includes its function as a solvent, facilitating the passage of carcinogens into cells or its effect of increased liver metabolism to activate carcinogenic substances (La

Vecchia, *et al.,* 1997). Inherited genes can also lead to host susceptibility factors, such as how efficiently carcinogen metabolism occurs in cells and the cells ability to repair DNA damage (McMahon and Chen, 2003).

1.2 Cancer and Inflammation

More recently, another possible cause of cancer has been found: inflammation. This link is beginning to have implications for the treatment and possible prevention of cancer (Balkwill and Mantovani, 2001). Inflammation is a normal part of an organism's reaction to various stimuli, with the function of counteracting them. Acute inflammation is a beneficial response that can be therapeutic if it is self-limiting or manifested for a short period of time (Lawrence, 2007; Aggarwal, *et al.,* 2006). It involves the recruitment of polymorphonuclear granulocytes followed closely by monocytes, which differentiate into macrophages. This process is usually initiated by the activation of resident macrophages, which release proinflammatory molecules such as eicosanoids, cytokines, chemokines and proteases, which recruit and activate leukocytes (Lawrence, 2002). On the other hand chronic, or long-lasting, inflammation may be harmful and can lead to disease. This perseverance can be due to a prolonged inflammatory stimulus or the dysregulation of an organism's own anti-inflammatory mechanisms (Lawrence, 2007; Aggarwal, *et al.,* 2006). Chronic inflammation has been found to play a key role in many diseases, including cardiovascular diseases, cancer, diabetes, arthritis, Alzheimer's disease, pulmonary diseases and autoimmune diseases (Aggarwal, 2004). More specifically to cancer however, studies have shown that chronic inflammation has been linked to many key steps which aid

in the progression of tumorigenesis, including cellular transformation, survival, proliferation, invasion, neovascularization and metastasis (Mantovani, 2005; Coussens and Werb, 2002). Also, supporting this idea is the fact that tumors are not made up completely of transformed cells, but that as much as 80% can be made up of stromal cells and inflammatory cells that have not been genetically modified (Leek, 1996).

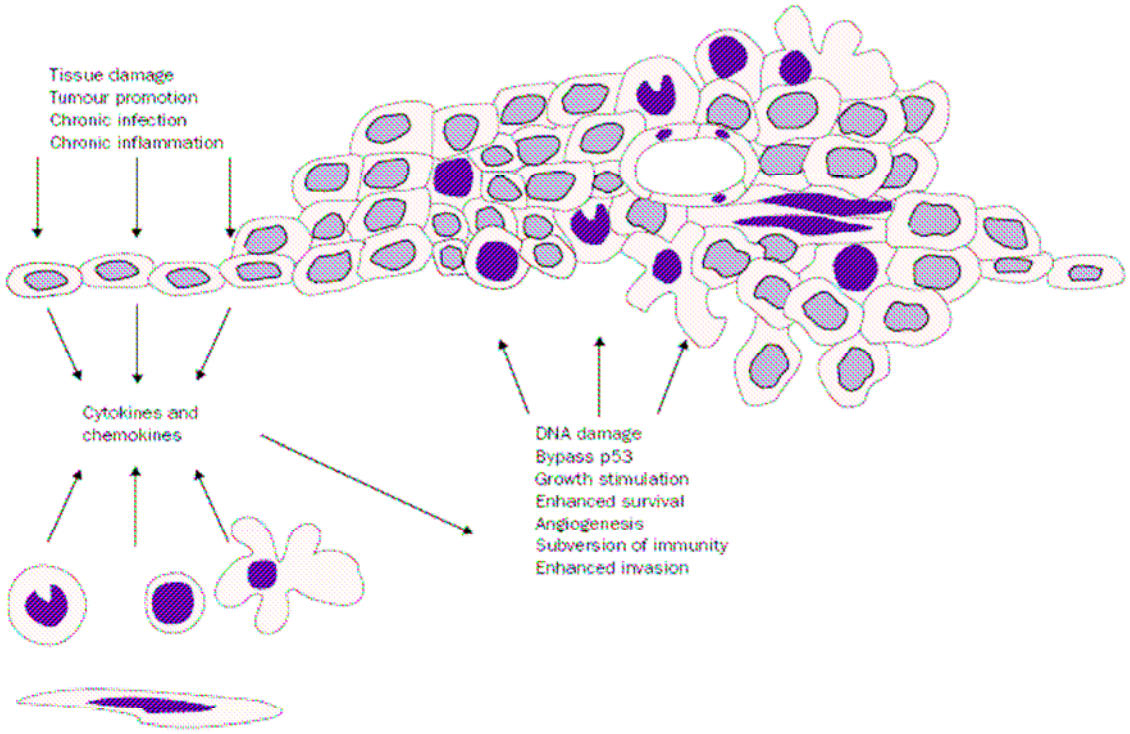


Figure 1: Chemokine production. Chronic inflammation, tissue damage and chronic infection may stimulate cytokines and chemokines that contribute to development of malignant disease (Balkwill and Mantovani, 2001).

1.3 Chemokines

One of the major groups of pro-inflammatory molecules that have been implemented in the progression of cancer are the chemokines. Chemokines, or ***chemo***tactic cyto***kines,*** are part of a large multifunctional family of molecules that induce the migration of leukocytes, and are induced by inflammatory cytokines, growth factors and pathogenic stimuli (Laing and Secombes, 2004; Balkwill, 2004). They are small molecules ranging anywhere from 8-10 kD in size (Laing and Secombes, 2004; Balkwill, 2004; Wilson and Slettenaar, 2006). Some chemokines are homeostatic in nature and are produced and secreted constitutively. Others are produced by cells during infection or following a pro-inflammatory stimulus (Laing and Secombes, 2004). Signaling by chemokines typically results in the transcription of various genes that are involved in many tumorigenic activities such as cell invasion, motility, extracellular matrix interactions and survival (Balkwill, 2004).

Chemokines are structurally divided into four main categories according to the number and spacing of the first two cysteine residues at the N-terminal end of the protein: C, CC, CXC and CX_3C (Laing and Secombes, 2004; Balkwill, 2004; Wilson and Slettenaar, 2006).

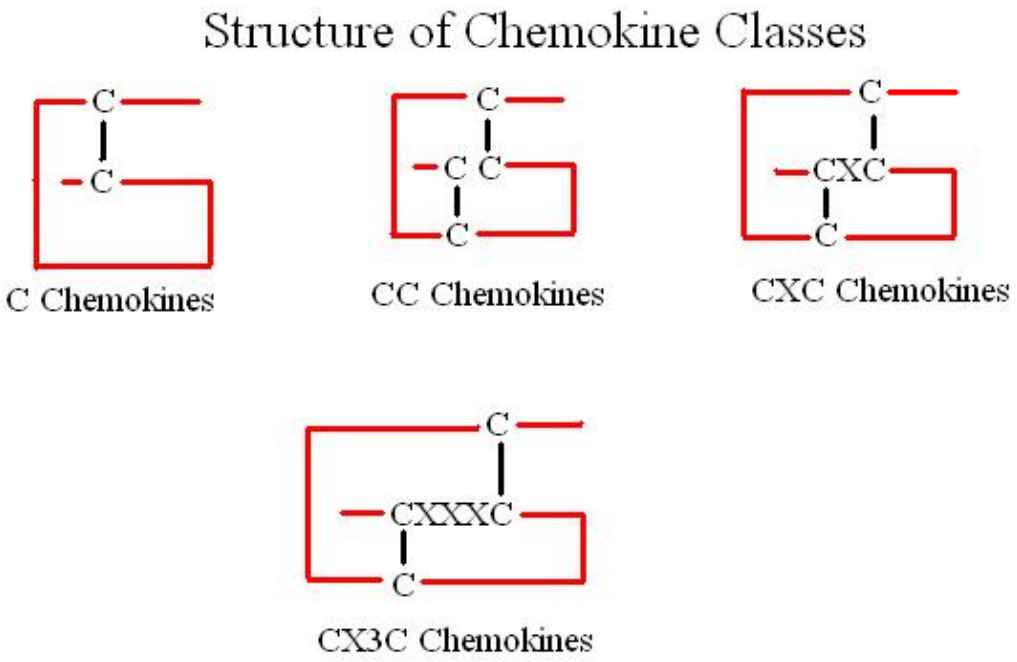


Figure 2: *Chemokine classes*. Representation of C, CC, CXC and CX_3C chemokine classes. Diagram shows consecutive cysteine residues, peptide chain and disulphide bridges

The ELR^+ CXC subgroup includes CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7, CXCL8 and CXCL15. The function of these CXC chemokines is to promote the adherence and migration of neutrophils closer to inflammatory sites. They also have angiogenic and chemotactic properties for endothelial cells (Laing and Secombes, 2004). The ELR^- CXC subgroup includes CXCL4, CXCL9, CXCL10, CXCL11, CXCL12, CXCL13, CXCL14 and CXCL16. These chemokines are responsible for attracting lymphocytes and monocytes, but unlike ELR^+ CXC chemokines, they have poor

chemotactic ability for neutrophils. Also opposite to the ELR^+ CXC chemokines, is this subgroup's ability to inhibit angiogenesis because most are believed to be angiostatic (Laing and Secombes, 2004). CC Chemokines are responsible for the attraction of mononuclear cells (macrophages) that are involved in either homeostatic or pro-inflammatory mechanisms as well as both chemoattractant and immunomodulatory roles. (Laing and Secombes, 2004; Yeudall and Miyazaki, 2007). The C and CX_3C groups are not as common and do not play as large of a role in inflammation and cancer as the CC and CXC groups.

Most chemokines, despite their differences in structure, exert their effects by binding to seven transmembrane domain G protein-coupled receptors (GPCRs). The CC chemokines bind to CCRs, the CXC chemokines bind to CXCRs and the C and CX_3C chemokines bind to CX_3CRs and XCRs (Yeudall and Miyazaki, 2007). There is considerable redundancy in the system with many chemokines binding to more than one receptor, each receptor may respond to more than one chemokine and exert various effects, as well as each cell type expressing several different receptors (see Figure 2) (Kulbe, *et al.,* 2004).

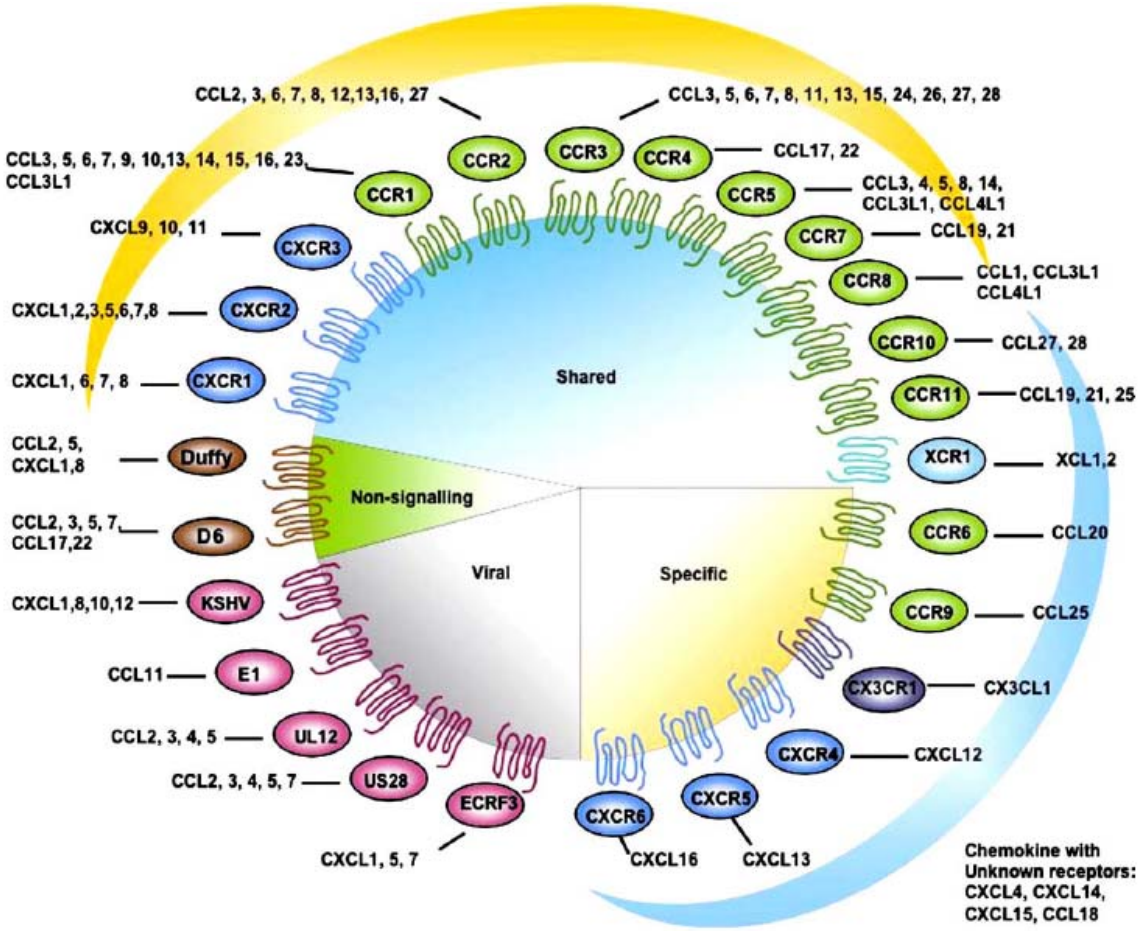


Figure 3: *Chemokine ligand and receptor interactions*. Representation of known specific, shared, viral, or non-signaling chemokine receptors and the chemokines that bind with and activate them (Wilson and Slettenaar, 2006)

1.4 GPCRs

G-protein-coupled receptors make up the vast majority of cell-surface receptors that are involved in signal transduction. They account for approximately 2% of the genes

encoded in the human genome (Dorsam and Gutkind, 2007). They all share a basic composition of seven-transmembrane α-helices that span across the plasma membrane (Dorsam and Gutkind, 2007). The G-proteins are associated with the cytoplasmic side of these receptors. They are composed of three basic subunits: G_α, G_β and G_γ. When a signaling molecule, such as a chemokine, binds the extracellular N-terminal domain of the GPCR, the receptor interacts with the G-protein on the C-terminal, intracellular side and catalyzes the exchange of GDP for GTP in the G_α subunit. This causes the dissociation of the G_α from the bound $G_{\beta/\gamma}$ subunits. This allows both sets of subunits to go on and activate other intracellular molecules. This activation causes the G_α subunit to hydrolyze its GTP, producing GDP, which causes it to deactivate and reassociate with the $G_{\beta/\gamma}$ subunits. This heterotrimer then goes and reattaches to the heptahelical receptor and awaits another extracellular signal (Dhanasekaran, *et al.,* 1998).

GPCR expression has been found in many different cells, including proliferating cells and contributing to cell growth and cancer, inflammation, neovascularization and tissue remodeling and repair (Dorsam and Gutkind, 2007). It has been observed that the normal physiological functions of GPCRs can by altered by malignant cells which leads to autonomous, uncontrolled proliferation, increased blood supply and invasion of other surrounding tissues and organs (Dorsam and Gutkind, 2007). Also, many GPCRs are overexpressed in several different cancer types, contributing to tumor cell growth. Since it has been found that several inflammatory mediators also stimulate GPCRs, this provides yet another possible link between inflammation and cancer (Dorsam and Gutkind, 2007).

1.5 CXCL8

Interleukin-8, more recently reclassified as CXCL8, was originally identified as a neutrophil chemotactic factor, meaning that it is an activator and chemoattractant for neutrophils (Xie, 2001). As its name implies, it is a member of the CXC chemokine family and also contains the glutamic acid-leucine-arginine motif making it an ELR^+ CXC chemokine (Roebuck, 1999). The *CXCL8* gene is located on human chromosome 4 in the q12-21 region. It contains four exons separated by three introns. When the *CXCL8* gene is transcribed the product is a 1.8 kb mRNA that is then translated into a precursor protein that is 99 amino acids in length (Figure 4) (Roebuck, 1999). After intracellular processing the active protein that is secreted from the cell is only 79 amino acids long. Further extracellular processing of the secreted protein results in a few different biologically active forms, however it is the 72 amino acid form, with a molecular mass of 8.5 kD, that predominates (Roebuck, 1999). This extracellular processing can be performed by MMP-9 (matrix metatalloproteinase-9 or gelatinase B) and has been found to actually increase CXCL8 activity by anywhere from 10 to 27 times (Van den Steen, *et al.,* 2000).

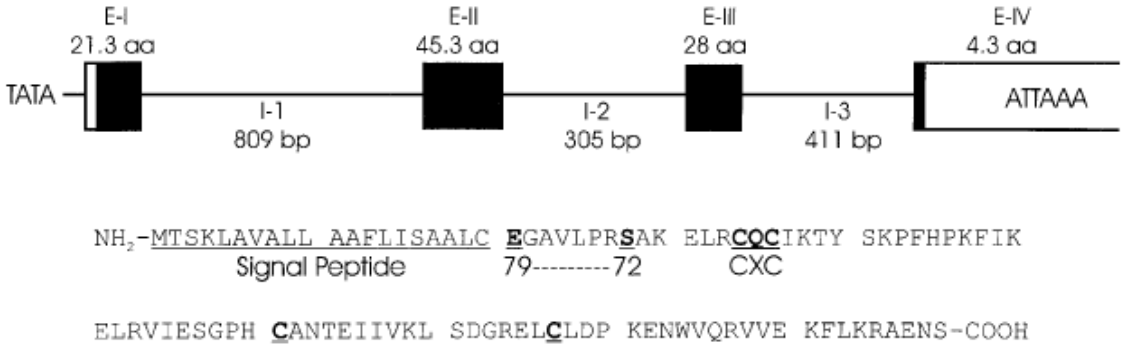


Figure 4: *Structure of CXCL8 gene and amino acid sequence*. CXCL8 gene contains four exons and three introns of varying lengths with a TATA box at the 5' end and polyadenylated tail at the 3' end. The amino acid sequence is shown along with the signal peptide region as well as the conserved CXC sequence (Roebuck, 1999).

CXCL8 expression is most strongly stimulated by the proinflammatory cytokines tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) (Brasier, *et al.,* 1998). Other moderate activators of CXCL8 are stress factors such as nitrous oxide (Remick and Villarete, 1996), hypoxia and subsequent acidosis (Shi, *et al.,* 1999). These factors rely on three basic transcription factors for CXCL8 expression: NF-κB, NF-IL-6 and AP-1 (Nakamura, *et al.,* 1991). For effective transcriptional responses in many cell types, it has been noticed that synergistic interactions among these three transcription factors need to take place (Mukaida, *et al.,* 1994).

As mentioned earlier, the first role identified for CXCL8 was as a chemoattractant cytokine for neutrophils during inflammation. Since then it has been implicated in several key roles in many human cancers. These include increased tumor cell proliferation, migration and angiogenesis (Yuan, *et al.,* 2000; Li, *et al.,* 2005; Li, *et al.,* 2003; Zhu, *et al.,* 2004; Huang, *et al.,* 2002; Itoh, *et al.,* 2005; Opdenakker and Van Damme, 2004, Luppi, *et al.,* 2006).

It has been observed that CXCL8 can work in an autocrine and paracrine manner to increase tumor cell proliferation (Schadendorf, *et al.,* 1993). Also, recent studies have shown that CXCL8 may stimulate proliferation through transactivation of epidermal growth factor receptors (EGFR) via G-protein activation and subsequent epidermal growth factor (EGF) shedding by matrix metalloproteinase (MMP) cleavage (Itoh, *et al.,* 2005; Luppi, *et al.,* 2006). Further proof of involvement with proliferation comes from CXCL8 gene knockdown experiments, which showed that a decrease in CXCL8 expression leads to decreased tumor cell growth (Miyamoto, *et al.,* 1998). Metastasis of tumor cells away from the primary site of growth has been shown to involve CXCL8. In order for migration of cells to occur the extracellular matrix surrounding tissues must be degraded. This is done in part by a group of enzymes known as matrix metalliproteinases (MMP). It was found that CXCL8 upregulates cellular expression of MMP-2 and MMP-9, which are both capable of degrading type-IV collagen, a major component of the basement membrane (Li, *et al.,* 2003; Li, *et al.,* 2005; Patel, *et al.,* 2005; Handsley and Edwards, 2005). At the same time, as mentioned earlier, MMP-9 cleavage of CXCL8 creates a more active chemokine (Van den Steen, *et al.,* 2000). From this information it would be safe to imply that a

positive feedback mechanism is created that would perpetuate tumor cell migration. All of these processes such as proliferation and migration, in which CXCL8 has been implicated, have high metabolic demands (Strieter, *et al.,* 2005). Tumor cells (and non-tumor cells) use several different strategies to accomplish this. Several findings have implicated CXCL8 as playing a role in increasing tumor angiogenesis. The processes used in order to accomplish this include the chemoattraction of endothelial cells by CXCL8. This increasing gradient of CXCL8 as proximity to tumor cell mass decreases causes the endothelial cells to increase their MMP-2 and MMP-9 expression which helps to pave the way by breaking down the extracellular matrix surrounding the tissues (Dorsam and Gutkind, 2007). Several studies have confirmed the increased expression of CXCL8 in tumor cell masses that show increased angiogenesis, as well as the concomitant increases in MMP production (Huang, *et al.,* 2002; Yuan, *et al.,* 2000; Li, *et al.,* 2003; Li, *et al.,* 2005).

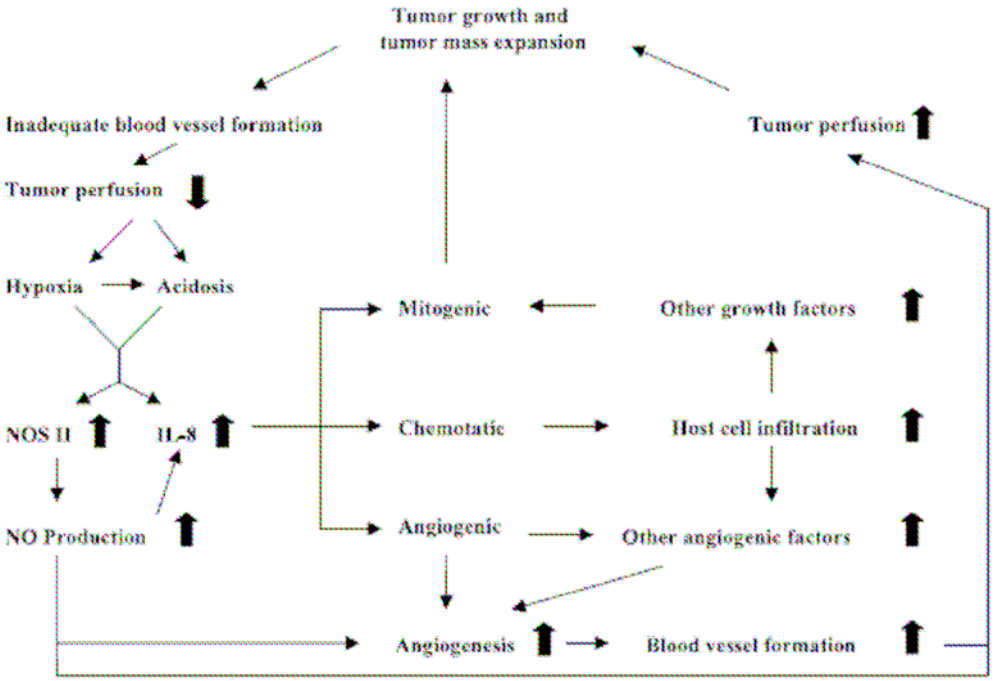


Figure 5: *Expression and function of IL-8 in tumor growth*. Diagram depicts a few factors that lead to increased IL-8 production and the subsequent effects it plays in tumor cell proliferation, metastasis and angiogenesis (Xie, 2001).

1.6 CXCL8 Receptors

As mentioned earlier, almost all chemokines are coupled to at least one seven transmembrane G-protein coupled receptor, this is true for CXCL8 as well. The two CXCL8 chemokine receptors are CXCR1 and CXCR2 (Bizzarri, *et al.,* 2006; Richards, *et al.,* 1997; Strieter, *et al.,* 2005). The CXCR1 receptor is more specific in its ligand binding and has been found to only interact with CXCL6 and CXCL8 with high affinity (Holmes, *et al.,* 1991). On the other hand, the CXCR2 receptor has been found to be more

promiscuous in its interactions and can bind all ELR^+ CXC chemokines, which include: CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7, CXCL8 and CXCL15 (Addison, *et al.,* 2000). These two receptors are associated with different aspects of CXCL8 induced changes in tumor cells. Although there is a lot of controversy as to the specific roles that each plays, it seems that several conclusions can be made. The first involves angiogenesis. Since it has been found that all ELR^+ CXC chemokines are pro-angiogenic (a function of the ELR motif), and all ELR^+ CXC chemokine ligands bind to CXCR2, it can be deduced that CXCR2 is involved in the pro-angiogenic effect of CXCL8. This conclusion has been supported by several different research groups (Strieter, *et al.,* 2005; Addison, *et al.,* 2000; Heidemann, *et al.,* 2003; Varney, *et al.,* 2006). The proliferative effects of CXCL8 have also been associated with ligand interactions with the CXCR2 receptor (Addison, *et al.,* 2000; Wuyts, *et al.,* 1998; Schraufstatter, *et al.,* 2000). The function of CXCR1 has not been as well defined but seems as if it may be involved with the chemoattractant properties of CXCL8 for neutrophils (Hammond, *et al.,* 1995; Bertini, *et al.,* 2004).

1.7 CXCL8 and HNSCC

In the current study, the effects of CXCL8 on head and neck squamous cell carcinoma were examined. Based on previous studies of CXCL8, we hypothesized that the overexpression of CXCL8 in head and neck squamous cell carcinoma causes an increase in proliferation and motility in HN4 and HN12 cells. This was examined using two different approaches: overexpressing CXCL8 in cells that express low endogenous levels of this

chemokine, and RNAi-mediated knockdown in cells expressing elevated endogenous levels of CXCL8. The overexpression of CXCL8 was performed in HN4 cells, which were derived from a primary squamous cell carcinoma of the tongue (Yeudall, *et al.,* 2005; Miyazaki, *et al.,* 2006). It was found that HN12 cells overexpressed CXCL8 (Miyazaki, *et al.,* 2006) and for this reason the knockdown experiments were performed in HN12 cells, which were derived from a nodal metastasis in the same patient that the HN4 cell line came from (Yeudall, *et al.,* 2005; Miyazaki, *et al.,* 2006).

Materials and Methods

2.1 Cell Cultures

HN12 and HN4 cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM) (4500 mg/L D-Glucose, L-Glutamine and 110 mg/L sodium pyruvate) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) and 1mL penicillin/streptomycin (10,000 u/mL penicillin, 10,000µg/mL streptomycin sulfate in 0.85% saline) (Invitrogen, Carlsbad, CA) for 293-T cells, in the presence of puromycin (1 µg/ml) for transfected HN12 cells or in the presence of G418 (400µg/ml) for HN4 cells. They were maintained in a humidified, 37º C incubator in 90% air and 10% CO_2.

2.2 Oligonucleotide Synthesis

The RNA sequence that was targeted in order to knock down expression of CXCL8 was identified using online software (www.clontech.com, 2007) and appropriate oligonucleotides designed (see Table 1). Oligonucleotides containing 5' phosphorylations were synthesized by Sigma-Genosys, (The Woodlands, TX) and were designed with BamHI and EcoRI compatible ends for ligation into a plasmid (see Table 1 for primer sequences).

2.3 Polymerase Chain Reaction

Reagents were thawed and set up as a 50 µL reaction mix in a 200 µL tube as follows: 40 µM dNTP mix, 10x PCR Buffer (200 mM Tris HCL pH 8.4 and 500 mM KCl)(Promega, Madison, WI), 10 µM Primers, 0.5 µg template DNA, 0.5 µL Taq DNA Polymerase (Promega, Madison, WI) and ddH_2O was add up to a total volume of 50 µL. The tube was briefly spun down and placed in the GeneAmp PCR System 9700 (40 cycles 95º C for 30 sec and 68ºC for 1.2 min)(Applied Biosystems, Foster City, CA).

2.4 Restriction Endonuclease Digestion

In order for the CXCL8 or shL8 sequences to be introduced into the plasmid of choice, restriction endonuclease digestions were performed. The pSirenRetroQ plasmid vector (Clontech) was digested with BamHI and EcoRI (New England Biolabs, Ipswich, MA) and the pcDNA3 plasmid vector was digested with BamHI (New England Biolabs, Ipswich, MA) and XbaI (Promega, Madison, WI) by addition of 20 u/µL of each enzyme, 10x BSA, 10x NEBuffer #2 (10mM Tris HCl, 10mM $MgCl_2$, 50mM NaCl, 1mM Dithiothreitol pH 7.9) (New England Biolabs, Ipswich, MA), 200 ng pSirenRetroQ plasmid and 6 µL ddH_2O. The solution was allowed to incubate in a 37º C water bath for at least 1h.

2.5 DNA Ligation

To a sterilized microcentrifuge tube the following were added: 1:3 molar ratio of oligonucleotide:vector, 4µl of 5x T4 DNA Ligase Buffer (250 mM Tris-HCl pH 7.6, 50 mM $MgCl_2$, 5 mM ATP, 5 mM DTT and 25% (w/v) polyethylene glycol-8000), ddH_2O to 19.5 µL and 0.5 µL T4 DNA Ligase (Invitrogen, Carlsbad, CA). Finally, the solution was incubated at room temperature for 2 h.

2.6 Agarose Gel Electrophoresis

A 1% agarose gel was prepared using 0.5 mg Agarose (ISC BioExpress, Kaysville, UT) in 50 mL 1x TAE buffer. The products were mixed with 6x DNA running buffer and run on the gel for 15-30 min at 120V side by side with a 1 kb Molecular Ruler (BioRad, Hercules, CA)

After the contents of the digestion were run on a 1% agarose gel the QIAquick Gel Extraction Kit (QIAGEN Sciences, MD) was used purify the digested plasmid DNA according to the manufacturer's protocol. First, the DNA fragment was excised from the gel using a clean, sharp scalpel. The slice was placed in a microcentrifuge tube with 300 µL Buffer QG. It was then incubated at 50º C for 10 min until the gel slice had completely dissolved. Vortexing every 2 min helped this process. Once completely dissolved, 100 µL of isopropanol was added and the entire solution was added to the QIAquick spin column in a 2 mL collection tube. The tubes were spun at top speed for 1 min in a microcentrifuge. The flow-through was discarded and 750 µL of Buffer PE was added to the column to

wash by centrifugation for another minute. The flow through was again discarded and the column spun again for 1 min at 13,000 rpm. The QIAquick column was placed in a clean 1.5 mL microcentrifuge tube and 50 µL of ddH_2O were added. The column was incubated at ambient temperature for 1 min and then centrifuged at top speed for 1 min to collect the eluted DNA.

2.7 Spectrophotometric Calculation of Nucleic Acid Concentration

Estimation of nucleic acid concentration was carried out in a DU 640 Spectrophotometer (Beckman Coulter, Fullerton, CA). The spectrophotometer was blanked at 260 nm and 280 nm using 50 µL of ddH_2O, then 50 µL of diluted sample (1:20 in ddH_2O) was placed in the spectrophotometer and the absorbances at 260 and 280 nm were read. Concentration of double-stranded DNA or single-stranded RNA is calculated by the following equations:

$$\text{DNA: } 1_{OD} @ 260 \text{ nm} = 50 \text{ ng}/\mu\text{L}$$

$$\text{RNA: } 1_{OD} @ 260 \text{ nm} = 34 \text{ ng}/\mu\text{L}$$

2.8 *E. coli* Transformation

Cells were removed from –80º C storage and thawed on wet ice. 2 µg of DNA solution were added to 50 µL of α-Select chemically competent cells (Bioline, Randolph, MA) and solution was gently swirled for a few seconds to mix. It was then incubated on

ice for 30 min at which time the tube was placed in a 42º C water bath for 45 s and then returned to ice for 2 min. The transformation reaction was then diluted by 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 $MgCl_2$ and 10 mM $MgSO_4$) (Bioline, Randolph, MA). The tubes were placed in a 37º C incubator and shaken at 200 rpm for 60 min. The cells were then plated by addition of 20 µL of cell transformation mixture on LB agar plates containing 50 µg/mL ampicillin and incubated overnight at 37º C.

2.9 Small Scale DNA Preparation

Miniprep DNA was purified using the Wizard Plus SV Miniprep DNA Purification System (Promega, Madison, WI). After *E. coli* colonies were picked and expanded in liquid culture, 1.5 mL of the resuspended solution was pelleted by centrifugation for 5 min. After drawing off the supernatant, the cells were thoroughly resuspended with 250 µL of Cell Resuspension Solution (50mM Tris-HCl pH 7.5, 10mM EDTA, 100µg/mL RNase A) (Promega, Madison, WI) and incubated at room temperature for 3-4 minutes. Next, 250 µL of Cell Lysis Solution (0.2M NaOH, 1% SDS) (Promega, Madison, WI) was added to each sample and inverted 4 times to mix. Samples were then incubated at ambient temperature for 4 min. 10 µL of Alkaline Protease Solution was added to each sample, inverted 4 times to mix and incubated at ambient temperature for 5 min. 350 µL Neutralization Solution (4.09M guanidine hydrochloride, 0.759M potassium acetate, 2.12M glacial acetic acid)

(Promega, Madison, WI) was then added to the solution, inverted 4 times to mix and centrifuged at 13,300 rpm for 10 min. Spin column (Promega, Madison, WI) was inserted into a vacuum adapter and the clear lysate added. A vacuum was applied until all the liquid had passed through. 750 µL of Wash Solution with ethanol (Promega, Madison, WI) was added and vacuum was again applied until all the solution passed through. This step was repeated again with 250 µL of Wash Solution (Promega, Madison, WI). The column was then dried by applying vacuum for 10 min. Next, the column was transferred to a 2 mL collection tube and centrifuged at 13,300 rpm for 2 minutes. The column was again transferred to a sterile 1.5 mL microcentrifuge tube and 100 µL of ddH_2O was added. It was then spun at top speed for 1 min to elute the DNA. The DNA was then ready to be stored at –20º C.

2.10 Cell Transfection

10 cm cell culture plates were plated with 293-T, HN4 or HN12 cells and were grown to 60% confluency. In a sterile microcentrifuge tube 10 µL/µg DNA of the TransIT-Keratinocyte Transfection Reagent (Mirus, Madison, WI) was added dropwise into 200 µL of serum-free media and was mixed thoroughly by vortexing. This was then incubated at ambient temperature for 20 min. DNA (2 µg) was then added to the diluted TransIT-Keratinocyte Transfection Reagent (Mirus, Madison, WI) and mixed by gentle pipetting. After a 20 min incubation, the transfection mix was added to the prepared cultures and gently rocked side to side to distribute the reagent/DNA mixture evenly.

2.11 RNA Extraction

Cells were lysed directly in the culture dish by addition of 1 mL of TRIsure (Bioline, Randolph, MA) per 10 cm^2 growth area. The plates were incubated at ambient temperature for 5-10 minutes and then scraped with a cell scraper and contents resuspended and transferred to a 1.5 mL microcentrifuge tube. 0.2 mL of chloroform per 1 mL of Trisure used was added, tubes were capped and shaken vigorously for 15 s. Samples were then incubated for 2-3 min at ambient temperature and then centrifuged at 12,000 x g for 15 min at 4º C. The upper aqueous phase was transferred to another tube and mixed with 0.5 mL isopropyl alcohol per 1 mL TRIsure used. The samples were then incubated at ambient temperature for 10 min and then centrifuged at 12,000 x g for 10 min at 4ºC. The supernatant was then removed and the pellet washed using 75% ethanol, adding at least 1 mL ethanol per 1 mL Trisure used. Samples were then vortexed and centrifuged at 7,500 x g for 5 min at 4º C. The supernatant was again removed and the pellet was allowed to air dry at ambient temperature for 5-10 min. The RNA was then dissolved in 20 µL of RNase-free H_2O and stored at –80º C.

2.12 Reverse Transcription

A mix was prepared containing 1 µg total RNA, 1 µL oligo$(dT)_{18}$ primer and ddH_2O up to 12 µL total. The mix was incubated for 5 min at 70º C, and then chilled on ice, after which 1 µL 40 mM dNTP mix (10 mM each), 4 µL 5x reaction buffer (2M Tris-

HCl pH 8.0, 5M NaCl, 0.5 EDTA) (Bioline, Randolph, MA) and ddH_2O up to 19.75 µL total were added to the mixture. This was then mixed by gently pipetting. Next, 0.25 µL of BioScript reverse transcriptase (200 u/µL; Bioline, Randolph, MA) was added and the solution was incubated at 37º C for 60 min. The reaction was then stopped by heating at 70º C for 10 min and stored at –20º C.

2.13 Quantitative Polymerase Chain Reaction (qPCR)

A master mix containing 5% 45µg (1/10x diluted) primers (see Table 1), 35% ddH_2O and 50% ABsolute SYBR Green ROX Mix (ABgene, Epsom, Surrey, UK) was made for each gene target to be analyzed. Actin primers were used as housekeeping genes to standardize the amount of cDNA initially added to each sample (see Table 1 for list of primer sequences). After 9 µL of the master mixes were added to the wells of a 96-well optical plate (Applied Biosystems, Foster City, CA) 1µL ddH_2O was added to the non-template controls, 1 µL of diluted amounts of known templates were added to additional wells to act as standards, which allowed relative standard curves to be constructed from which relative cDNA copy number could be determined. For test (unknown) samples, 1 µL of desired cDNA was added to triplicate wells. The 96-well plate was covered with an optical cover and then inserted into the 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA) and the run initiated using the 7500 Fast System SDS Software (Applied Biosystems, Foster City, CA).

Table 1. *Primer Sequences*

Primer name	Sequence
shL8- sense	5'GATCCGTCCTGATTTCTGCAGCTCTTTCAAGAGA AGAGCTGCAGAAATCAGGATTTTTTACGCGTG-3'
shL8- antisense	5'AATTCAGCGTAAAAAATCCTGATTTCTGCAGCTC TTCTCTTGAAAGAGCTGCAGAAATCAGGACG-3'
CXCL8- sense	5'-TTTTGCCAAGGAGTGCTAAAG-3'
CXCL8- antisense	5'-AACCCTCTGCACCCAGTTTTC-3'
Actin- sense	5'-CATGTACGTTGCTATCCAGGC-3'
Actin- antisense	5'-CTCCTTAATGTCACGCACGAT-3'

2.14 Conditioned Media

Cells were washed with PBS, removed from the plate using 0.1% Trypsin EDTA (Greiner Bio-One, Monroe, NC) and counted. The cells were then replated ensuring equal numbers of cells in all plates. After 24 h the cells were washed twice with PBS and 10 mL DMEM/1% BSA (Invitrogen, Carlsbad, CA) was added and cells were returned to the 37º C incubator for 48 h. At that time the media were removed, aliquoted into microcentrifuge tubes and stored at –20º C.

2.15 Immunoprecipitation

Mouse anti-human IL-8 monoclonal antibody (1 µg per 1 mg of protein) (GenScript Corp., Piscataway, NJ) was added to 1 mL of conditioned media and incubated for 24-48 h at 4º C with end-over-end rotation. Then 30 µL of 50% Gammabind G Sepharose (Pharmacia Biotech AB, Uppsala, Sweden) in PBS was added to each sample and incubated for a further 1 h at 4º C with rotation. Samples were then centrifuged at 10,000 rpm for 5 min at 4º C. The supernatant was then decanted and 1 mL of PBS was added. The tube was gently flicked to resuspend and wash the pellet and then recentrifuged for 1 min. This step was repeated twice. After the final wash, the tube was recentrifuged without addition of buffer and a micropipette was used to remove all the remaining supernatant. The sepharose-antibody-protein complexes were resuspended in 1x SDS-PAGE loading buffer, heated to 95º C for 10 min, then either run on a polyacrylamide gel or stored at –20º C.

2.16 Western Blot

Immunoprecipitated proteins, or concentrated conditioned media samples, were resolved in 15% denaturing polyacrylamide gels containing 0.1% SDS (Molecular Cloning: A Laboratory Manual 2001). Proteins were resolved for 1.5 h at 120 V in 1x SDS-PAGE running buffer (20 mM Tris-Cl (pH 7.9), 100 mM NaCl, 70 mM EDTA, 2% (w/v) SDS). Next, proteins were transferred onto PVDF membrane (Immobilon-P; Millipore Corp., Bedford, MA) for 16h in 1x transfer buffer (20 mM Tris-HCl (pH 7.9),

100 mM NaCl, 70 mM EDTA, 20% MeOH). The membrane was dried, rehydrated, then blocked with 5% non-fat dried milk in 0.05% tween-TBS (T-TBS) for 1 h on a shaker at ambient temperature. After three 5 min washes in T-TBS the membrane was incubated in a 1:1000 (1µg/mL) mouse anti-human IL-8 monoclonal antibody (GenScript Corp., Piscataway, NJ) diluted in 1% BSA (EMD Chemicals Inc., Gibbstown, NJ) overnight at 4°C. After three 5 min T-TBS washes the membrane was incubated in horseradish peroxidase-conjugated (HRP) goat anti-mouse monoclonal antibody diluted 1:1000 in blocking buffer for 1 h on a shaker at ambient temperature. The interaction of the primary and secondary antibodies was detected using Western Lightning Chemiluminescence Reagent Plus (PerkinElmer Life Sciences Inc., Boston, MA)

2.17 MTT Assay

To determine proliferation, cells were trypsinized using 0.1% trypsin-2.21 mM EDTA (Mediatech, Herndon, VA) and counted. The cells were replated in triplicate in 12-well cell culture plates (Greiner Bio-One, Monroe, NC) at a density of $1x10^4$ cells per well. The cells were incubated for 5 days (HN12) or 7 days (HN4). At that time 100 µL of 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. Next, all media and MTT solution was removed from each well and 1 mL of MTT solubilization solution (10% SDS in 0.01M HCl) was added and incubated overnight at 37° C. After all crystals had been dissolved the optical density of the solution was determined spectrophotomettically at 570 nm.

Alternatively, $1x10^4$ HN12 cells or $2x10^4$ HN4 cells, or derivative cell lines were plated in triplicate in eight 12-well cell culture plates. On each of 8 consecutive days, 1 plate was removed, 100 µL of MTT solution added to the media and incubated at 37° C for 4 h. At this point media were removed completely and the plates were stored at -80° C until all 8 plates had been collected. Crystals were solubilized and the optical density determined as before.

2.18 Migration Assay

Cells were detached from culture plates in 1-2 mL of HBSS/5 mM EDTA/25 mM Hepes pH 7.2 (+/- 0.01% trypsin) (Mediatech, Herndon, VA). Cells were then washed twice in DMEM/0.1% BSA (Invitrogen, Carlsbad, CA) and resuspended at a density of $2.5x10^5$/mL. 200µL of the cell suspension was added to the upper chamber of an 8.0µm pore size Transwell insert (Corning Inc, Corning, NY) in triplicate. 1 mL of serum-free DMEM/0.1% BSA/10 ng/mL EGF was added to the lower chamber of each well . The cells were placed in a 37º C incubator for 20 h and allowed to migrate. The plate was then removed from the incubator and the cells were fixed with 0.025% glutaraldehyde (Sigma-Aldrich, St. Louis, MO) in PBS. The membranes were then stained with 0.1% crystal violet in PBS for a minmimum of 20 min, then destained thoroughly with ddH_2O. The non-migratory cells on the upper surface of the membrane were removed by wiping with a Q-tip and the membranes were mounted on a glass microscope slide. Cells were counted in 5 random fields per membrane at 200x magnification.

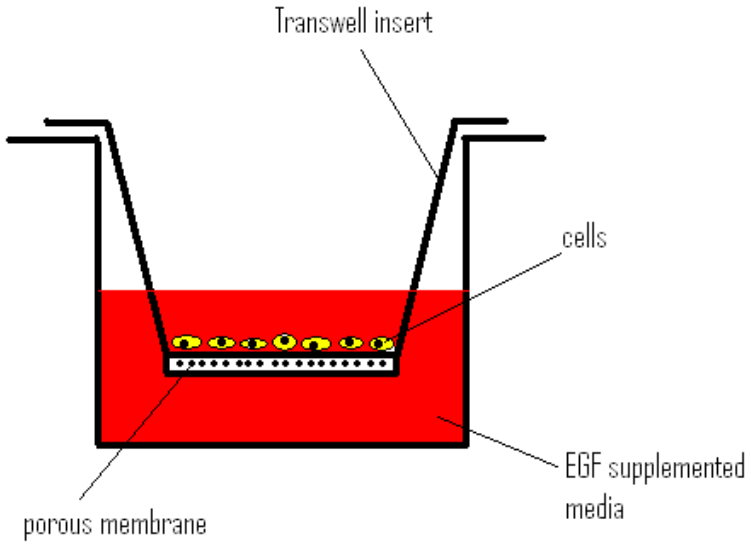


Figure 6: *Migration Assay.* Representation of an individual cell culture well used for the migration assays performed. Cells were plated on top portion of porous membrane and media supplemented with EGF was placed in the bottom chamber.

2.19 Invasion Assay

Invasion assays were carried out in a similar manner to migration assays. Transwell inserts with 12.0 µm pores (Corning Inc., Corning, NY) were coated with 200 µL Matrigel, which was diluted 1:6 in ice-cold DMEM, and allowed to gel at 37º C for 20-30 min. Subconfluent cell cultures were then washed with PBS and incubated at 37º C in 1-2 mL HBSS/5 mM EDTA/25 mM Hepes pH 7.2 (Mediatech, Herndon, VA) until detached. They were then resuspended in DMEM/0.1% BSA at a density of $2x10^5$ cells/2 mL. 1 mL of DMEM/0.1% BSA/10 ng per mL EGF was added to the lower chamber of each well; the

Transwells were placed in each well ensuring that no bubbles formed below the membrane, and 500 µL of cell suspension ($5x10^4$ cells) were added to the upper chamber. The plates were then incubated in a 37º C incubator for 16-20 h, after which the plates were removed from the incubator and cells fixed, stained and counted as for migration assays (above).

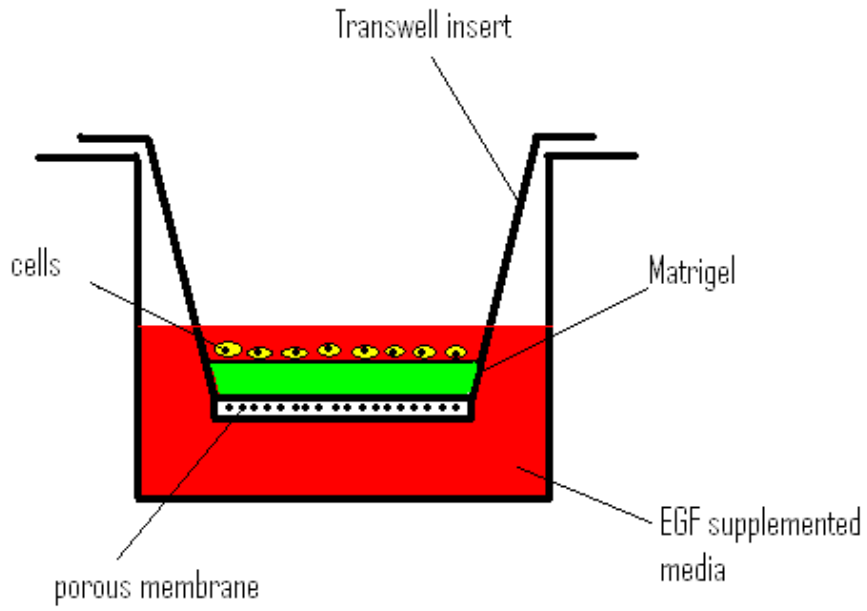


Figure 7: *Invasion Assay*. Representation of the setup used for the invasion assays. A porous membrane is first covered with Matrigel, placed in the chamber, cells of choice are plated on top of the membrane and media supplemented with EGF was placed in the bottom chamber.

2.20 Statistical Analysis

All data was analyzed using an unpaired t-test comparing two means using GraphPad QuickCalcs software (http://www.graphpad.com/quickcalcs/). All p-values were compared to an α-value of either 0.05 or 0.005.

Results

3.1 Targeted Suppression of CXCL8 Expression

It has been previously reported that the HN12 cell line, which was derived from a nodal metastasis in a patient with head and neck squamous cell carcinoma, overexpressed CXCL8 approximately 2 fold when compared to HN4 cells, which were derived from the primary tumor in the same patient as the HN12 cells (Miyazaki 2006). In order to study the biological role of CXCL8 in HN12 cells we used RNA interference (RNAi) to specifically inhibit the expression of CXCL8. This was done by stably transfecting HN12 cells with a pSiren-RetroQ plasmid that synthesizes shRNA targeted to degrade CXCL8 mRNA, or a non-targeting control (NTC) plasmid. Individual clones (HN12-shL8, HN12-NTC) were selected for resistance to puromycin and screened for CXCL8 expression by extracting RNA, reverse transcription and subsequent analysis by qPCR using cDNA as a template. In addition, conditioned media were collected and levels of secreted CXCL8 determined by immunoprecipitation and western blot analysis as described in Materials and Methods. As shown in Figure 8 (top panel) CXCL8 expression was decreased 2.5 fold when compared to the control, as judged by qPCR. This was a significant difference having a p-value of 0.002. Western blot analysis (lower panel) confirmed a decrease of CXCL8 in the

shL8 cells. Thus, stable targeted suppression of CXCL8 expression was achieved in HN12 cells.

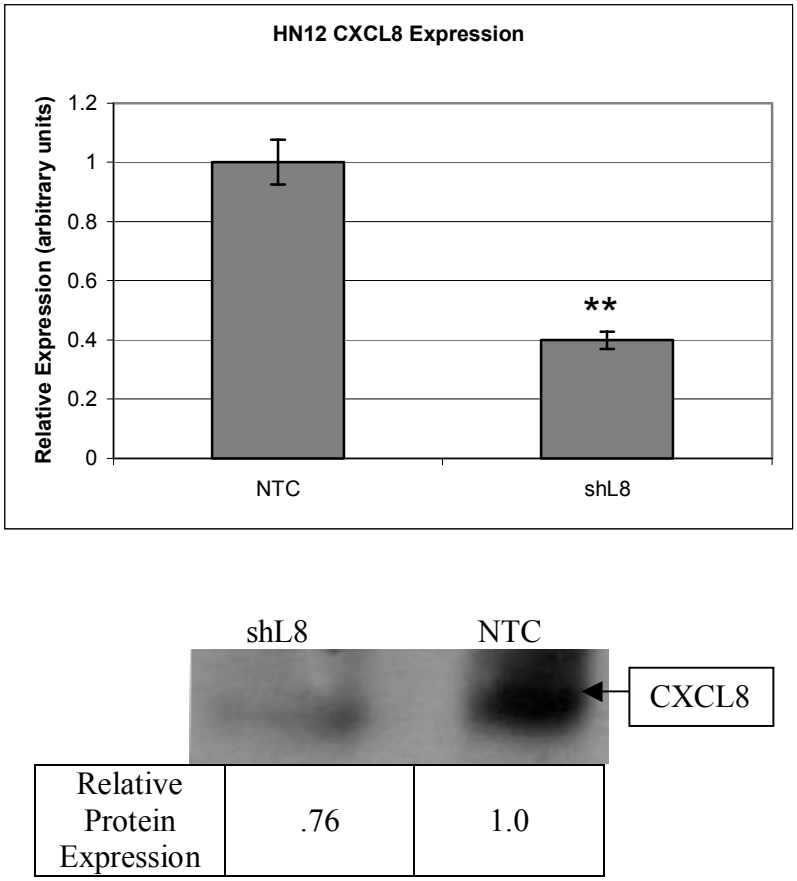
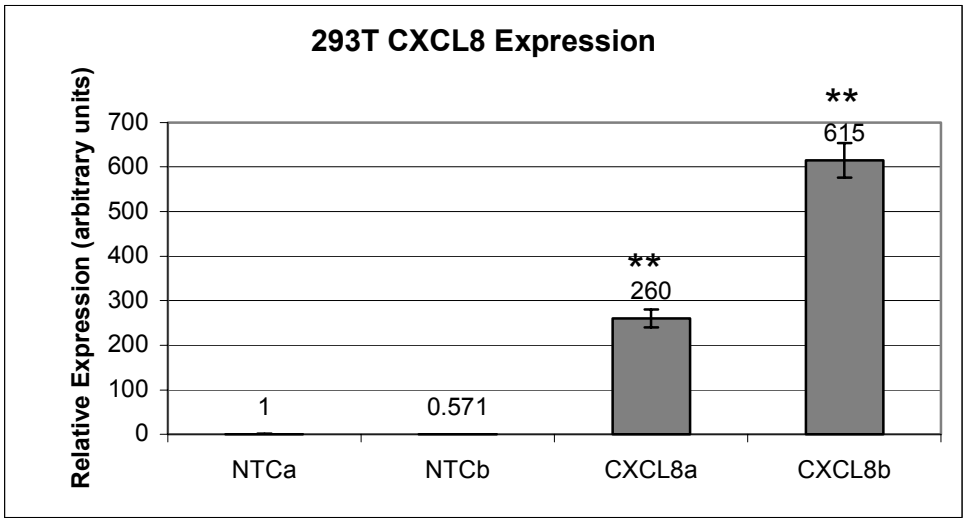


Figure 8: RNA was extracted from subconfluent cultures of the indicated cells, reverse-transcribed and qPCR as performed as described in Materials and Methods. The expression of CXCL8 was standardized to the internal standard, actin (top panel). The assay was performed in triplicate and relative means ± 1 SEM are shown. In parallel, equal numbers of HN12-NTC and HN12-shL8 cells were serum deprived for 48 h and conditioned media collected. CXCL8 was immunoprecipitated and western blotted with anti-CXCL8 antibody. (** represents $p<0.005$)

3.2 Overexpression of CXCL8

HN4 cells express low basal levels of CXCL8 and, thus, are a good target cell in which to test the effects of CXCL8 overexpression. To facilitate this, a full-length CXCL8 cDNA was generated by PCR using HN12 cDNA as a template. CXCL8 cDNA was obtained and the CXCL8 sequence was restriction digested using BamHI and Xba1 and then ligated into the pcDNA3 plasmid, similarly digested. Recombinant clones were obtained and the presence of an insert of the predicted size was determined by restriction digestion. To further confirm the identity and integrity of PCR-generated sequences, individual clones were subjected to nucleotide sequence determination. To confirm that CXCL8 was expressible from these plasmids, 293T cells were used as a preliminary test due to this cell line's high transfectability and ability to express a large amount of protein. 293T cells were transiently transfected with pcDNA3-CXCL8 or pcDNA empty vector as control. Forty-eight hours later, RNA was prepared, reverse-transcribed and CXCL8 expression determined by qPCR. As shown in Fig. 9(top panel) CXCL8 was almost undetectable in vector-transfected cells, but highly expressed in cells transfected with pcDNA-CXCL8. These data were found to be extremely statistically significant ($p= 0.001$). Western blot analysis (Fig. 9, lower panel) confirmed these findings, with a similar trend in CXCL8 expression between the control and CXCL8-transfected cells.



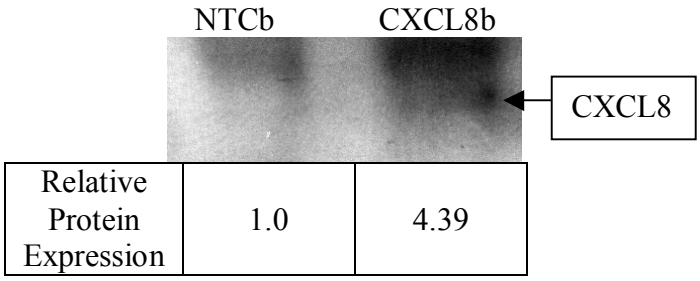


Figure 9: RNA was extracted from subconfluent cultures of 293T cells, reverse-transcribed qPCR analysis was performed as described earlier in Materials and Methods. CXCL8 expression was standardized to the internal standard, actin (top panel). The assay was performed in triplicate for both controls and both CXCL8-transfected cells and relative means ± 1 sem are shown. In parallel, equal numbers of 293T cells, similarly transfected, were allowed to condition the media for 48h, and CXCL8 immunoprecipitated and expression determined by western blotting (lower panel)

(** represents $p<0.005$)

After the ability of pcDNA3-CXCL8 plasmids to express CXCL8 was confirmed, a stable transfection was performed in HN4 cells. Individual clones were selected in G418 and, again, CXCL8 expression levels were analyzed by qPCR after RNA extraction and reverse transcription. Figure 10 shows CXCL8 qPCR results obtained for HN4 cells. A 2-fold difference between the control and CXCL8 overexpressing clone was observed. This difference was found to be significant ($p= 0.001$). As before, conditioned media were also tested for CXCL8 expression by immunoprecipitation and western blotting. However, the levels of CXCL8 in these cells lines were not readily detectable by this methodology (data not shown).

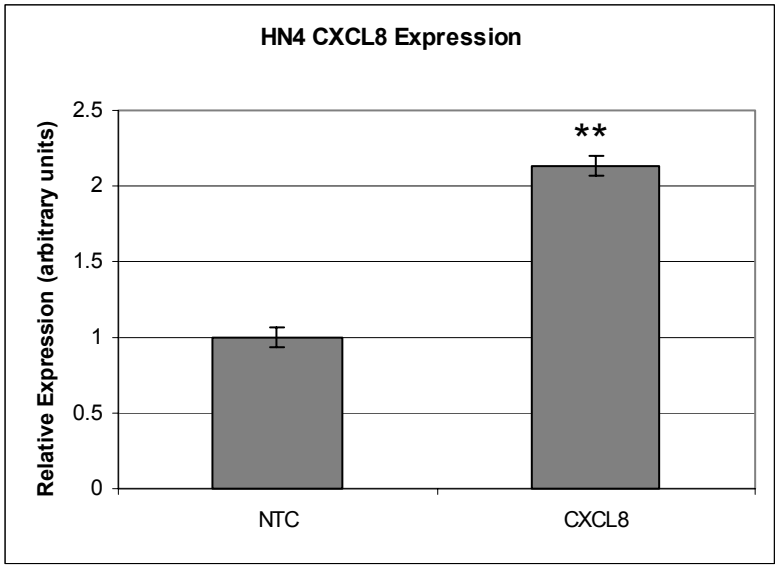
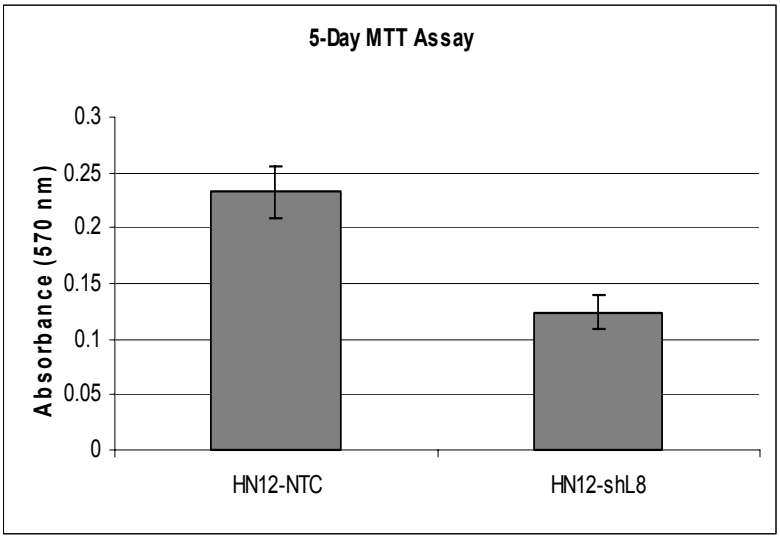


Figure 10: RNA was extracted from subconfluent cultures of the indicated cells, reverse-transcribed and qPCR analysis performed as described earlier in Materials and Methods. CXCL8 expression was standardized to the internal standard, tubulin (top panel). The assay was performed in triplicate and relative means ± 1 SEM are shown. (** represents $p<0.005$)

3.3 CXCL8 overexpression leads to increased cellular proliferation

Increased cellular proliferation plays an integral role in tumor progression. To better understand the role that CXCL8 plays in this critical process we used the HN12-NTC and HN12-shL8 cells, as well as the HN4-NTC and HN4-CXCL8 cell lines described above. Equal numbers of cells were plated in triplicate ($1x10^4$ per well for HN12 and $2x10^4$ per well for HN4) in 12-well cell culture plates, and cultured for 5 or 8 days (HN12 and HN4, respectively) and then subjected to MTT Assay. This allowed us to observe a final difference in proliferation between the control and experimental groups of both cell lines. As shown in Fig. 11A, a 3-fold decrease in the proliferation of HN12-shL8 cells was observed when compared to the control. These data were statistically significant ($p=0.022$). Similarly, overexpression of CXCL8 in HN4 cells (Fig. 11B) resulted in an increase in proliferation of 2-fold compared to controls. Again, this is statistically significant ($p= 0.003$).

A



B

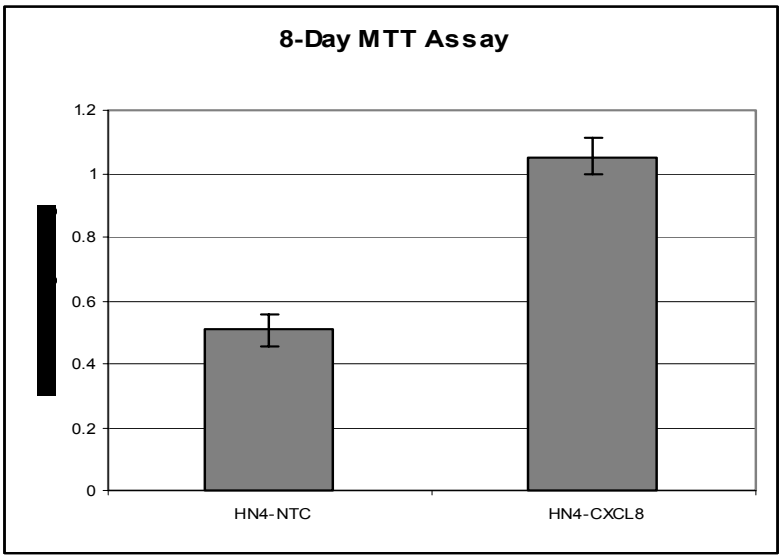
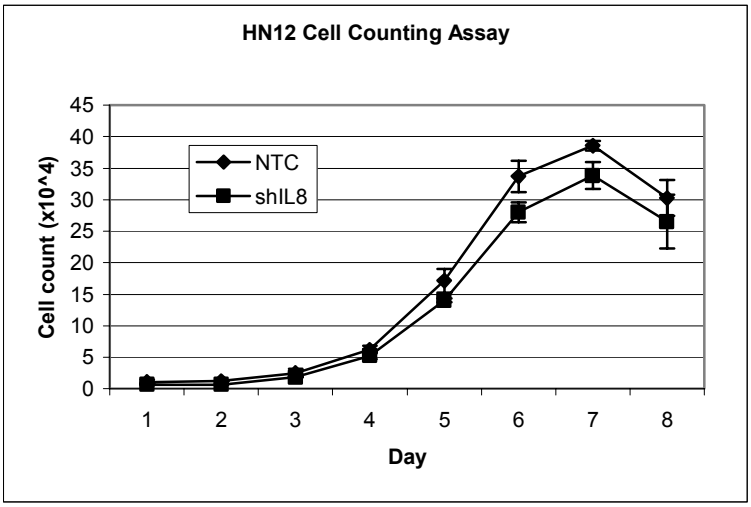


Figure 11: Equal numbers of the indicated cells were plated in triplicate and incubated under standard culture conditions. MTT assay was carried out after 5 days (A) or after 8 days (B) as described in Materials and Methods. Values shown are means ± SEM. (** represents $p<0.005$)

This difference in proliferation was further confirmed by daily cell counting, as well as by daily MTT assay. Figure 12A shows the results of HN12-NTC and HN12-shL8 proliferation assay where the cells were counted for eight consecutive days. The same general trend was observed, with HN12-shL8 cells showing less proliferation. As can be seen from Fig.12B, there is a statistically significant difference between the cell numbers of the HN12-shL8 cells compared to control, with the eight day showing around a 50% decrease in proliferation. Similarly, figure 12C shows a similar trend for HN4-CXCL8 compared to control cells. At almost all timepoints a significant increase in proliferation between the HN4-CXCL8 cells was observed compared to control, with the eighth day showing an 2-fold increase in growth.

A



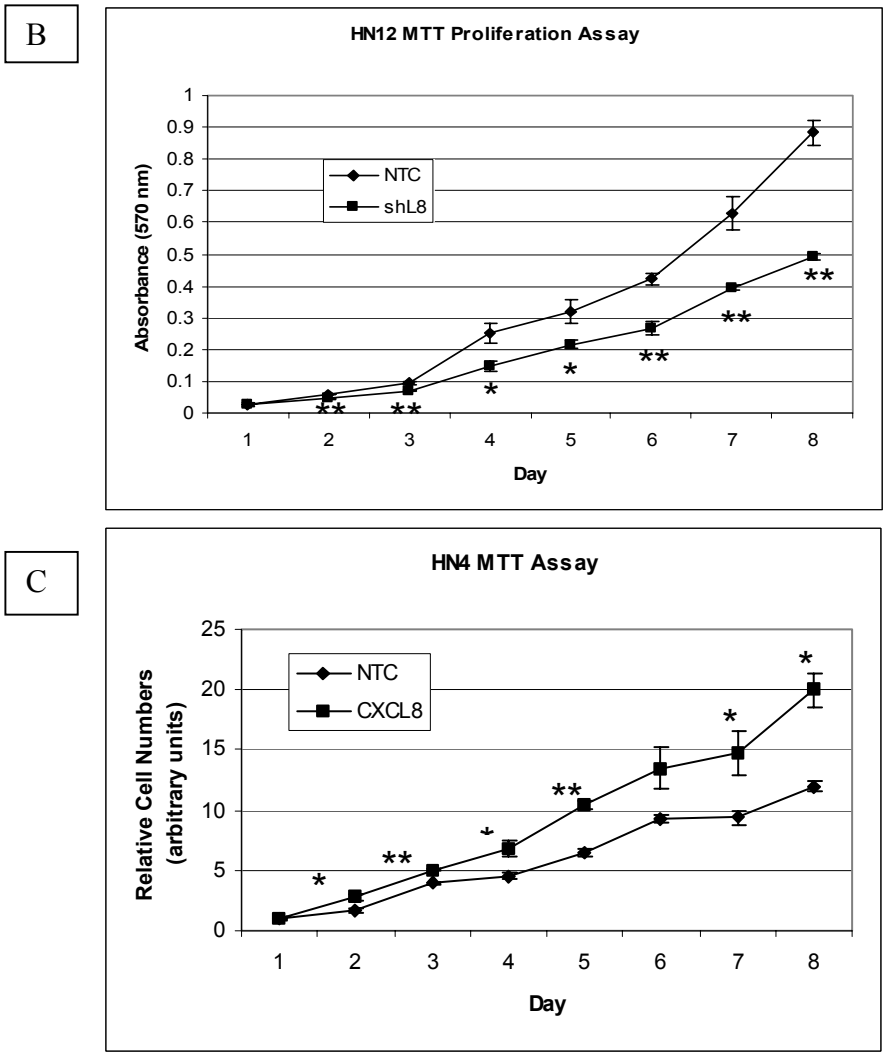
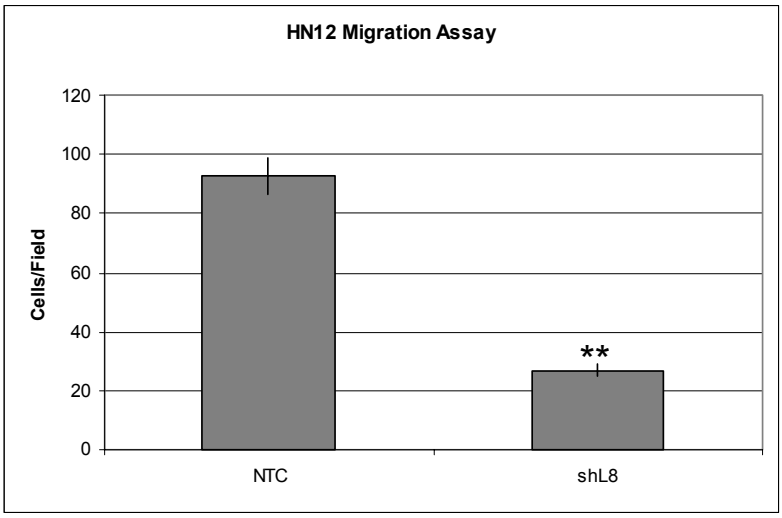


Figure 12: Equal numbers of the indicated cells were plated in triplicate and incubated under standard culture conditions. (A) Cells were trypsinized and counted daily for eight consecutive days. (B&C). MTT assay was carried out as described in Methods. Values shown are means of cell number ± 1 SEM. (* represents $p<0.05$ and ** represents $p<0.005$)

3.4 CXCL8 Stimulates Cell Migration and Invasion

Along with increased proliferation, CXCL8 has been implicated in increasing cellular migration, and might, potentially, affect invasion into other tissues leading to metastasis of the tumor. To test this, both the HN12-NTC and HN12-shL8 cells, as well as the HN4-NTC and HN4-CXCL8 cells were used. Equal numbers of cells were plated in triplicate in the upper chamber of a Transwell cell culture plate as described in Materials and Methods. In the bottom chamber, cell culture media containing EGF, which acts as a chemoattractant, was added and cells incubated under standard conditions for 16-20 h, after which migratory cells were stained and counted. Figure 13A shows approximately a 3 fold decrease in migration in the HN12-shL8 cells when compared to the control. These data were statistically significant with a p-value of 0.001. Similarly, Figure 13B shows the same trend in HN4 cells. The HN4-CXCL8 cells showed a 3-fold increase in migration when compared to the control. These data are also statistically significant ($p=0.006$). These results support the hypothesis that CXCL8 leads to increased cell migration.

A



B

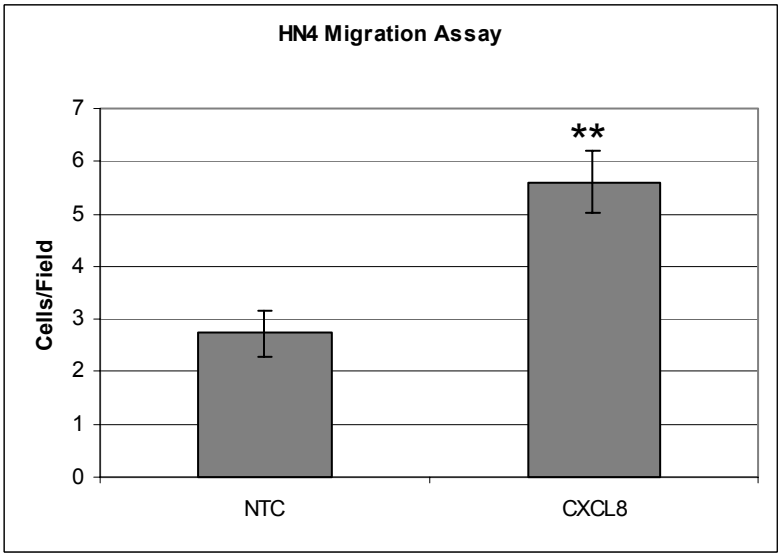


Figure 13: The indicated cells were detached from culture plates in the absence of trypsin, washed and plated in upper chamber of Transwell inserts as described in Materials and Methods. After 16 h (A) or 20 h (B), migrated cells were stained and counted in 15 random high power fields. Data shown represent mean ± SEM. (** represents $p<0.005$).

HN12 cells are highly invasive *in vitro* (Li, *et al.,* 2003; Zhu, *et al.,* 2004). To test the hypothesis that CXCL8 leads to increased invasion, the HN12-NTC and HN12-shL8 cells were plated in triplicate on the upper chamber of Matrigel-coated Transwells and allowed to migrate. After 16 h invading cells were stained and counted. Figure 14 shows the data obtained from a representative experiment. A clear difference between the HN12-shL8 and control cells is apparent, with a statistically significant 3-fold decrease in invasion observed for the HN12-shL8 cells ($p= 0.001$). These data indicate that CXCL8 contributes to the invasive phenotype of HN12 cells.

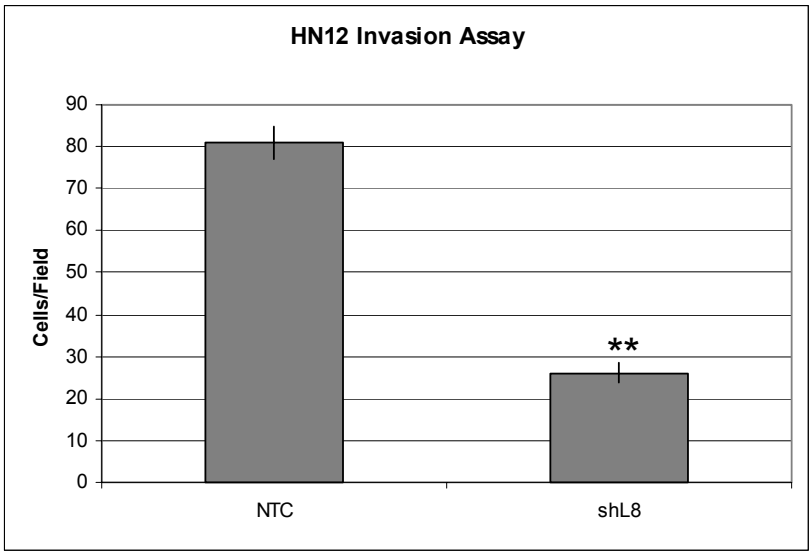


Figure 14: HN12-shL8 and HN12-NTC cells were plated in Matrigel-coated Transwell chambers as described in Materials and Methods. 16 h later, invading cells were stained and counted in 15 random high power fields. Data shown represent mean ± SEM. (** represents $p<0.005$)

Discussion

4.1 Current Study

The aims of the current study were to determine what role CXCL8 plays in the proliferative and metastatic characteristics of primary (HN4) and metastatic (HN12) HNSCC cells. Previous reports in other cell lines lead to testing the hypothesis that increased levels of CXCL8 leads to increased cellular proliferation as well as an increased ability of the cells to migrate and invade into other nearby tissues.

4.2 HNSCC Model

In order to study the effects of CXCL8 on HNSCC cells an *in vitro* approach was taken. Previously, HNSCC cell lines had been isolated and maintained in culture. The first of these cell lines was the HN4 cell line, which was generated from the primary tumor of the tongue. The second cell line was the HN12 cell line, which was isolated from a lymph node metastasis in the same patient from which the HN4 cells were obtained (Miyazaki, *et al.,* 2006; Yeudall, *et al.,* 2005). To find out what differences did exist in the two cell lines a microarray analysis was performed (Miyazaki, *et al.,* 2006), which showed that approximately 60 genes total were either overexpressed or downregulated in the HN12

cells when compared to the HN4 cells. More specifically, two genes that were found to be upregulated were the CXC chemokines CXCL5 and CXCL8. Proliferation and migration/invasion assays were previously performed on both cells lines while genetically modifying CXCL5 levels and it was reported that increased levels of CXCL5 lead to increased proliferation and metastatic ability of cells (Miyazaki, *et al.,* 2006). The previously mentioned microarray study showed that CXCL8 was increased over 2 fold in HN12 cells when compared to HN4 cells. This data along with previous reports of the role of CXCL8 in various cancers lead to the idea that CXCL8 may play a prevalent role in HNSCC progression, and is the basis for the current study.

In order to test the role of CXCL8 in HNSCC using the HN4 and HN12 cells, two approaches were taken. In the first approach, HN12 cells, which were found to overexpress CXCL8, were subjected to RNA interference, which allows the knockdown of the CXCL8 gene. The second approach used was by overexpressing CXCL8 in HN4 cells, which have a baseline level of the protein. However, before this second approach was taken with the HN4 cells, 293T cells, which are good at expressing large amounts of protein, were used for a transient transfection in order to ensure that CXCL8 could be overexpressed in HN4 cells.

After genetic modification, the expression levels of CXCL8 in HN12 cells was confirmed using both qPCR and western blot analysis. Both sets of experiments showed similar knockdown trends for CXCL8, with about a 2.5 fold decrease in the HN12-shL8 knockdown when compared to the control. In the transient transfection with 293T cells, the qPCR and western blot results show that overexpression of the protein is possible with

qPCR results showing a 260 and 615 fold increase in two separate test lines. Once HN4 cells were stably transfected the expression levels were tested using qPCR only. These results showed approximately a 2-fold increase in the HN4-CXCL8 overexpressing cell line when compared to the control. Western blot analysis of this cell line could not detect the presence of CXCL8, even with the various concentrations of primary antibody used to probe the transfer membrane. This lead us to believe that the since the baseline levels of CXCL8 are low in this cell line, even the overexpression of the protein would not cause the increase to be of detectable levels using our methods.

4.3 CXCL8 Expression and Proliferation

Several studies have been done which link CXCL8 to increased tumor growth and proliferation (Yuan, *et al.,* 2000; Li, *et al.,* 2005; Li, *et al.,* 2003; Zhu, *et al.,* 2004; Huang, *et al.,* 2002; Itoh, *et al.,* 2005; Opdenakker and Van Damme, 2004, Luppi, *et al.,* 2006). More specifically it is believed to function via an autocrine and paracrine mechanism (Schadendorf, *et al.,* 1993). Results from the current study have confirmed these results in both HN12 and HN4 tumor cell lines. In the HN12 line, when comparing the HN12-shL8 knockdown to the control there was significantly decreased cellular proliferation. Similarly, in the HN4 cell line, the HN4-CXCL8 overexpressing cells lead to a significant increase in cellular proliferation when compared to the control.

Although some of the results may be debatable, it seems as though the predominant CXCL8 receptor that is responsible for its proliferative effects on cells is the CXCR2

receptor and to a lesser extend CXCR1(Addison, *et al.,* 2000; Wuyts, *et al.,* 1998, Schraufstatter, *et al.,* 2000; Hammond, *et al.,* 1995; Bertini, *et al.,* 2004). Both of these receptors are coupled to G-proteins and have many downstream proteins that they interact with upon ligand binding and activation. One possible mechanism responsible for the increased proliferation associated with CXCL8 is the interaction of the GPCR activation of phosphoinositide-3 kinase (PI3K) and Akt (Hammond*, et al.,* 2000). When PI3K is activated and subsequent activation of Akt occurs. This protein then phosphorylates BAD, increases its affinity for 14-3-3. By increasing this affinity, BAD can no longer form a heterodimer with Bcl-X_L (anti-apoptotic), which allows it to inhibit the actions of Bax, a pro-apoptotic protein (see Figure 15) (Hammond*, et al.,* 2000; Lodish, *et al.,* 2004). This therefore allows for the inhibition of the subsequent apoptosis pathway and permits the cell to survive. Similarly activation of Ras by these GPCR's leads to downstream mitogen activating protein kinase (MAPK) cascade and cell survival (Hammond*, et al.,* 2000; Lodish, *et al.,* 2004)

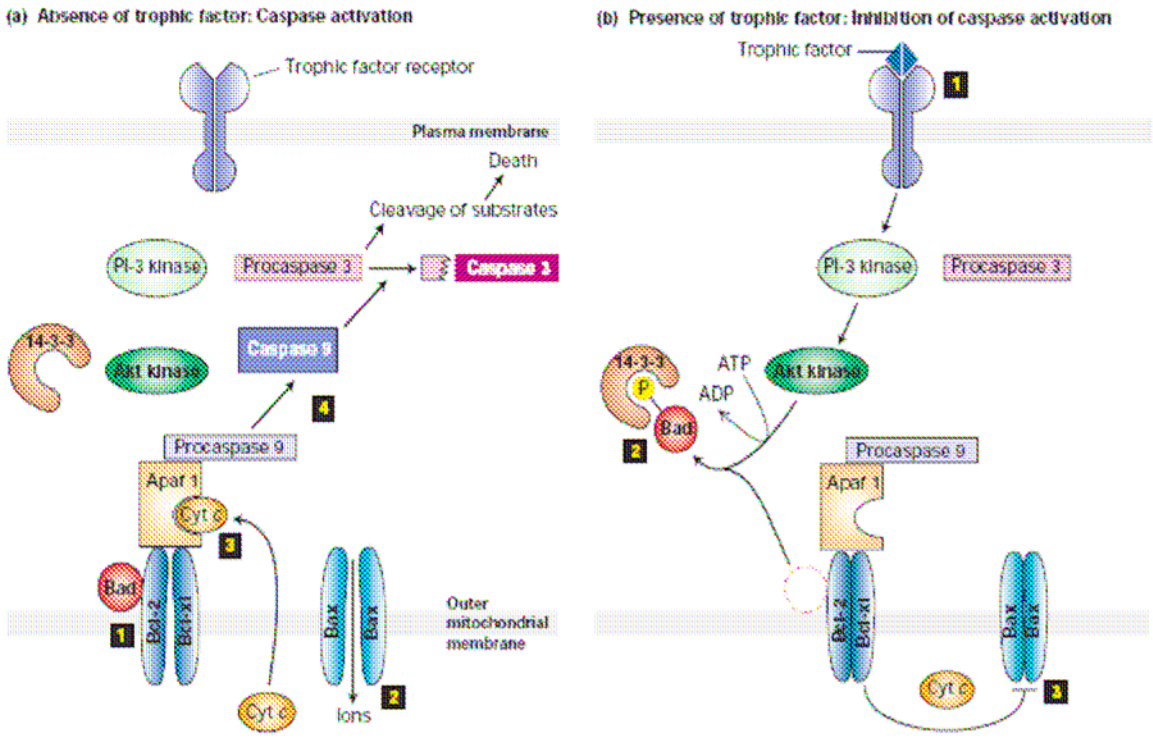


Figure 15: *Apoptosis Pathway.* A representation of the pathways that are activated in the apoptotic (absence of trophic factors) pathway (A) and the inhibition of the same pathway in the presence of trophic factors (B) (Lodish, *et al.,* 2004)

Another suggested mechanism for increased proliferation by CXCL8 is by transactivation of the epidermal growth factor receptor (EGFR) by G-protein coupled receptor (GPCR) stimulation (Itoh, *et al.,* 2004, Luppi, *et al.,* 2006). It has been previously reported that CXCL8 causes an increase in matrix metalloprotease 2 and 9 (MMP-2 and

MMP-9) (Li, *et al.,* 2003; Li, *et al.,* 2005; Patel, *et al.,* 2005; Handsley and Edwards, 2005). It is then feasible that stimulation of CXCR2 by CXCL8 would cause an increase in levels of MMP-2 and MMP-9. These MMPs would then cleave the membrane bound epidermal growth factor (EGF), which would subsequently activate its receptor, EGFR (Itoh, *et al.,* 2004; Luppi, *et al.,* 2006). EGF, a known growth factor has been found to work via the mitogen activating protein kinase (MAPK) cascade to increase cellular proliferation among other cellular changes (Lodish, *et al.,* 2004).

4.4 CXCL8 Expression and Metastasis

Metastasis occurs when tumors gain the ability to invade into other nearby tissues or into circulation to migrate to distant sites in the body. Along with increased proliferation, CXCL8 has been implicated in the phenomenon of tumor cell metastasis as well, with increased CXCL8 levels leading to a higher incidence of metastasis (Yuan, *et al.,* 2000; Li, *et al.,* 2005; Li, *et al.,* 2003; Zhu, *et al.,* 2004; Huang, *et al.,* 2002; Itoh, *et al.,* 2005; Opdenakker and Van Damme, 2004, Luppi, *et al.,* 2006). Results from the current study support this hypothesis. When *in vitro* experiments were performed with the HN12 cell line it was found that the HN12-shL8 knockdown line had significantly fewer numbers of cells migrating across the semi-permeable membrane. Similarly, when this same membrane was covered with a solution that mimics the composition of the extracellular matrix (ECM), the number of cells that invaded through this layer for the HN12-shL8 knockdown line was far fewer than for the control cell line. When the same migration

assay was performed for the HN4 cells, the HN4-CXCL8 overexpressing line had significantly more cells migrating across the membrane than did the control. This difference was not as profound as for the HN12 cells however. An invasion assay was not performed for the HN4 cells due to the low number of cells that migrated with the semi-permeable membrane alone. It was believed that adding the layer of Matrigel to the membrane would completely inhibit any sort of migration or invasion.

The increase in metastasis by CXCL8 has been attributed to several downstream signaling pathways which are predominantly associated with activation of the CXCR2 receptor (Strieter, *et al.,* 2005; Addison, *et al.,* 2000; Heidemann, *et al.,* 2003; Varney, *et al.,* 2006). This GPCR has been shown to activate Rac and Rho as well as the MAPK pathway mentioned earlier (Strieter, *et al.,* 2005; Bizzarri, *et al.,* 2006). Rac and Rho, along with increased intracellular Ca^{2+} influx, function to allow cellular migration through rearrangement of the actin cytoskeleton. Rac is involved in lamellipodia formation and Rho with focal adhesion and stress fiber assembly. Together, these two play a key role in the cell motility that is needed for tumor metastasis.

Although one would assume that there is no link between the mechanisms of cellular proliferation and cellular migration and invasion, it seems as though this is not the case. The common denominator underlying these two mechanisms is the matrix metalloproteases (MMPs) that are upregulated in response to CXCL8 binding to its receptor. As mentioned earlier, when CXCL8 binds to its G-protein coupled receptor (GPCR) it activates a signaling cascade that leads to an increase in transcription and subsequent translation of the gene for MMP-2 and MMP-9 (Li, *et al.,* 2003; Li, *et al.,*

2005; Patel, *et al.,* 2005; Handsley and Edwards, 2005). MMP-2 and -9 are part of a group of proteases that specifically break down and degrade type-IV collagen, which is a major component of the basement membrane (Li, *et al.,* 2003; Li, *et al.,* 2005; Patel, *et al.,* 2005; Handsley and Edwards, 2005). By breaking down this membrane, these specific MMPs help to pave the way for the migration of cells. Another interesting finding is that by Van den Steen, *et al.,* (2000) which shows that post-translational CXCL8 processing by MMP-9 leads to a 10-27 fold increase in CXCL8 activation. This would imply that there is a positive feedback loop between CXCL8 and MMP-9, which would not only increase the amount of MMP which can degrade the extracellular matrix, but may also be a cause of the increased proliferation via transactivation of epidermal growth factor receptor (EGFR), as mentioned earlier.

4.5 HNSCC Model Limitations

The HNSCC model that was used for the current study has certain limitations that could have possible effects on the outcomes of the experiments performed on them. Although both the HN4 and HN12 cell lines were derived from the same patient, their genetic background is not as similar as we would hope. Microarray analysis performed by Miyazaki, *et al.,* (2006) shows that approximately 30 genes were upregulated and 30 genes were downregulated in HN12 metastatic cells when compared to HN4 primary tumor cells. These genes include an assortment of growth factors and tumor antigens as well as molecules involved in signal transduction, angiogenesis, cell survival, transcriptional regulation and metabolism. These variables could possibly have an effect on CXCL8 that

is not well understood. For example, they may act synergistically to enhance the effects of CXCL8 or perhaps inhibit its actions.

Also, the fact that this is an *in vitro* study in itself has limitations. With this sort of study only one specific tissue is tested outside of the host organisms body. This has its downfalls because there may be factors which affect and interact with CXCL8 that would be present in *in vivo* studies, but which are absent in ours. With that being said, since we have only just begun to look at the effects of CXCL8 on tumorigenesis, an *in vitro* study is a good starting point, which would give a general idea of the trends that we should expect to see when the experiments progress into an *in vivo* study. Previous studies in both *in vitro* and *in vivo* models have reported similar results as our own in respect to CXCL8 function and effects on tumor progression. These studies, however, have not been performed in HNSCC, and mainly focused on tumors of the lung, colon and prostate where inflammation seems to play a role (Yuan, *et al.,* 2000; Li, *et al.,* 2005; Li, *et al.,* 2003; Zhu, *et al.,* 2004; Huang, *et al.,* 2002; Itoh, *et al.,* 2005; Opdenakker and Van Damme, 2004, Luppi, *et al.,* 2006).

4.6 shRNA Limitations

shRNA has proved to be an effective method for testing biological function of specific genes by degradation of their mRNA. In the current study using our shRNA protocol we were able to decrease the levels of expressed CXCL8 by approximately 2.5 fold. Previous shRNA studies performed in our lab on CXCL5, another CXC chemokine, showed a decrease in expression of almost 15 fold (Miyazaki, *et al.,* 2006). This is a much

more drastic difference than the decrease observed in the current study. The question then arises of how, if possible, could the knockdown of CXCL8 be performed in a greater, more efficient manner. It is possible that by varying the conditions of the actual transfection itself, the cells would be more likely to take up the plasmid with the incorporated shL8 sequence. Also, reconstructing several different shL8 sequence and testing each separately, although costly, could help to find an optimal level of downregulation of the gene of interest. This sequencing issue may be responsible for less drastic knockdown of CXCL8 than that seen in CXCL5. Perhaps the RNA specificity was not as great as expected.

shRNA mediated gene downregulation is one of the newer methods of decreasing gene expression. This procedure works great for *in vitro* studies, however, the use of shRNA for *in vivo* studies is fairly new and has a greater chance for failure. When transfecting the shRNA containing plasmid into a cell culture, one of the main concerns is the ability to cross the plasma membrane of the cell. When working with an *in vivo* model more factors come into play. The silencing depends on the efficiency of the delivery as well as the retention of the plasmid in the tissue of interest. Another concern is the stability of the plasmid once placed in the system (www.ambion.com, 2007).

Another method that has been used to inhibit specific protein function, other than gene knockdown, is the antibody method. By using specific antibodies to bind to and sequester the active protein or comepitively binding to and inhibiting its receptor the biological functions of the protein of choice can be effectively elucidated.

4.7 Future Studies

In the current study CXCL8 has been shown to play a role in the increased proliferation and migration/invasion that is associated with certain types of cancers. Another known function of CXCL8 in tumor progression has to do with its involvement in neovascularization of tumors (Yuan, *et al.,* 2000; Li, *et al.,* 2005; Li, *et al.,* 2003; Zhu, *et al.,* 2004; Huang, *et al.,* 2002; Itoh, *et al.,* 2005; Opdenakker and Van Damme, 2004, Luppi, *et al.,* 2006). A possible future direction for this current study would be to perform *in vitro* angiogenesis assays using human umbilical vein endothelial cells (HUMVEC) and testing to see if CXCL8 causes increased migration of these cells towards the secreting HN12 or HN4 cells.

Once the results of this assay are confirmed *in vitro* these assays, including proliferation, migration and angiogenesis can be tested on an *in vivo* mouse model. By using the xenograft method of introducing tumors to the tongue or cheek of the mouse it would be possible to test *in vivo* to see if CXCL8 plays a role in tumor proliferation, metastasis and increased neovascularization.

As mentioned earlier, rather than using shRNA to knockdown our gene of interest, perhaps using antibodies directed to CXCL8 or its receptors, CXCR1 and CXCR2, would be more effective, or could be run in parallel to confirm the results of the shRNA study. Also, using the antibody method could allow us to inhibit known downstream molecules that are activated or inhibited by CXCL8 so as to elucidate in more detail the signal transduction pathways that are involved in this process.

Literature Cited

Literature Cited

Addison CL, Daniel TO, Burdick MD, Liu H, Ehlert JE, Xue YY, The CXC Chemokine receptor 2, CXCR2, is the putative receptor for ELR^+ CXC Chemokine-induced angiogenic activity. J Immunol 165(9):5269-5277 (2000)

Aggarwal B, Nuclear factor-kappaB: the enemy within. Cancer Cell 6(3):203-8 (2004)

Aggarwal B, Shishodia S, Sandur S, Pandey M, Sethi G, Inflammation and cancer: How hot is the link? Biochemical Pharmacology 72 1605-1621 (2006)

Balkwill, F. Mantovani, A. Inflammation and cancer: back to Virchow? The Lancet, 357: 539-45 (2001)

Balkwill F, Cancer and the Chemokine Network. Nature. 4: 540-550 (2004)

Bertini R, Allegretti M, Bizzarri C, Moriconi A, Locati M, Zampilla G, Noncompetitive allosteric inhibitors of the inflammatory Chemokine receptors CXCR1 and CXCR2: prevention of reperfusion injury. PNAS 101(32) 11791-11796 (2004)

Bizzarri C, Beccari AR, Riccardo B, Cavicchia MR, Giorgini S, Allegretti M, ELR^+ CXC Chemokines and their receptors (CXC Chemokine receptor 1 and CXC Chemokine receptor 2) as new therapeutic targets. Pharmacology and Therapeutics 112 139-149 (2006)

Brasier AR, Jamaluddin M, Casola A, Duan W, Shen O, Garofalo RP, A promoter recruitment mechanism for tumor necrosis factor-alpha-induced interleukin-8 transcription in type II pulmonary epithelial cells. Dependence on nuclear anundance of Rel A, NF-kappaB1, and c-Rel transcription factors. J. Biol. Chem. 273, 3551-3561 (1998)

Coussens LM, Werb Z, Inflammation and cancer. Nature 420(6917):860-7 (2002)

Dhanasekaran N, Tsim S, Dermott J, Onesime D, Regulation of cell proliferation by G-proteins. Oncogene 17: 1383-94 (2002)

Dorsam R, Gutkind JS, G-protein-coupled receptors and cancer. Nature Rev. Cancer. 7 :79-94 (2007)

Hammond ME, Lapointe GR, Feucht PH, Hilt S, Gallegos CA, Gordon CA, IL-8 induces neutrophil chemotaxis predominantly via type I IL-8 receptors. J Immunol 155(3):1428-1433 (1995)

Handsley MM, Edwards DR, Metalloproteinases and their inhibitors in tumor angiogenesis. Int. J. Cancer: 115, 849-860 (2005)

Heidemann J, Ogawa H, Dwinell MB, Rafiee P, Maaser C, Gockel HR, Otterson MF, Ota DM, Lugering N, Domschke W, Binion DG, Angiogenic effects of interleukin 8 (CXCL8) in human intestinal microvascular endothelial cells are mediated by CXCR2. J Biol Chem. Mar 7;278(10):8508-15 (2003)

Holland and Frei. Cancer Medicine- 5th Edition. B.C Becker Inc. (2000)

Holmes WE, Lee J, Kuang WJ, Rice GC, Wood WI, Structure and functional expression of human interleukin-8 receptor. Science 253 (5025): 1278-1280 (1991)

Huang S, Mills J, Mian B, Tellez C, McCarty M, Yang XD, Gudas JM, Menashe BE, Fully humanized neutralizing antibodies to Interleukin-8 (ABX-IL8) inhibit angiogenesis, tumor growth and metastasis of human melanoma. American Journal of Pathology, 161;1:125-134 (2002)

Itoh Y, Joh T, Tanida S, Sasaki M, Kataoka H, Itoh K, Oshima T, Ogasawara N, Togawa S, Wada T, Kubota H, Mori Y, Ohara H, Nomura T, Higashiyama S, Itoh M, IL-8 promotes cell proliferation and migration through metalloptroteinase-cleavage proHB-EGF in human colon carcinoma cells. Cytokine 29: 275-282 (2005)

Kim M, Califano J, Molecular Pathology of Head-and-Neck Cancer, Int. J. Cancer: 112, 545-553 (2004)

Kulbe H, Levinson N, Balkwill F, Wilson J, The Chemokine network in cancer- much more than directing cell movement. Int. J. Dev. Biol. 48: 489-496 (2004)

Laing K Secombes C, Chemokines. Developmental and Comparative Immunology 28 443-460 (2004)

La Vecchia C, Tavani A, Franceshi S, Levi F Corrao G, Negri E, Epidemiology and Prevention of Oral Cancer, Oral Oncology, 33: 5, 302-312 (1997)

Lawrence T, Anti-inflammatory lipid mediators and insights into the resolution of inflammation. Nat. Rev. Immunol. 2, 787-795 (2002)

Lawrence T, Inflammation and cancer: a failure of resolution?, Trends Pharmacol. Sci. in press (2007)

Leek RD, Association of macrophage infiltration with angiogenesis and prognosis in invasive breast carcinoma. Cancer Res. 56, 4625-4629 (1998)

Li A, Dubey S, Varney M, Dave BJ, Singh R, IL-8 directly enhanced endothelial cell survival, proliferation, and matrix metalloproteinases production and regulated angiogenesis. The Journal of Immunology, 170:3369-3376 (2003)

Li A, Varney M, Valasek J, Godfrey M, Dave B, Singh R, Autocrine role of Interleukin-8 in induction of endothelial cell proliferation, survival, migration and MMP-2 production and angiogenesis. Angiogenesis 8:63-71 (2005)

Lodish H, Berk A, Matsudaira P, Kaiser C, Krieger M, Scott M, Ziprusky S, Darnell J, Molecular Cell Biology- Fifth Edition, W.H. Freeman and Company (2004)

Luppi F, Longo AM, de Boer WI, Rabe KF, Hiemstra PS, Interleukin-8 stimulates cell proliferation in non-small cell lung cancer through epidermal growth factor receptor transactivation. Lung Cancer in press (2006)

Mantovani A, Cancer: inflammation by remote control. Nature 435(7043):752-3 (2005)

McMahon S, Chen A, Head and Neck Cancer, Cancer and Metastasis Reviews 22:21-24, (2003)

Miyamoto M, Shimizu Y, Okada K, Kashii Y, Higuchi K, Watanabe A, Effect of interleukin-8 on production of tumor-associated substances and autocrine growth of human liver and pancreatic cancer cells. Cancer Immunol Immunother, Sep;47(1):47-57 (1998)

Miyazaki H, Patel V, Wang H, Ensley JF, Gutkind JS, Yeudall WA, Growth factor-sensitive molecular targets identified in primary and metastatic head and neck squamous cell carcinoma using microarray analysis. Oral Oncology 42, 230-256 (2006)

Mukaida N, Morita M, Ishikawa Y, Rice N Okamoto S, Kasahara T, Matsushima K, Novel Mechanism of glucocorticoid-mediated gene repression. Nuclear factor-kappaB is target for glucocorticoid-mediated interleukin-8 gene repression. J. Biol. Chem. 269, 13289-13295 (1994).

Nakamura H, Yoshimura K, Jaffe HA, Crystal RG, Interleukin-8 gene expression in human bronchial epithelial cells. J. Biol. Chem. 266:19611-19617 (1991)

Opdenakker G, Van Damme J, The countercurrent principle in invasion and metastasis of cancer cells. Recent insights on the roles of Chemokines. Int. J. Dev. Biol. 48:519-527 (2004)

Patel BP, Shah PM, Rawal UM, Desai AA, Shah SV, Rawal RM, Patel PS, Activation of MMP-2 and MMP-9 in patients with oral squamous cell carcinoma. Journal of Surgical Oncology; 90:81-88 (2005)

Remick DG, Villarete L, Regulation of cytokine gene expression by reactive oxygen and reactive nitrogen intermediates. J Leukocyte Biol. 59:471-5 (1996)

Richards BL, Eisman RJ, Sprio JD, Lindquist RL, Kreutzer DL, Coexpression of Interleikin-8 Receptors in Head and Neck Squamous Cell Carcinoma. The American Journal of Surgery 174: 507-512 (1997)

Roebuck K, Regulation of Interleukin-8 Gene Expression. Journal of Interferon and Cytokine Research 19:429-438 (1999)

Schadendorf D, Moller A, Algermissen B, Worm M, Sticherling M, Czarnetzki BM, IL-8 produced by human malignant melanoma cells in vitro is an essential autocrine growth factor. J Immunol. Sep 1;151(5):2667-75 (1993)1

Schraufstatter IU, Chung J, Burger M, IL-8 activates endothelial cell CXCR1 and CXCR2 through Rho and Rac signaling pathways. Am. J Physiology Lung Cell Mol Physiol 280:1094-1103 (2001)

Shi Q, Abbruzzese J, huang S, Fidler IJ, Xie K, Constitutive and inducible interleukin-8 expression by hypoxia and acidosis renders human pancreatic cancer cells more tumorignic and metastatic. Clin. Cancer Res. 5:3711-21 (1999)

Streieter RM, Burdick MD, Gomperts BN, Belperio JA, Keane, MP. CXC Chemokines in angiogenesis. Cytokine & Growth Factor Reviews 16: 593-609 (2005)

Van den Steen P, Proost P, Wuyts A, Van Damme J, Opdenakker G, Neutrophil Gelatinase B potentiates interleukin-8 tenfold by aminoterminal processing, whereas it degrades CTAP-III, PF-4, and Gro-a and leaves RANTES and MCP-2 intact. Blood. 96 (8): 2673-2681 (2000)

Varney ML, Johansson SL, Singh RK, Distinct Expression of CXCL8 and Its Receptors CXCR1 and CXCR2 and Their Association With Vessel Density and Aggressibeness in Malignant Melanoma. Am. J. Clin. Pathol. 125:209-216 (2006)

Wilson J, Slettenaar V, The Chemokine network: A target in cancer biology? Advanced Drug Delivery Reviews 58 :962-974 (2006)

Wuyts A, Proost P, Lenaerts JP, Ben-Baruch A, Van Damme J, Wang JM, Differential usage of the CXC Chemokine receptors 1 and 2 by interleukin-8, granulocyte Chemotactic protein-2 and epithelial-cell-derived neutrophil attractant-78. Eur. J. Biochem. 255, 67-73 (1998)

Xie K, Interleukin-8 and human cancer biology. Cytokine and Growth Factor Reviews 12 : 375-391 (2001)

Yeudall WA, Miyazaki H, Ensley JF, Cardinali M, Gutkind JS, Patel V, Uncoupling of epidermal growth factor-dependent proliferation and invasion in a model of squamous carcinoma progression. Oral Oncology 41, 698-708 (2005)

Yeudall WA, Miyazaki H, Chemokines and Squamous cancer of the head and neck: targets for therapeutic intervention? Expert Rev Anticancer Ther. Mar;7(3):351-60. Review (2007)

Yuan A, Yang PC, Yu CJ, Chen WJ, Lin FY, Kuo SH, Luh KT, Interleukin-8 messenger ribonucleic acid expression correlates with tumor progression, tumor angiogenesis, patient survival, and timing of relapse in non-small-cell lung cancer. Am. J. Respir. Crit. Care Med. 162:1957-1963 (2000)

Zhu YM, Webster SJ, Flower D, Woll PJ, Interleukin-8/CXCL8 is a growth factor for human lung cancer cells. British Journal of Cancer 81;11: 1970-1976 (2004)

VITA

Emil Christofakis was born in Yonkers, New York on February 1, 1983. He has been in Richmond, VA since the age of four. He attended the University of Mary Washington in Fredericksburg, VA where he graduated with a B.S in Biology in the spring of 2005. He went on get his M.S in Physiology at the VCU School of Medicine and will attend the VCU School of Dentistry as part of the class of 2011 in the fall of 2007.