CROSSTALK BETWEEN ENDOTHELIAL CELLS AND TUMOR CELLS IN HEAD AND NECK CANCER

by

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DEDICATION

To Victoria,

who showed me the meaning of unconditional love…

who makes everything worthwhile!

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ABSTRACT

Tumor angiogenesis is necessary for cancer progression and requires active interaction between endothelial cells and tumor cells. It is well established that cancer cells secrete angiogenic factors to recruit and sustain tumor vascular networks. However, little is known about the effect of endothelial cell-secreted factors on the phenotype and behavior of tumor cells. The identification and characterization of signaling events initiated by tumor-associated endothelial cells may have important implications in cancer therapy. The hypothesis underlying this dissertation is that factors secreted by endothelial cells initiate signaling pathways in head and neck squamous cell carcinoma (HNSCC) cells that enhance tumor growth.

Here, we observed that soluble mediators from primary human dermal microvascular endothelial cells activate STAT3, Akt, and ERK signaling in HNSCC cells. HNSCC cells adjacent to blood vessels showed increased phosphorylation of STAT3, Akt, and ERK in xenograft human tumors. IL-6, CXCL8, and EGF are upregulated in endothelial cells co-cultured with HNSCC, and blockade of endothelial cell-derived IL-6, CXCL8, or EGF inhibited the activation of STAT3, Akt, or ERK in tumor cells, respectively. Notably, activation of these pathways by endothelial cells enhanced migration and inhibited anoikis of tumor cells. It is known that Bcl-2 is upregulated in tumor microvessels of patients with HNSCC. Here, we observed that Bcl-

2 signaling induces expression of IL-6, CXCL8, and EGF, providing a mechanism for the upregulation of these cytokines in tumor-associated endothelial cells.

We also observed that endothelial cell-induced Akt or ERK signaling in HNSCC has a compensatory effect whereas STAT3 pathway is activated independent of Akt or ERK. Among these three pathways, STAT3 presented the higher phosphorylation levels, and was mainly induced by endothelial cell-secreted IL-6. Interestingly, downregulation of IL-6 in tumor-associated endothelial cells inhibited tumor growth in xenograft human tumors. These results suggest that patients with HNSCC might benefit from targeted inhibition of signaling events initiated by tumor associated-endothelial cells.

Collectively, this work expands the contribution of vascular endothelial cells to the pathobiology of cancer. It shows that endothelial cells function as the initiators of molecular signaling events that enhance head and neck tumor growth.

CHAPTER I

INTRODUCTION

Squamous cell carcinoma (SCC) is the most common malignancy of the head and neck region. Head and neck squamous cell carcinoma (HNSCC) is a significant public health challenge and represents one of the sixth most common cancers in the world (Parkin et al., 2005). Despite many advances in prevention and treatment of other types of cancer, the five-year survival rate after diagnosis for HNSCC remains low, approximately 50%, which is considerably lower than that for most cancers (Jemal et al., 2008). The limited survival of HNSCC patients is likely due to a high proportion of patients presenting with advanced disease stages, lack of suitable markers for early detection, and failure to respond to available chemotherapy (Forastiere et al., 2001; Mao et al., 2004). Their poor prognosis is also the reflection of the incomplete knowledge of the mechanisms underlying the malignant progression of this cancer type (Molinolo et al., 2009). Therefore, the ability to gain insights into the complex molecular events leading to the development and progression of HNSCC will have important implications for the early diagnosis, therapy, and prognosis of HNSCC patients.

Head and neck squamous cell carcinoma

Head and neck cancer includes malignant lesions arising in the oral cavity, larynx, and pharynx, with more than 500,000 new cases reported annually worldwide (Jemal et al., 2008; Parkin et al., 2005). Although most patients with stage I disease can be cured, around 10% relapse and die. For more advanced stages, the proportion of patients with relapses increases to 30% for stage II, 50% for stage III, and more than 70% for stage IV (Prince and Ailles, 2008).

Several risk factors contribute to the development of HNSCC. Among them, carcinogen exposure, diet, oral hygiene, infectious agents, family history, and preexisting medical conditions all play a role, individually or in combination. Tobacco smoking is well established as the dominant risk factor for HNSCC, and this risk is correlated with the intensity and duration of smoking (Brennan et al., 1995; Ho et al., 2007; Zhang et al., 2000). Heavy alcohol consumption is also an independent risk factor for HNSCC, particularly for cancers of the hypopharynx. Alcohol consumption, however, is most relevant for its ability to amplify the effects of tobacco smoke in a synergistic manner (Sturgis et al., 2004; Talamini et al., 2002). Although tobacco exposure and alcohol consumption are responsible for the vast majority of HNSCC that occur in the oral cavity, larynx, and hypopharynx, their role in promoting tumorigenesis of the oropharynx is less consequential. Instead, human papillomaviruses (HPV), and in particular HPV-16, has been established as a causative agent in up to 70% of oropharyngeal cancers (Gillison et al., 2000; Herrero et al., 2003; Hobbs et al., 2006; Mork et al., 2001; Wiest et al., 2002).

The progression of head and neck cancer, from a simple squamous hyperplasia through the advancing stages of squamous dysplasia to invasive squamous cell carcinoma is driven by the progressive accumulation of genetic alterations (Forastiere et al., 2001). Histologically, dysplasia refers to neoplastic alterations of the surface epithelium prior to invasion of the subepithelial connective tissues. These changes include abnormal cellular organization, increased mitotic activity, and nuclear enlargement with pleomorphism. These alterations are typically graded on a scale of 1 to 3 based on the severity of the atypia (mild, moderate, or severe dysplasia/carcinoma *in situ*). With progression, the carcinoma *in situ* breaks through the basement membrane and infiltrates the subepithelial connective tissue as cohesive nests and cords. With advanced tumor growth, nests of invasive tumor invade skeletal muscle, craniofacial bones, and facial skin. Invasion may be associated with tumor extension along nerves and involvement of lymphatic spaces (Pai and Westra, 2009).

The initiation and progression of head and neck cancer is a complex multistep process that entails a progressive acquisition of genetic and epigenetic alterations (Califano et al., 1996b). An early model of head and neck tumorigenesis based on microsatellite analysis of allelic imbalances showed that the p53 gene is almost universally disrupted in HNSCC (Califano et al., 1996a; Poeta et al., 2007). In fact, 40% to 60% of patients with HNSCC present with a mutation of the p53 gene (Hollstein et al., 1991; Somers et al., 1992). The loss of p53 function is associated with the progression from premalignancy to invasive disease (Forastiere et al., 2001) and increases the likelihood of further genetic progression (Boyle et al., 1993). Amplification of the oncogene cyclin D1, which constitutively activates cell cycle progression, is present in

30% to 50% of patients with HNSCC and is usually associated with invasive disease, early recurrence, and shortened survival (Callender et al., 1994; Sidransky, 1995). Loss of chromosomal region 9p21 is one of the most common genetic changes and occurs early in the progression of HNSCC (Cairns et al., 1995; van der Riet et al., 1994). The main effect of this loss is the inactivation of the CDKN2A (formerly p16) gene, an inhibitor of cyclin-dependent kinase (CDK) that is important in regulating the cell cycle (Kamb et al., 1994; Reed et al., 1996). This early inactivation is consistent with the findings that keratinocytes in culture often loose p16 function and thus escape senescence (Munro et al., 1999; Papadimitrakopoulou et al., 1997). More recently, epidermal growth factor receptor (EGFR) was found to be highly expressed in more than 95% of HNSCC. Notably, increased expression of the protein and its ligand is associated with poor prognosis (Kalyankrishna and Grandis, 2006).

The model of the molecular progression of HNSCC demonstrates that tissue with a normal or benign appearance can also contain clonal genetic changes (Califano et al., 1996b). Several studies suggest that early genetic changes do not necessarily correlate with observable changes in morphology (Rosin et al., 2000). Therefore, testing for these genetic alterations in early lesions may identify patients who are at the greatest risk for progression and lead to more definitive therapy (Forastiere et al., 2001).

Field cancerization is the term used to describe the lateral spread of pre-malignant or malignant disease, often fatal, that develop in the head and neck, lungs, esophagus, or other regions of 10–40% of patients with HNSCC (Ha and Califano, 2003; Jones et al., 1995). Field cancerization was first described more than 50 years ago as histologically altered epithelium surrounding tumors that were taken from the upper aerodigestive tract

(Slaughter et al., 1953). According to the field cancerization concept, multiple cell groups independently undergo neoplastic transformation under the stress of regional carcinogenic activity. Molecular genetic approaches have challenged the notion that independent transforming events are common in the epithelium of patients with HNSCC. Indeed, when a primary HNSCC is compared with a second tumor elsewhere in the respiratory tract, the paired tumors often harbor identical patterns of genetic alterations (Bedi et al., 1996; Worsham et al., 1995). Presumably, a critical genetic alteration in a single cell provides a growth advantage over its neighboring cells. At some point after transformation, cells harboring these early genetic alterations migrate to populate contiguous tracts of mucosa, accumulate other alterations, acquire additional growth advantages, and ultimately transform into aggressive subclones separated by time and space (Califano et al., 2000). Several reports have demonstrated that the epithelium of the upper respiratory tract may become populated by these clones of genetically damaged cells despite the lack of histopathologic evidence of dysplasia (Mao et al., 1996; Westra and Sidransky, 1998). The presence of morphologically intact but genetically damaged cells not only explains the phenomenon of field cancerization, but accounts for certain patterns of tumor behavior, such as local tumor recurrence following complete surgical resection, since pre-malignant or malignant clones have the ability to migrate and persist outside the filed of treatment. Future studies to refine the relationship among these lesions is needed to identify key molecular alterations that can be used as targets for therapy (Ha and Califano, 2003).

Metastases arise following the spread of cancer from a primary site and the formation of new tumors in distant organs. At least 50% of patients with locally advanced

HNSCC develop locoregional or distant metastases, which are usually detected within the first 2 years of treatment (Argiris et al., 2008). When cancer is detected at an early stage, before it has spread, it can often be treated successfully by surgery or local irradiation, and the patient is frequently cured. However, when the cancer is detected after it is known to have metastasized, treatments are much less successful. Furthermore, for many patients in whom there was no evidence of metastasis at the time of their initial diagnosis, metastases can be detected at a later time. The metastatic process consists of a series of steps. As a primary tumor grows, it needs to develop a blood supply that can support its metabolic needs, through a process called angiogenesis (Folkman, 1971; 2002). These new blood vessels can also provide an escape route by which cells can leave the tumor and enter into the circulatory blood system (Wyckoff et al., 2000). Tumor cells might also enter the blood circulatory system indirectly via the lymphatic system (Stacker et al., 2002). The cells need to survive in the circulation until they can arrest in a new organ, extravasating from the circulation into the surrounding tissue. Once in the new site, cells initiate and maintain growth to form preangiogenic micrometastases. This growth is sustained by the development of new blood vessels in order for a macroscopic tumor to form (Chambers et al., 2002).

Tumor angiogenesis

In the early seventies Judah Folkman proposed the hypothesis that tumor growth is angiogenesis-dependent (Folkman, 1971). In this revolutionary report, Folkman showed preliminary evidence that tumors could not enlarge beyond 1 to 2 millimeters in

diameter without recruiting new blood vessels. His hypothesis stated that tumors secrete a diffusible substance that could stimulate endothelial cell proliferation in host capillary blood vessels. This was also the first report to introduce the concept of a novel form of tumor dormancy caused by blockade of angiogenesis. Indeed, most of us harbor microscopic tumors in various organs throughout life, and the vast majority of these tumors never expand further (Black and Welch, 1993). They may contain up to 1 million tumor cells that are proliferating and undergoing apoptosis, but the microscopic tumors do not invade locally and do not metastasize. They remain nonangiogenic, dormant, and harmless (Folkman and Kalluri, 2004). In the event of a shift in the balance of proangiogenic and antiangiogenic proteins, the total output of these proteins renders the tumor mass proangiogenic, and the tumor is enabled to switch to an angiogenic phenotype (Almog et al., 2006; Hanahan and Folkman, 1996). After the angiogenic switch (Rastinejad et al., 1989), both benign and malignant tumors can expand their tumor mass. Malignant angiogenic tumors, however, invade locally, metastasize to remote sites, and are potentially lethal. In the absence of the angiogenic switch, even tumors that are malignant by all other criteria may be harmless to the host (O'Reilly et al., 1994). Folkman's discovery that tumor growth is angiogenesis-dependent led to a profound paradigm shift in cancer therapy and cancer biology. Before his hypothesis, the cancer cell itself was the only target of cancer therapies. Now it is widely accepted that microvascular endothelial cells recruited by tumors are viable and effective therapeutic targets (Cao and Langer, 2008).

The vascular endothelial growth factor (VEGF) family and the receptor tyrosine kinases are key mediators of proangiogenic effects in tumor angiogenesis. This family of

structurally related molecules includes VEGF-A, VEGF-B, VEGF-C, VEGF-D, and placental growth factor (PlGF) (Ferrara, 2002; Hicklin and Ellis, 2005). The major mediator of tumor angiogenesis is VEGF-A, referred to as VEGF, which is expressed as various isoforms due to alternative splicing that leads to 121-, 165-, 189- and 206-amino-acid proteins. $VEGF_{165}$ is the predominant isoform and is commonly overexpressed in a variety of human solid tumors. The VEGF ligands bind to and activate three structurally similar type III receptor tyrosine kinases, namely VEGFR1 (also known as FLT1), VEGFR2 (also known as KDR), and VEGFR3 (also known as FLT4). Each VEGF ligand has distinctive binding specificities for each of these tyrosine kinase receptors, which contributes to their diversity of function (Ellis and Hicklin, 2008). VEGF signals mainly through VEGFR2, which is expressed at elevated levels by endothelial cells engaged in angiogenesis and by circulating bone marrow-derived endothelial progenitor cells. The role of VEGFR1 is less clear. It binds VEGF with higher affinity than VEGFR2, but its signaling properties are weaker (Shibuya and Claesson-Welsh, 2006). VEGFR3 preferentially binds VEGF-C and VEGF-D and its expression in the adult is primarily on lymphatic endothelial cells. More recent data has demonstrated the expression and function of VEGFR3 on vascular endothelial cells (Laakkonen et al., 2007). VEGFR3 is important in cardiovascular development and remodeling of primary vascular networks during embryogenesis, and has a crucial role in post-natal lymphangiogenesis (Alitalo and Carmeliet, 2002). The neuropilins (NP1 and NP2, also known as NRP1 and NRP2) act as co-receptors for the VEGFRs, increasing the binding affinity of VEGF (Bielenberg and Klagsbrun, 2007; Klagsbrun et al., 2002; Soker et al., 1998). NP1 and NP2 have been postulated to signal independently of their association with VEGFR tyrosine kinase

receptors, but the role of VEGF activation of NP-mediated signaling is not fully understood. In fact, recent studies suggest that dual targeting of the vasculature with antibodies to VEGF and NP1 is more effective than single-agent therapy (Batchelor et al., 2007). Differences in VEGFR biology are important, as various VEGF-targeting approaches differ in their ability to block receptor function (Ellis and Hicklin, 2008).

Most types of human cancer cells express elevated levels of VEGF, as a consequence of the numerous and diverse genetic and epigenetic ways in which VEGF can be induced (Kerbel and Folkman, 2002). Hypoxia is a characteristic of solid tumors and is an important inducer of VEGF. Its effect is mediated through the hypoxia-inducible transcription factors 1α and 2α (Semenza, 2003). The VEGF action is attributed mainly to a paracrine mechanism, since tumor cells produce VEGF but do not respond to it directly due low levels of VEGF receptors. In contrast, endothelial cells engaged in angiogenesis express numerous VEGF receptors, but they produce very little or no detectable VEGF. It is now clear, however, that VEGF in amounts sufficient to drive tumor angiogenesis originates from various host cells in the body such as endothelial cells (Kaneko et al., 2007), platelets (Klement et al., 2009), muscle cells (Kut et al., 2007), and tumor-associated stromal cells (Liang et al., 2006). The fact that tumor cells of many types express VEGF receptors (especially VEGFR1) (Dallas et al., 2007; Dong et al., 2007; Fan et al., 2005; Kessler et al., 2007; Wey et al., 2005) and also produce VEGF, indicates that VEGF may act as an autocrine growth factor for tumor cells (Kerbel, 2008). Furthermore, it has been shown that VEGFR1 is expressed within the cell rather than on the surface of breast cancer cells, promoting cell survival by an intracrine mechanism (Lee et al., 2007b). In addition, it has been reported that very low

levels of autocrine VEGF mediate endothelial cell survival and vascular homeostasis by signaling through intracellular VEGFR2 (Lee et al., 2007a).

Based on this knowledge, several angiogenesis inhibitors have been developed to target vascular endothelial cells and block tumor angiogenesis. Recent reports have also shown that inhibiting tumor angiogenesis increases the effectiveness of coadministered chemotherapy (Escudier et al., 2007a; Faivre et al., 2007; Hurwitz et al., 2004; Motzer et al., 2006; Sandler et al., 2006).

Antiangiogenic therapy

Targeting cells that support tumor growth rather than cancer cells themselves is a relatively new approach to cancer therapy. Endothelial cells are more stable genetically and therefore less likely to accumulate mutations that allow them to develop drug resistance (Kerbel and Folkman, 2002). In a human colorectal carcinoma, for example, it has been reported up to 11,000 total genomic alterations per tumor cell (Stoler et al., 1999). In contrast, endothelial cells in a tumor bed reveal differences only in gene expression, but not genomic alterations (Folkman et al., 2000; St Croix et al., 2000).

Angiogenesis inhibitors can act directly or indirectly. Direct angiogenesis inhibitors prevent vascular endothelial cells from proliferating, migrating, or avoiding cell death in response to a spectrum of pro-angiogenic proteins, including VEGF, basic fibroblast growth factor (bFGF), interleukin-8 (CXCL8), platelet-derived growth factor (PDGF), and platelet-derived endothelial growth factor (PD-EGF). Tumors treated with direct angiogenesis inhibitors are less likely to acquire drug resistance (Boehm et al.,

1997; Kerbel, 1991). Indirect angiogenesis inhibitors generally prevent the expression or the activity of a tumor protein that activates angiogenesis, or block the expression of its receptor on endothelial cells. Many of these tumor cell proteins are the products of oncogenes that drive the angiogenic switch (Kerbel et al., 1998; Rak et al., 2002).

In 2004, the U.S. Food and Drug Administration (FDA) approved the first antiangiogenic drug for cancer treatment in combination with conventional chemotherapy: bevacizumab (Avastin®). Bevacizumab was the first angiogenesis inhibitor proven to delay tumor growth and, more importantly, increased the overall survival of patients. Phase III clinical trials for patients with metastatic colorectal cancer demonstrated improved response rates, increased progression-free survival and increased overall survival when bevacizumab was added to irinotecan and 5-fluorouracil (Hurwitz et al., 2004). A survival benefit from bevacizumab in combination with chemotherapy has also been observed in patients with advanced non-small cell lung cancer (Manegold, 2008; Sandler et al., 2006), metastatic breast cancer (Miller et al., 2005) and metastatic renal cell carcinoma (Escudier et al., 2007b). Additionally, single agent bevacizumab led to an increase of time to tumor progression in advanced renal cell cancer (Yang, 2004). Several other clinical trials are currently underway to investigate the activity of this agent in various other cancer types. A recent phase I study in which bevacizumab was used in combination with fluorouracil/hydroxyurea/radiation therapy showed anti-tumor activity in HNSCC patients (Seiwert et al., 2008).

There are currently eight FDA approved anticancer therapies with recognized antiangiogenic properties, comprising two primary categories: 1) monoclonal antibodies directed against specific proangiogenic growth factors and/or their receptors; and 2) small

molecule tyrosine kinase inhibitors (TKIs) of multiple proangiogenic growth factor receptors. Inhibitors of mammalian target of rapamycin (mTOR) represent a third, smaller category of antiangiogenic agents. In addition, at least two other approved angiogenic agents may indirectly inhibit angiogenesis through mechanisms that are not completely understood.

Four monoclonal antibody therapies are approved to treat several tumor types: Bevacizumab (Avastin®), cetuximab (Erbitux®), panitumumab (Vectibix™), and trastuzumab (Herceptin®). Bevacizumab is a humanized monoclonal antibody that binds biologically active forms of VEGF and prevents its interaction with VEGFR1 and VEGFR2, thereby inhibiting endothelial cell proliferation and angiogenesis (Hicklin and Ellis, 2005). The approved indications for bevacizumab are metastatic colorectal cancer, non-small cell lung cancer, and advanced breast cancer. Cetuximab is a chimeric IgG1 monoclonal antibody that binds the extracellular domain of EGFR, preventing ligand binding and activation of the receptor. This blocks downstream signaling of EGFR, inhibiting cell proliferation and angiogenesis. Cetuximab was the first targeted agent proved to be clinically effective in combination with radiation therapy for patients with locally advanced HNSCC (Bonner et al., 2006; Choong and Cohen, 2006; Harari and Huang, 2006; Harari et al., 2009; Le Tourneau and Siu, 2008). Cetuximab is also approved for use in metastatic colorectal cancer. Panitumumab is a fully humanized IgG2 anti-EGFR monoclonal antibody, also approved for use in metastatic colorectal cancer. Trastuzumab is a humanized IgG1 monoclonal antibody that binds the extracellular domain of HER-2, which is overexpressed in 25-30% of invasive breast cancer tumors.

Three small molecule tyrosine kinase inhibitors are currently approved as

anticancer therapies: Erlotinib (Tarceva®), sorafenib (Nexavar®), and sunitinib (Sutent®). Erlotinib inhibits EGFR and is indicated for the treatment of non-small cell lung cancer and pancreatic cancer. Sorafenib inhibits VEGFR1, VEGFR2, VEGFR3, PDGFR-β, and Raf-1; and it is approved for treatment of advanced hepatocellular and renal-cell carcinoma (Escudier et al., 2007a). Sunitinib inhibits VEGFR1, VEGFR2, VEGFR3, PDGFR-β, and RET; and its indications are advanced renal-cell carcinoma and gastrointestinal stromal tumor (Motzer et al., 2007b).

One mTOR inhibitor is currently approved as anti-cancer therapy. Temsirolimus (Torisel®) is a small molecule inhibitor of PI3K/Akt/mTOR pathway involved in tumor cell proliferation and angiogenesis, and is approved for treatment of advanced renal cell carcinoma (Hudes et al., 2007; Motzer et al., 2007a).

Most recently, two drugs were approved as cancer therapies for multiple myeloma and mantle cell lymphoma. First, bortezomib (Velcade®) is a proteasome inhibitor that disrupts signaling of the cancer cell, leading to cell death and tumor regression, and may have indirect antiangiogenic properties (Ma and Adjei, 2009). Lastly, thalidomide (Thalomid®) possesses immunomodulatory, anti-inflammatory, and antiangiogenic properties (Breitkreutz and Anderson, 2008). The precise mechanisms of action of these two drugs are also not fully understood.

Despite all the advances in antiangiogenic therapies, several challenges still remain. The survival benefits of these treatments are relatively modest, usually measured in months (Kerbel, 2008). In addition, the treatments are extremely expensive (Berenson, 2006; Schrag, 2004) and have significant side effects (Eskens and Verweij, 2006; Verheul and Pinedo, 2007). Although promising results have been obtained with

antiangiogenic therapies in phase II trials (Fujita et al., 2007; Liang et al., 2008), cetuximab is the only agent proved by phase III clinical trials to improve survival, either in combination with radiation therapy or in first-line treatment for recurrent or metastatic HNSCC. Cetuximab was approved by the FDA in 2006, and constitutes the only targeted therapy available for this malignancy (Bozec et al., 2009; Le Tourneau and Siu, 2008). Discovery of novel molecular targets, and the inclusion of these agents in combined treatment regimes, is likely to increase therapeutic efficacy. Therefore, understanding of the molecular mechanisms involved in the development and progression of HNSCC may help to identify better tractable targets for pharmacological intervention in this disease.

Signaling pathways and molecular targeted therapy

The aberrant expression and function of molecules regulating cell signaling, growth, survival, motility, cell cycle, and angiogenesis underlie the progressive acquisition of a malignant phenotype in HNSCC (Molinolo et al., 2009). Key signaling networks deregulated in HNSCC include, among others: the EGFR (Dietz et al., 2008; Pomerantz and Grandis, 2003), the transcription factor signal transducer and activator transcription 3 (STAT3) (Grandis et al., 1998; Leeman et al., 2006), the phosphatidylinositol-3-kinase (PI3K)/Akt (Moral and Paramio, 2008), and the mitogen activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) pathways (Mishima et al., 2002; Zhang et al., 2004).

Many human cancers express high levels of growth factors and corresponding receptors, and many malignant cells exhibit highly active receptor tyrosine kinases due to

their activation by an autocrine or paracrine mechanism. Among the best-studied group of these receptors is the EGFR family (also known as type I receptor tyrosine kinases or ErbB tyrosine kinase receptors), which is essential for numerous normal cellular processes. The aberrant activity of this receptor family has also been linked to the development and growth of numerous tumor types including 80 to 90% of all HNSCC (Grandis and Tweardy, 1993). Indeed, EGFR overexpression may represent an independent prognostic marker correlating with increased tumor size, decreased radiation sensitivity, and increased risk of recurrence (Ang et al., 2002; Gupta et al., 2002). Constitutive EGFR activation in HNSCC is also caused by its autocrine stimulation through the co-expression of EGFR with one of its ligands, transforming growth factor-α (TGFα), which is frequently observed in HNSCC and correlates with a poor prognosis (Quon et al., 2001). The presence of truncated mutant forms of EGFR, which causes its constitutive activation, has also been detected in some HNSCC cases and may be resistant to EGFR target therapy (Sok et al., 2006). Interestingly, G protein-coupled receptor (GPCR)-induced cleavage of EGF-like growth factors leads to EGFR transactivation and EGFR-related signaling in cancer cells, suggesting that GPCR–EGFR cross-communication may play a role in the development and progression of HNSCC and other human cancers (Dorsam and Gutkind, 2007). Once activated, EGFR stimulates a number of downstream signaling events that contribute to normal and aberrant cell growth. Among them, EGFR activates the STAT3, the PI3K/Akt, and the MAPK/ERK pathways, which in turn contribute to the malignant growth and metastatic potential of HNSCC (Molinolo et al., 2009).

Currently, seven STAT family members have been identified, STAT 1, 2, 3, 4, 5a,

5b and 6, which participate in the transcription of genes involved in immune responses, growth, and cell fate decisions (Darnell, 2002). While STAT activity is essential for normal cellular functions, deregulation of the STAT pathway contributes to a number of human diseases. Indeed, gain of function of STATs is often associated with cellular transformation and oncogenic potential (Reich and Liu, 2006). Cytokines and growth factors stimulate STAT proteins by acting on their cognate receptors, which leads to the recruitment and phosphorylation of Janus kinase 1 and 2 (JAK-1 and JAK-2) that in turn phosphorylate STAT proteins at specific tyrosine residues, leading to the formation of homo- and heterodimers. STAT dimers translocate to the nucleus where they bind to consensus DNA sequences and activate the expression of growth promoting genes (Reich and Liu, 2006). Constitutive activation of STAT3 has been demonstrated in many cancers, including breast cancer, leukemia, lymphoma, lung, and thyroid cancers (Bromberg, 2002; Darnell, 2005). Notably, early studies indicated that HNSCC also exhibit remarkably elevated levels of the phosphorylated active forms of STAT3 (Grandis et al., 1998). Moreover, studies have shown that blockade of STAT3 activity leads to growth inhibition of HNSCC, supporting the importance of signaling through STAT3 in HNSCC (Grandis et al., 1998; Grandis et al., 2000; Rubin Grandis et al., 2000). Elevated levels of STAT3 alter cell cycle progression, and promote the proliferation and survival of HNSCC cells (Leeman et al., 2006). In fact, STAT3 activation may represent an early event in oral carcinogenesis, as both tumor and the adjacent normal epithelia of HNSCC patients show higher levels of STAT3 expression and phosphorylation (Grandis et al., 2000). Activated STAT3 also correlates with lymph node metastasis and poor prognosis (Grandis et al., 1998; Leeman et al., 2006). Many

mechanisms may converge to promote the persistent activation of STAT3 in HNSCC. While the direct activation of STAT3 by EGFR has been clearly shown in HNSCC cells (Grandis et al., 1998; Leeman et al., 2006), STAT3 can also be activated by an EGFR-independent mechanism (Sriuranpong et al., 2003). This mechanism often involves the autocrine activation of the gp130 cytokine receptor in HNSCC cells by tumor-released cytokines such as interleukin-6 (IL-6), which activates STAT3 independently from the activation status of EGFR. Furthermore, interfering with this cytokine-initiated pathway of STAT3 activation can result in the reduced growth and apoptotic death of HNSCC cells (Sriuranpong et al., 2003). Other cytokines and growth factors, such as erythropoietin (Lai et al., 2005), growth hormone (Gronowski et al., 1995), TGF-α (Grandis et al., 1998), thrombopoietin (Gurney et al., 1995), and PDGF (Vignais et al., 1996) have been demonstrated to stimulate STAT3 in HNSCC and other malignancies (Aggarwal et al., 2006). Several therapeutic approaches are being developed to block STAT3, which may prove to be an efficacious treatment for HNSCC (Leeman et al., 2006).

The PI3K/Akt signaling is one of the most frequently targeted pathways in human cancers, since deregulation in any component of PI3K leads to tumorigenesis (Cully et al., 2006). Genomic aberrations in the PI3K components include mutation, amplification, and/or rearrangements, which result in deregulated cell growth control and survival. These alterations contribute to a competitive growth advantage, metastatic potential, and resistance to therapy (Hennessy et al., 2005). The direct product of PI3K activity, the lipid second messenger PtdIns(3,4,5)P_3 (PIP_3), is a constituent of the inner leaflet of the plasma membrane and serves as docking sites for proteins including Akt. The Akt family

of kinases consists of three members (Akt1, Akt2 and Akt3), which possess conserved phosphorylatable threonine and serine residues (T308 and S473) that are critical for Akt function (Cantley, 2002). Akt is a central regulator of widely divergent cellular processes including proliferation, differentiation, migration, survival, and metabolism (Tokunaga et al., 2008). The importance of Akt in cancer is underscored by the fact that many oncogenic mutations result in the constitutive activation of Akt, and that many tumor suppressor proteins act by inhibiting the activity of Akt and its downstream targets (Luo et al., 2003; Vivanco and Sawyers, 2002). Recent reports support the importance of Akt in HNSCC. Akt has been shown to be persistent activated in HNSCC, and blockade of PDK1, which acts upstream of Akt, has been demonstrated to inhibit tumor cell growth (Amornphimoltham et al., 2004; Patel et al., 2002). Moreover, Akt is detected in nearly 50% of tongue preneoplastic lesions, and its activation represents an independent prognostic marker of poor clinical outcome in tongue and oropharyngeal HNSCC (Massarelli et al., 2005; Yu et al., 2007). Multiple genetic and epigenetic events may converge to promote the activation of the PI3K/Akt pathway in HNSCC, including EGFR overexpression, Ras mutations, PI3K gene amplification, and defective PTEN activity. Indeed, the presence of multiple convergent pathways resulting in enhanced Akt function may explain why activation of the Akt pathway represents one of the most frequent events in HNSCC (Molinolo et al., 2007; Molinolo et al., 2009).

Several agents have been developed to target the MAPK pathway for cancer treatment, based on its aberrant activity in tumor cells (Sebolt-Leopold and Herrera, 2004). The MAPK cascade is a key intracellular signaling pathway that regulates diverse cellular functions including cell proliferation, cell cycle regulation, cell survival,

angiogenesis, and cell migration. The MAPK pathway is generally described as a linear cascade of events initiated by receptor tyrosine kinases at the cell surface and culminates in the regulation of gene transcription in the nucleus. These cascades are comprised of three protein kinases controlled by protein phosphorylation: a MAPK kinase kinase (MAPKKK), a MAPK kinase (MAPKK), and a MAPK (Johnson and Lapadat, 2002). The terminal serine/threonine kinases (MAPKs) are the ERK1/2 (p44 ERK1 and p42 ERK2), the c-Jun amino-terminal kinases (JNK1/2/3), and p38 kinases. Generally, the ERK pathway is activated by growth factor-stimulated cell surface receptors tyrosine kinases that are important in cancer biology, such as EGFR, PDGFR, and VEGFR (Roberts and Der, 2007); whereas the JNK and p38 pathways are activated by stress (UV light, osmotic stress, γ-irradiation, heat shock, and growth factor withdraw) and cytokines (TNF-α and IL-1) (Raingeaud et al., 1995; Xia et al., 1995). The role of ERK pathway in tumorigenesis is well established because of its critical involvement in the regulation of cell proliferation and survival, making ERK a focus of intense investigation for therapeutic targeting (Benson et al., 2006). On the other hand, JNK and p38 inhibitors are mainly studied as a therapy for inflammatory diseases (Dominguez et al., 2005; Hynes and Leftheri, 2005), although JNK inhibitors have also been explored for the treatment of vascular, neurodegenerative, metabolic, and oncological diseases (Manning and Davis, 2003). During the past few years, the understanding of the Ras/Raf/MEK/ERK signaling in HNSCC has increased (Das et al., 2000; Mishima et al., 2002; Yamatodani et al., 2009; Zhang et al., 2008); therefore, molecular therapies targeting MAPK/ERK are likely to emerge for the treatment of HNSCC.

In summary, the knowledge of the complex signaling network in cancer has

increased significantly leading to the discovery of novel molecular targets for drug development. Emphasis is given on targeting multiple signaling pathways for more effective disease management. Therefore, a better understanding of the crosstalk between signaling pathways involved in HNSCC will provide the opportunity for the development of novel strategies for targeting multiple molecular components for more effective prevention and treatment of HNSCC (Matta and Ralhan, 2009).

Cytokines and growth factors

Cytokines and growth factors play pivotal roles in a variety of biological responses, including the immune response, hematopoiesis, neurogenesis, embryogenesis, and oncogenesis (Heldin, 1995; Hibi and Hirano, 1998; Hirano, 1998; Hunter, 1997). They exert their effects through specific receptors, and activate several signaling pathways through distinct regions of each receptor's cytoplasmic domain, including STAT3, PI3K, and MAPK (Birge et al., 1996; Darnell, 1997; Heldin, 1995; Heldin et al., 1997; Hirano et al., 1997; Hunter, 1997; Ihle, 1996; Taniguchi, 1995).

Cytokines are low molecular weight, soluble proteins, that generally act over short distances, within narrow time frames at very low concentrations and must therefore be produced de novo in response to a stimulus (Pries and Wollenberg, 2006). They are known to possess pleiotropic as well as redundant characteristics, which means that a particular cytokine can influence various cell types as well as different cytokines can share the same function. Furthermore cytokines are able to function in an antagonistic or synergistic manner by inhibiting or stimulating particular functions of other cytokines,

respectively. Thus, a highly complex network of cytokine interactions exists *in vivo* (Rakesh and Agrawal, 2005; Taniguchi, 1995).

Cytokines and growth factors are produced by various cell types including cells of the immune system, endothelial cells, epithelial cells, and fibroblasts, and deregulation in this network lead to several diseases (Bendall, 2005; Yan et al., 2006). HNSCC cells develop molecular strategies in order to evade growth inhibitory effects of cytokines present in the tumor microenvironment; thus, the malignant transformation process is strongly associated with an altered response to cytokine stimulation (Douglas et al., 2004; Pries and Wollenberg, 2006). Several cytokines and growth factors are highly secreted in HNSCC including IL-4, IL-6, IL-8 (CXCL8), IL-10, prostaglandin E_2 (PGE_2), granulocyte macrophage-colony-stimulating factor (GM-CSF), VEGF, EGF, and bFGF (Chen et al., 1999; Eisma et al., 1997; Mann et al., 1992; Wilson et al., 2009).

IL-6 is a multifunctional cytokine that is important for immune responses, cell survival, apoptosis, and proliferation (Kishimoto, 2005), and it plays an important role in the pathophysiology of cancer (Hodge et al., 2005). IL-6 signals via a heterodimeric IL-6R/gp130 complex, whose engagement triggers mainly the activation of JAK/STAT pathway, although it can also activate PI3K/Akt and Ras/MAPK (Kishimoto, 2005). Several studies describe a tumorigenic role of IL-6 and its major effector, STAT3, in many cancers, including breast, lung, colon, prostate, ovarian, melanoma, and hematological cancers (Hodge et al., 2005). Notably, recent studies have demonstrated a correlation between high levels of IL-6 and poor prognosis of HNSCC patients (Chakravarti et al., 2006; Duffy et al., 2008; Kanazawa et al., 2007; Riedel et al., 2005). In addition, IL-6 has been shown to directly influence the proliferation and the invasion

potential of HNSCC cells (Kanazawa et al., 2007). Given the importance of IL-6/gp130/IL-6R/STAT3 signaling in HNSCC, IL-6 may be an attractive target for HSCCC therapy.

Chemokines compose a large family of inflammatory cytokines. CXCL8 is a chemokine of the Glu-Leu-Arg (ELR)+ CXC family with potent angiogenic effects (Strieter et al., 2006). CXCL8 activates multiple intracellular signaling pathways downstream of two cell-surface, G protein-coupled receptors, CXCR1 and CXCR2 (Holmes et al., 1991; Murphy and Tiffany, 1991). Increased expression of CXCL8 and/or its cognate receptors has been characterized in cancer cells, endothelial cells, infiltrating neutrophils, and tumor-associated macrophages, suggesting that CXCL8 may function as a significant regulatory factor within the tumor microenvironment (Waugh and Wilson, 2008). CXCL8 has been demonstrated to activate PI3K/Akt (Cheng et al., 2008), MAPK/ERK (Venkatakrishnan et al., 2000), and STAT3 (Burger et al., 2005) pathways. As a consequence of the diversity of effectors and downstream targets, CXCL8 signaling promotes angiogenic responses in endothelial cells, increases proliferation and survival of endothelial and cancer cells, and potentiates the migration of cancer cells, endothelial cells, and infiltrating neutrophils at the tumor site (De Larco et al., 2004; Li et al., 2003). Accordingly, CXCL8 expression correlates with the angiogenesis, tumorigenicity, and metastasis of tumors in numerous xenograft and orthotopic *in vivo* models (Bendre et al., 2005; Huang et al., 2002; Inoue et al., 2000; Karashima et al., 2003). Thus, inhibiting the effects of CXCL8 signaling may be a significant therapeutic intervention in targeting HNSCC.

In addition to cytokines, growth factors represent a subset of extracellular stimuli from the surrounding microenvironment that regulates cell proliferation, differentiation, migration, and survival. Soluble growth factor peptide ligands bind to their cognate receptors and initiate a cascade of intracellular events. Epidermal growth factor (EGF) is the prototypic member of the EGF family, and is a key regulatory factor in promoting cell survival (Jost et al., 2000). EGF binds to a family of receptors (ErbB receptor family) and illicits specific cellular response through signal transduction pathways. These signaling pathways are regulated by the amount of EGF present, the expression level of ErbB receptors, and the presence of both positive and negative signaling regulators, indicating the complexity and specificity of EGF signaling (Burgess et al., 2003; Herbst, 2004). The ErbB family consists of four closely related tyrosine kinase transmembrane receptors: ErbB1/EGFR, ErbB2/HER2/neu, ErbB3/HER3, and ErbB4/HER4 that sense their microenvironment by binding to an array of extracellular ligands (Normanno et al., 2005). EGF binds exclusively to EGFR, which forms homo- or heterodimers with the other three ErbB receptors (Muthuswamy et al., 1999; Olayioye et al., 2000). This dimerization activates the tyrosine kinase domain through phosphorylation of the receptors, which ultimately leads to phosphorylation of serine or threonine residues on other protein kinases and/or transcription factors (Olayioye et al., 2000). This kinase cascade leads to amplification of a network of signaling pathways resulting in changes in protein function and activation of gene transcription (Henson and Gibson, 2006). Among the signaling pathways activated by EGF signaling are STAT3 (Leaman et al., 1996), PI3K/Akt (Gibson et al., 2002), and MAPK/ERK (Johnson et al., 2005). In cancer, intracellular signaling pathways regulated by EGF are often altered. The constitutively

activated STAT3 in cancer cells is mainly attributed to deregulation of upstream pathways, such as activation of EGFR, which elevates EGF-induced transcriptional activity, and cell survival (Olayioye et al., 1999). EGF-mediated deregulation of the PI3K/Akt pathway is present in approximately 25% of solid tumors (Sansal and Sellers, 2004). Moreover, EGF-mediated activation of ERK signaling is deregulated through mutations in Ras in many cancers (Duursma and Agami, 2003). Since the EGF signaling network can be deregulated at many levels during cancer progression, it also makes the EGF pathway a potential target for HNSCC therapy.

The understanding of the complex network and interaction of cytokines, chemokines, and growth factors in HNSCC may provide new therapeutic targets for the treatment of HNSCC patients. Cells from the tumor microenvironment play a key role in the malignant progression since they secrete high levels of cytokines and growth factors, making them another potential target for HNSCC therapy.

Tumor microenvironment

Increasing evidence shows that the interaction between cancer cells and the surrounding stroma is a critical factor in tumor growth (Anton and Glod, 2009). As the understanding of cancer biology continues to expand, there is a tendency to treat tumors as a functional organ, composed of various cell types and molecules that interact in a dynamic and interdependent manner. The recognition of the tumor as a complex organ may lead to more specific and elegant methods of treating cancer that may minimize toxicity and improve patient survival (Hanna et al., 2009).

In addition to tumor cells, the tumor microenvironment is composed of endothelial cells, pericytes, invading inflammatory cells and leucocytes, fibroblasts, and extracellular matrix (ECM) components. Recent studies demonstrated that the tumor stroma does not exist simply as a passive support structure, but rather plays an active role in tumor progression (Blavier and Declerck, 2005; Derynck et al., 2001; Shekhar et al., 2001). In fact, the activation of autocrine and paracrine signaling pathways by stroma-derived cytokines and growth factors has been implicated in promoting tumor cell proliferation and metastasis (Aharinejad et al., 2009). Therefore, elements of the tumor microenvironment are promising targets for novel therapies that may overcome many of the limitations of current treatment options that are primarily targeted to the cancer cell. Combinatorial therapy that targets multiple components of the tumor microenvironment may allow for lower doses of each individual agent, and may overcome compensatory escape mechanisms that tumors usually rely upon for survival (Blansfield et al., 2008; Dorrell et al., 2007; Mizukami et al., 2005). In addition, anticancer agents directed at the tumor stroma, which is genetically more stable than tumor cells themselves, may exhibit less associated toxicity and drug resistance (Hanna et al., 2009). Although, recent studies in several experimental models suggest that both tumor and stromal cells may be involved in the reduced responsiveness to the treatments, as a consequence of acquired resistance to antiangiogenic drugs, similar to other anticancer therapies (Shojaei and Ferrara, 2008).

Several unique targets for cancer therapy are present in the tumor microenvironment. Recent studies of endothelial cells (Arap et al., 2002; Benezra et al., 2001; Hoffman et al., 2003; Joyce et al., 2003; Trepel et al., 2002), and stromal

fibroblasts (Bhowmick et al., 2004a; Bhowmick et al., 2004b; Park et al., 1999) have identified molecular differences between nontumor cells in the microenvironment of preneoplastic lesions compared to tumors. These proteins themselves could be therapeutically targeted or used as "zip codes" to specifically deliver drugs to these cells. Thus, targeting selectively modified cells in the tumor microenvironment, would not affect their unmodified precursors in normal tissues (Joyce, 2005). Finally, the ultimate goal of targeting tumor-stroma interactions is to develop improved therapies that translate into prolonged survival for the HNSCC patients (Hanna et al., 2009).

Statement of purpose

More than half a million new cases of HNSCC are diagnosed each year worldwide. Despite extensive basic and clinical research, the 5-year survival rate for these patients is 50%, one of the lowest among major cancers, and has not improved significantly over the past three decades. Molecular targeted therapy is a promising approach to treat HNSCC, and it has been shown to improve overall patient survival. Although recent drugs are proven to have a positive effect in the treatment of cancer, the benefits are still modest, the prices are extremely high, these agents have many side effects, and the tumors may acquire drug resistance. Therefore, discovery of novel therapeutic targets may improve clinical outcomes specially if combined with existing therapies.

Tumor angiogenesis is crucial for cancer progression and requires active interaction between endothelial cells and tumor cells. It is well established that cancer

cells secrete angiogenic factors to recruit and sustain tumor vascular networks. However, little is known about the effect of endothelial cell-secreted factors on the phenotype and behavior of tumor cells. The identification and characterization of the signaling events initiated by tumor-associated endothelial cells may have important implications in cancer therapy. Emerging knowledge has shed light on signaling cascades that regulate HNSCC progression. Among them, STAT3, PI3K/Akt, and MAPK/ERK pathways are known to play critical roles in the control of cell cycle, survival, proliferation, and migration of tumor cells. However, the impact of endothelial cell-initiated signaling on the activation of the STAT3, Akt, and ERK pathways in HNSCC is unclear. In this dissertation we will explore molecular signaling events in HNSCC cells initiated by endothelial cells and the biological effects of this crosstalk.

In chapter II, we demonstrated that factors secreted by endothelial cells namely IL-6, CXCL8, and EGF activate STAT3, Akt, and ERK signaling pathways in HNSCC cells, increasing tumor cell survival and migration. In chapter III we explored in more depth this network of signaling events in HNSCC initiated by endothelial cells. More specifically, we demonstrated that downregulation of IL-6 in tumor-associated endothelial cells is sufficient to inhibit tumor growth.

Hypothesis

The central hypothesis addressed in this dissertation is that factors secreted by endothelial cells initiate signaling pathways in head and neck squamous cell carcinoma cells that enhance tumor growth.

Specific Aims

This central hypothesis will be tested by addressing the following specific aims:

- **Specific Aim 1:** To evaluate the impact of endothelial cell-secreted factors on the gene expression profile of head and neck squamous cell carcinoma cells.

- **Specific Aim 2:** To study molecular signaling events initiated by endothelial cells that result in the activation of STAT3, Akt, and ERK signaling pathways in head and neck squamous cell carcinoma cells.

- **Specific Aim 3:** To study the effect of endothelial cell-derived IL-6 on head and neck squamous cell carcinoma growth and microvessel density.

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CHAPTER II

CROSSTALK INITIATED BY ENDOTHELIAL CELLS ENHANCES MIGRATION AND INHIBITS ANOIKIS OF SQUAMOUS CELL CARCINOMA CELLS THROUGH STAT3/AKT/ERK SIGNALING

Abstract

It is well known that cancer cells secrete angiogenic factors to recruit and sustain tumor vascular networks. However, little is known about the effect of endothelial cell-secreted factors on the phenotype and behavior of tumor cells. The hypothesis underlying this study is that endothelial cells are able to initiate signaling pathways in tumor cells. Here, we observed that soluble mediators from primary human dermal microvascular endothelial cells (HDMEC) induce phosphorylation of STAT3, Akt, and ERK in a panel of head and neck squamous cell carcinoma (HNSCC) cells (OSCC3, UM-SCC-1, UM-SCC-17B, UM-SCC-74A). Gene expression analysis demonstrated that interleukin-6 (IL-6), interleukin-8 (CXCL8), and epidermal growth factor (EGF) are upregulated in endothelial cells co-cultured with HNSCC. Blockade of endothelial cell-derived IL-6, CXCL8, or EGF by gene silencing or with neutralizing antibodies inhibited the activation of STAT3, Akt, and ERK in tumor cells, respectively. It has been demonstrated that Bcl-2 is upregulated in tumor microvessels in patients with HNSCC. Here, we observed that Bcl-2 signaling induces expression of IL-6, CXCL8, and EGF, providing a mechanism

for the upregulation of these cytokines in tumor-associated endothelial cells. This study expands the contribution of endothelial cells to the pathobiology of tumor cells. It unveils a new mechanism in which endothelial cells function as the initiators of a molecular crosstalk that enhances the survival and promotes the migration of tumor cells.

Introduction

Tumor angiogenesis requires active interaction between endothelial and tumor cells, and plays an important role in cancer progression (Folkman, 2002). However, emphasis has been placed on the assumption that tumor cell-initiated signals are the dominant events in tumor angiogenesis and tumor growth. Recent studies have demonstrated signaling crosstalks between tumor cells and endothelial cells that promote tumor growth. In cell contact-dependent interactions, Jagged1 expressed by squamous cell carcinoma (SCC) cells activates Notch signaling in adjacent endothelial cells and enhances tumor growth (Zeng et al., 2005). In a cell contact-independent system, vascular endothelial growth factor (VEGF) secreted by tumor-associated endothelial cells induces Bcl-2, growth-related oncogene (GRO)-α (CXCL1), and interleukin-8 (CXCL8) expression in SCC cells (Kaneko et al., 2007). Notably, these endothelial cell-initiated signals significantly enhanced tumor growth *in vivo* (Kaneko et al., 2007). Collectively, this work indicates that the impact of endothelial cells in the pathobiology of cancer is not limited to making angiogenic blood vessels in response to tumor cell-initiated signals. The identification and characterization of the signaling events initiated by tumor-associated endothelial cells may have important implications in human cancer therapy.

Squamous cell carcinoma is a common cancer in the gastro-intestinal track, lung, skin, and head and neck region. For example, more than 500,000 new patients with head and neck squamous cell carcinomas (HNSCC) are diagnosed each year worldwide (Chin et al., 2006; Jemal et al., 2008). The 5-year survival rate for patients with HNSCC is one of the lowest among major cancer sites, and has not improved significantly over the last 30 years despite extensive basic and clinical research (Forastiere et al., 2001; Jemal et al., 2008; Mao et al., 2004). Today, we know that several intracellular signaling molecules are key orchestrators of SCC progression. Among them, the signal transducer and activator of transcription 3 (STAT3) has been described to be constitutively active in HNSCC and in several other epithelial malignancies (Grandis et al., 1998; Leeman et al., 2006). Altered expression or mutation of components of the phosphoinositol 3-kinase (PI3K)/Akt pathway has also been implicated in tumorigenesis (Luo et al., 2003; Vivanco and Sawyers, 2002). In addition, the mitogen-activating protein kinase (MAPK) and extracellular signal-regulated kinase (ERK) pathway has been described as an important regulator of tumor growth and a key target for cancer therapy (Friday and Adjei, 2008; Roberts and Der, 2007). These three signaling molecules, *i.e.* STAT3, Akt, and ERK, play critical roles in the control of cell cycle, survival, proliferation, and migration of tumor cells. Notably, deregulation of any of these pathways has been shown to drive oncogenic transformation (Chang et al., 2003; Song and Grandis, 2000; Zeng et al., 2002). However, the impact of endothelial cell-initiated signaling on the activation of STAT3, Akt, and ERK signaling in tumor cells is unclear.

It is known that vascular endothelial growth factor (VEGF) induces Bcl-2 expression in endothelial cells, and that Bcl-2 expression level in tumor-associated

endothelial cells is directly correlated with tumor angiogenesis and tumor growth (Kaneko et al., 2007; Nor et al., 1999; Nor et al., 2001a). Notably, Bcl-2 gene expression is approximately 60,000-fold higher in the endothelial cells lining tumor blood vessels in patients with HNSCC, as compared to the endothelial cells from normal oral mucosa (Kaneko et al., 2007). It is also known that Bcl-2 induces CXC chemokines expression by endothelial cells, which results in enhanced HNSCC invasiveness and local recurrence (Karl et al., 2005; Warner et al., 2008). It has been shown that Bcl-2 signals through nuclear factor kB (NF-kB) to induce CXCL1 and CXCL8 expression in endothelial cells (Karl et al., 2005). However, the effect of Bcl-2 in the expression of IL-6 (known to activate the STAT3 signaling pathway) and EGF (known to activate the ERK signaling pathway) is unknown. In this study, we unveil a signaling pathway that is initiated by endothelial cells and that results in the activation of STAT3, Akt, and ERK in HNSCC cells. Notably, blockade of endothelial cell-initiated signaling had a direct impact on tumor cell survival and migration.

Materials and Methods

Cell Culture

Oral squamous cell carcinoma (OSCC3, gift of M. Lingen, University of Chicago), University of Michigan-squamous cell carcinoma (UM-SCC-1, UM-SCC-17B, and UM-SCC-74A, gift of T. Carey, University of Michigan) were cultured in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin

(Invitrogen). Primary human dermal microvascular endothelial cells (HDMEC; Cambrex, Walkersville, MD) were cultured in endothelial growth medium-2 (EGM2-MV; Cambrex). Pools of HDMEC stably transduced with Bcl-2 (HDMEC-Bcl-2) and HDMEC-LXSN (empty vector controls) were generated with retroviruses and cultured in EGM2-MV supplemented with 250 μg/ml G418 (Cellgro, Mediatech Inc., Herndon, VA), as described (Nor et al., 1999; Nor et al., 2001a). Conditioned medium (CM) from HDMEC was prepared in endothelial cell medium (EBM) without supplementation with growth factors or serum from 24-hour cultures.

Non-contact co-culture assays

2 x 10^5 tumor cells (OSCC3, UM-SCC-17B, UM-SCC-1, or UM-SCC-74A) were plated in 1 μm-pore cell culture inserts containing PET (polyethylene terephthalate) track-etched membranes (Becton Dickinson, Franklin Lakes, NJ) in 6-well plates (Multiwell; Becton Dickinson). HDMEC (1 x 10^5) were cultured in the bottom wells of the Transwell system. Serum-free endothelial basal medium (EBM) or EGM2-MV was used for these co-culture experiments. Total RNA and protein from both endothelial and tumor cells was collected 24 hours after cells were combined, and microarrays or western blots were performed.

Stable short hairpin RNA (shRNA) transduction

Lentiviruses expressing a short hairpin RNA (shRNA) construct for silencing IL-6, CXCL8, or EGF (Vector Core, University of Michigan) were generated in human embryonic kidney cells (293T) transfected by the calcium phosphate method, as

described (Kaneko et al., 2007). A scrambled oligonucleotide sequence (shRNA-C) was used as control. Supernatants were collected 48 hours after transfection and used to infect HDMEC in 1:1 dilution medium containing 4 μg/ml polybrene (Sigma-Aldrich, St. Louis, MO). Cells were selected and maintained in EGM2-MV supplemented with 1 μg/ml puromycin (InvivoGen, San Diego, CA). Downregulation of IL-6, CXCL8, or EGF expression was confirmed by ELISA.

Western blots

8 x 10^5 OSCC3, UM-SCC-1, UM-SCC-17B, or UM-SCC-74A were plated in 60 mm dishes, starved overnight, and exposed to HDMEC conditioned medium (CM) or EBM for the indicated time points. Alternatively, tumor cells were exposed to HDMEC CM containing 0.1-2 μg/ml anti-IL-6, anti-CXCL8, anti-EGF, or IgG isotype control (R & D Systems); or CM collected from HDMEC-shRNA-IL-6, HDMEC-shRNA-CXCL8, HDMEC-shRNA-EGF, or HDMEC-shRNA-C. Tumor cells were also exposed to 1-50 ng/ml rhIL-6 (Biosource, Camarillo, CA), 1-100 ng/ml rhCXCL8, or 1-100 rhEGF (R & D Systems, Minneapolis, MN) for 30 minutes. Signaling pathways were blocked by pre-incubating tumor cells for 1 to 2 hours with 0.5-20 μM Stattic (STAT3 inhibitor V, Calbiochem, San Diego, CA), 5-100 μM LY294002 (PI3 kinase inhibitor, Cell Signaling Technology, Danvers, MA), or 5-100 μM U0126 (MEK1/2 inhibitor, Cell Signaling), and exposed to HDMEC CM. Protein (30 μg) was electrophoresed on SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Primary antibodies were: mouse anti-human phospho-STAT3, rabbit anti-human STAT3, rabbit anti-human phospho-Akt, rabbit anti-human Akt, rabbit anti-human phospho-ERK1/2, mouse anti-human ERK1/2

(Cell Signaling); rabbit anti-human VEGFR1, rabbit anti-human IL-6R, rabbit anti-human EGFR (Santa Cruz Biotechnology, Santa Cruz, CA); mouse anti-human gp130 (Biosource, Camarillo, CA); mouse anti-human CXCR1, rabbit anti-human CXCR2 (Abcam, Cambridge, MA); hamster anti-human Bcl-2 (BD Biosciences, San Jose, CA); mouse anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Chemicon, Millipore, Billerca, MA). Immunoreactive proteins were visualized by SuperSignal West Pico chemiluminescent substrate (Thermo Scientific, Rockford, IL).

GEArray RNA Microarrays

Gene expression of tumor cells (OSCC3, UM-SCC-17B, UM-SCC-1A, or UM-SCC-74A) co-cultured with HDMEC was compared with tumor cells cultured alone using GEArray Q serties (SuperArray Bioscience Corporation, Frederick, MD). RNA was purified using the RNeasy kit (Qiagen, Valencia, CA) and 3 μg total RNA was analyzed using Human Signaling Transduction in Cancer and Human Angiogenesis gene arrays, according to the manufacturer's instructions.

Affymetrix Microarray

Total RNA was isolated from HDMEC cultured alone or co-cultured with OSCC3 using Trizol (Invitrogen, Carlsbad, CA). The RNA was purified using the RNeasy kit (Qiagen, Valencia, CA) and 10 μg total RNA was used to perform microarray analysis on Human Genome U133 Plus 2.0 (Affymetrix, Santa Clara, CA). Briefly, cDNA was synthesized from total RNA (One-Cycle cDNA Synthesis Kit, Affymetrix) and in vitro transcription was followed to make biotin labeled cRNA (IVT Labeling Kit, Affymetrix).

Fragmented cRNA was hybridized with microarray. After microarray washing, staining, and scanning, data was collected and further analyzed using Data Mining software and NetAffx Analysis Center (Affymetrix).

Enzyme-linked immunosorbent assay (ELISA)

Supernatants of 24-hour endothelial or tumor cell cultures were collected and centrifuged to eliminate debris. IL-6, CXCL8, and EGF expression was determined using ELISA kits (Quantikine; R & D Systems, Minneapolis, MN) according to the manufacturer's instructions. Data were normalized by cell number.

Sulforhodamine B Cell Proliferation Assays

OSCC3 (2×10^3) were seeded in 96-well plates (Becton Dickinson) and exposed to EBM, HDMEC conditioned medium (CM) containing 1 μg/ml anti-IL-6, anti-CXCL8, anti-EGF neutralizing antibodies, or IgG isotype control. Alternatively, tumor cells were exposed to CM from HDMEC-shRNA-IL-6, HDMEC-shRNA-CXCL8, HDMEC-shRNA-EGF, or HDMEC-shRNA-C. Tumor cells were also exposed to HDMEC CM containing 5 μM Stattic (Calbiochem), 10 μM LY294002, 10 μM U0126 (Cell Signaling), or DMSO vehicle control. After 24, 48, or 72 hours, cells were fixed with 10% trichloracetic acid and stained with 0.4% sulforhodamine B (SRB) solution as previously described (Zeitlin et al., 2006). Plates were read in a microplate reader at 565 nm (TECAN, Salzburg, Austria).

Migration Assays

HDMEC conditioned medium (CM) was pre-incubated with 1 μg/ml anti-IL-6, anti-CXCL8, anti-EGF, or IgG control (R & D Systems) for 1 hour, and 400 μl of CM was added to 24-well companion plates (Fisher Scientific, Pittsburgh, PA). Alternatively, CM collected from HDMEC-shRNA-IL-6, HDMEC-shRNA-CXCL8, HDMEC-shRNA-EGF, or HDMEC-shRNA-C was used. OSCC3 (2×10^5) were serum-starved overnight, loaded onto 8 μm pore-sized cell culture inserts (Becton Dickinson), and allowed to migrate for 24 hours towards HDMEC CM. In addition, OSCC3 were pre-incubated for 1 to 2 hours with 5 μM Stattic (Calbiochem), 10 μM LY294002, 10 μM U0126 (Cell Signaling), or DMSO vehicle control. Unconditioned EBM was used as negative control. Migrated cells were trypsinized, collected, and stained with 2 μM Cell Tracker Green (Invitrogen) for 1 hour. Fluorescence was read at 485/535 nm in a microplate reader (Tecan, Salzburg, Austria).

Survival Assays

OSCC3 (3×10^5) were seeded in 6-well ultra-low attachment plates (Corning Incorporated, Corning, NY) using EBM, HDMEC conditioned medium (CM) containing 1 μg/ml anti-IL-6, anti-CXCL8, anti-EGF, or IgG control (R & D Systems); CM from HDMEC-shRNA-IL-6, HDMEC-shRNA-CXCL8, HDMEC-shRNA-EGF, or HDMEC-shRNA-C; or HDMEC CM containing 5 μM Stattic (Calbiochem), 10 μM LY294002, 10 μM U0126 (Cell Signaling), or DMSO vehicle control. OSCC3 seeded in regular 6-well plates (Becton Dickinson) were used as positive control. After 24 hours, propidium iodide staining followed by flow cytometry was used to determine the percentage of dead cells, as described (Nor et al., 2002). In addition, percentage of apoptotic cells was

determined by Trypan blue (Gibco, Invitrogen Corporation, Grand Island, NY) exclusion method after 24 to 48 hours.

Statistical analyses

T-tests or one-way analysis of variance was performed using the SigmaStat 2.0 software (SPSS; Chicago, IL). Statistical significance was determined at $p<0.001$.

Results

Endothelial cell-secreted factors induce phosphorylation of STAT3, Akt, and ERK in head and neck squamous cell carcinoma cells

The overall hypothesis underlying this study is that endothelial cell-secreted factors initiate signaling pathways in tumor. To begin to address this hypothesis, we exposed a panel of head and neck tumor cells, *i.e.* OSCC3 (Figure 2.1A), UM-SCC-17B (Figure 2.1B), UM-SCC-1 (Figure 2.1C), or UM-SCC-74A (Figure 2.1D) to serum-free endothelial cell (HDMEC) conditioned medium (CM) and analyzed the phosphorylation levels of key signaling molecules over time. We observed that STAT3, Akt, and ERK were consistently phosphorylated in the tumor cells upon exposure to endothelial cell CM, as compared to tumor cells treated with unconditioned medium (Figure 2.1). The induction of phosphorylation by endothelial cell CM was observed primarily between 15 minutes and 1 hour, decreasing after 4 to 24 hours as expected. The four tumor cell lines that we tested showed different phosphorylation levels upon exposure to HDMEC CM, but the trends among the cell lines were similar. To verify the results of the CM

experiments with a second experimental model, we used a non-contact co-culture system with HDMEC and HNSCC cells. Within one hour, STAT3, Akt, and ERK phosphorylation was enhanced in OSCC3 co-cultured with HDMEC, as compared to OSCC3 in single culture (Figure 2.2A). Notably, even after 24 hours in co-culture the phosphorylation status of STAT3 was still upregulated in two (out of four) HNSCC cell lines tested here (Figure 2.2B). Collectively, these results showed that endothelial cell-secreted factors induce STAT3, Akt, and ERK phosphorylation in HNSCC cells.

IL-6, CXCL8, and EGF are upregulated in endothelial cells co-cultured with head and neck squamous cell carcinoma cells

In search for putative factors secreted by endothelial cells that could lead to STAT3, Akt, and ERK phosphorylation, we performed an Affymetrix microarray comparing the gene expression profile of HDMEC cultured alone with HDMEC co-cultured with OSCC3. We observed that IL-6, CXCL8, and EGF were upregulated in HDMEC when co-cultured with OSCC3 (Figure 2.3A). We then analyzed whether the tumor cells express the receptors for IL-6, CXCL8, and EGF. All tumor cells tested here express IL-6R and gp130 (receptors for IL-6); CXCR1 and CXCR2 (receptors for CXCL8); and EGFR (receptor for EGF) (Figure 2.3B). We selected OSCC3 cells for most of the remaining experiments presented here, and used a second HNSCC cell line to verify reproducibility of results of key experiments. Next, we compared the expression levels of IL-6, CXCL8, and EGF in endothelial and tumor cells. Since it is known that HNSCC cells secret high levels of VEGF (Eisma et al., 1997), we exposed HDMEC to VEGF and evaluated the impact of this treatment on the expression of IL-6, CXCL8, and

EGF. Notably, the expression of IL-6 and CXCL8, but not EGF, was upregulated in endothelial cells treated with VEGF (Figure 2.3C). We observed that IL-6 induced phosphorylation of STAT3, CXCL8 enhanced phosphorylation of Akt and ERK compared with untreated group (first lane), and EGF enhanced phosphorylation of STAT3, Akt, and ERK (Figure 2.3D). We also exposed OSCC3 to the combination of IL-6, CXCL8, and EGF and observed similar signaling trends as compared to CM treatment (Figure 2.3E)

Effect of endothelial cell-secreted IL-6, CXCL8, and EGF on the phosphorylation of STAT3, Akt, and ERK in head and neck squamous cell carcinoma cells

To evaluate the role of these cytokines on endothelial cell-initiated signaling pathways in tumor cells, we exposed OSCC3 to HDMEC CM in the presence of neutralizing antibodies to IL-6, CXCL8, or EGF, and analyzed phosphorylation of STAT3, Akt, and ERK. Blockade of IL-6 in HDMEC CM blocked STAT3 phosphorylation in OSCC3 but had no effect on the phosphorylation levels of Akt and ERK, as compared with IgG controls (Figure 2.4A). Blockade of CXCL8 in HDMEC CM slightly decreased phosphorylation levels of STAT3 and Akt, but had no significant effect on ERK (Figure 2.4A). Finally, blockade of EGF in HDMEC CM eliminated ERK phosphorylation in OSCC3 compared with IgG control, while had no effect on STAT3 and Akt phosphorylation levels (Figure 2.4A). To evaluate specifically the role of endothelial cell initiated events, we silenced expression of IL-6, CXCL8, or EGF in HDMEC. The effectiveness of the downregulation was verified by ELISA (Figure 2.4B). OSCC3 were then exposed to CM from HDMEC-shRNA-IL-6, HDMEC-shRNA-

CXCL8, HDMEC-shRNA-EGF, or control HDMEC-shRNA-C. Downregulation of IL-6 and EGF in endothelial cells resulted in inhibition of phosphorylation of STAT3 and ERK in tumor cells, respectively (Figure 2.4C). However, downregulation of CXCL8 in endothelial cells did not result in an observable inhibition of Akt in the tumor cells, presumably because HDMEC-shRNA-CXCL8 cells still secreted substantial amounts of this protein.

Bcl-2 signaling induces IL-6, CXCL8, and EGF expression in endothelial cells

To begin to understand the mechanisms involved in the upregulation of IL-6, CXCL8, and EGF in the endothelial cells, we evaluated a potential role for Bcl-2 signaling. Previous work has shown that tumor cell-secreted VEGF induces Bcl-2 expression in endothelial cells, and that upregulation of Bcl-2 in microvascular endothelial cells is sufficient to enhance tumor progression (Nor et al., 1999; Nor et al., 2001a). Notably, Bcl-2 is significantly upregulated in the endothelial cells of head and neck tumor microvessels (Kaneko et al., 2007). We have also shown that Bcl-2 upregulates CXCL1 and CXCL8 expression in endothelial cells through the IKK/I-kB/NF-kB pathway (Karl et al., 2005), and it is known that NF-kB directly stimulates the transcription of several cytokines and growth factors (Karin et al., 2002). These observations led to the hypothesis that Bcl-2 induces expression of IL-6 and EGF in endothelial cells. To test this hypothesis, we generated pools of endothelial cells overexpressing Bcl-2 (HDMEC-Bcl-2) and empty vector controls (HDMEC-LXSN) (Figure 2.5A). HDMEC-Bcl-2 secreted significantly higher levels of IL-6, CXCL8, and EGF than HDMEC-LXSN (Figure 2.5B). Next, we analyzed phosphorylation levels of

STAT3, Akt, and ERK in OSCC3 when exposed to CM collected from HDMEC-LXSN and HDMEC-Bcl-2. At initial time points (*i.e.* 15 minutes) the phosphorylation levels induced by HDMEC-LXSN and HDMEC-Bcl-2 CM were similar (Figure 2.5C). However, at later time points, phosphorylation of STAT3 (4 and 24 hours) and ERK (4 hours) was higher when OSCC3 cells were exposed to HDMEC-Bcl-2 CM than in OSCC3 exposed to HDMEC-LXSN CM (Figure 2.5C). Collectively, these results showed that Bcl-2 signaling induces expression of IL-6, CXCL8, and EGF in endothelial cells. Moreover, Bcl-2 expression levels in the endothelial cells had a significant impact on the phosphorylation status of STAT3 and ERK, but not Akt, in the tumor cells.

Endothelial cell-secreted factors enhance the motility and prevent anoikis of head and neck squamous cell carcinoma cells

To understand the biological impact of endothelial cell-induced tumor cell STAT3, Akt, and ERK phosphorylation, we exposed the OSCC3 to HDMEC CM containing inhibitors of these pathways and evaluated tumor cell proliferation, survival, and migration. We used increasing concentrations of Stattic (STAT3 inhibitor), LY294002 (PI3K/Akt inhibitor), or U0126 (MEK/ERK inhibitor) (Figure 2.6A), and selected the lowest dose able to inhibit activation of STAT3, Akt, and ERK to carry out these experiments. Viability experiments showed that these dosages were not cytotoxic (data not shown). No difference was observed in HNSCC proliferation when cells were exposed to HDMEC CM for up to 72 hours, as compared to exposure to unconditioned medium. Blockade of IL-6, CXCL8, or EGF in the CM using neutralizing antibodies (Figure 2.7A) or shRNA in HDMEC (Figure 2.7B) did not affect HNSCC proliferation.

As expected, direct blockade of STAT3, Akt, or ERK pathways with chemical inhibitors decreased tumor cell proliferation (Figure 2.7C). Notably, HDMEC CM had a significant effect on tumor cell survival. To analyze the effect of endothelial cell derived factors on tumor cell survival we cultured OSCC3 cells in extra low attachment plates. The percentage of dead cells was analyzed by flow cytometry (Figure 2.6) or by cell counting (Figure 2.8). The number of dead cells was significantly higher in low attachment plates in EBM compared to normal cell culture plates (Figure 2.6B; Figure 2.8). CM from HDMEC protected OSCC3 from anoikis induced by preventing the attachment of the tumor cells to the plate (Figure 2.6B; Figure 2.8). Notably, blockade of IL-6, CXCL8, and EGF in the CM using neutralizing antibodies (Figure 2.6B, a; Figure 2.8A, a-b) or gene silencing in HDMEC (Figure 2.6B, b; Figure 2.8B, a-b) inhibited the protective effect mediated by endothelial cells. Blockade of STAT3, Akt, or ERK activity with Stattic, LY294002, or U0126 showed similar results (Figure 2.6B, c; Figure 2.8C, a-b). To evaluate the effect of endothelial cell-secreted IL-6, CXCL8, or EGF on tumor cell migration we also used two experimental strategies. Blockade of IL-6, CXCL8, or EGF with neutralizing antibodies (Figure 2.6C, a), or shRNA in HDMEC (Figure 2.6C, b) inhibited migration of OSCC3, as compared to controls. In addition, blockade of STAT3, Akt, or ERK using Stattic, LY294002, or U0126 also inhibited OSCC3 migration (Figure 2.6C, c). Collectively, these data demonstrate that endothelial cell-initiated signaling has a significant impact on tumor cell survival and migration, two critical elements of the pathobiology of HNSCC.

Discussion

This study suggests a new paradigm for endothelial cell and tumor cell interactions in the tumor microenvironment. We demonstrate here that endothelial cells play an active role in establishing tumor cell phenotypes that are critical for the pathobiology of cancers, namely, tumor cell survival and migration. Several reports suggest a role for inflammation, extracellular matrix, macrophages, and stromal fibroblasts in the initiation and progression of carcinomas (Bhowmick and Moses, 2005; Coussens and Werb, 2002; Halin et al., 2009). However, the role of endothelial cells in activating oncogenic signaling pathways in tumor cells is just starting to be unveiled. Notably, the current paradigm is that tumor cell-initiated signaling are the predominant. Yet, this study provides evidence for endothelial cells as key players in the determination of the tumor cell phenotype.

We observed that endothelial cell-initiated signals activate three key intracellular signaling molecules, namely STAT3, Akt, and ERK in HNSCC. The role of these pathways in cancer has been extensively studied. STAT3 is activated in a wide variety of cancers including HNSCC. Notably, studies have consistently demonstrated an essential role for STAT3 in tumor progression (Buettner et al., 2002; Masuda et al., 2007; Nikitakis et al., 2004). The Akt signaling network is considered a key determinant of the biological aggressiveness of tumors. PI3K/Akt is often activated in HNSCC (Fenic et al., 2007; Opel et al., 2007). And finally, ERK is one of the most important signaling molecules in the regulating cell proliferation and is overexpressed in a variety of tumors, including HNSCC (Mishima et al., 2002). The results of our study suggest that

endothelial cells play an active role in the activation of these pathways in HNSCC. Our results demonstrated that STAT3, Akt, and ERK were phosphorylated in tumor cells without requiring cell contact. These results suggested that endothelial cell-secreted factors were able to activate these signaling pathways in the tumor cells. This is in line with observations from other research groups that have characterized soluble factors as key regulators of tumorigenesis (Balkwill, 2004; van der Horst et al., 2008; Vicari and Caux, 2002). One of the challenges of our study was to find out which factors were mediating these effects in the tumor cells. Affymetrix microarrays revealed several genes upregulated in endothelial cells when co-cultured with tumor cells. Several validation experiments, together with the existing knowledge of the effects of IL-6, CXCL8, and EGF on STAT3, Akt, and ERK signaling, led us to focus on these three soluble mediators. Although previous studies have already shown that endothelial cells overexpressing Bcl-2 secrete high levels CXCL8 (Karl et al., 2005), the role of endothelial cell-secreted IL-6, CXCL8, and EGF on activation of signaling pathways in tumor cells have not been described. IL-6 is a cytokine that affects a variety of biological functions including immune response, inflammation, hematopoiesis, and oncogenesis by regulating cell growth, survival, and differentiation (Hirano et al., 2000; Van Snick, 1990). IL-6 is one of the major activators of STAT3 signaling (Hirano et al., 2000; Hodge et al., 2005; Huang, 2007), although it can also stimulate PI3K/Akt and ERK pathways (Kamimura et al., 2003). Recent studies correlate IL-6 levels in HNSCC patients with poor prognosis (Duffy et al., 2008; Heimdal et al., 2008; Squarize et al., 2006). CXCL8 is a member of CXC chemokine family that contributes to cancer progression through several mechanisms, including the promotion of angiogenesis (Koch et al., 1992; Strieter

et al., 1995). Many cancer cells constitutively secrete CXCL8 and its receptors CXCR1 and CXCR2, and it has been established that CXCL8 is an autocrine growth factor for a variety of human cancer cells (Zhu and Woll, 2005). CXCL8 can also activate STAT3, PI3K/Akt, and ERK signaling pathways (Burger et al., 2005; Fuhler et al., 2005). Overexpression of EGFR has been reported in the majority of HNSCC cases (Grandis and Tweardy, 1993; Grandis et al., 1998). Notably, downstream intracellular targets of EGFR include STAT3, PI3K/Akt, and ERK pathways (Kijima et al., 2002; Squarize et al., 2006; Sriuranpong et al., 2003). One concludes from these studies that each one of these three cytokines has effects that may converge towards these three pathways in tumor cells. However, under our experimental conditions we observed that the primary effect of endothelial cell-derived IL-6 was the activation of STAT3, the primary effect of CXCL8 was on the activity of Akt, and the primary effect of EGF was on ERK activity in HNSCC.

Previous work has shown that Bcl-2 expression levels in endothelial cells have a direct effect on tumor growth (Kaneko et al., 2007; Nor et al., 2001a). Here, we demonstrate that IL-6 and EGF are upregulated in endothelial cells overexpressing Bcl-2, and that CM from these cells further enhanced phosphorylation of STAT3 and ERK, two pathways implicated in the survival of tumor cells. Notably, it has been previously demonstrated that the apoptotic index of tumors vascularized with endothelial cells overexpressing Bcl-2 was lower than the index in tumors vascularized with control endothelial cells (Nor et al., 2001a). At that time, the data were interpreted as simply an increase in endothelial cell survival and tumor microvessel density directly due to the upregulation of Bcl-2 expression in these cells. However, the data presented here suggest

that the activation of major survival pathways for SCC mediated by endothelial cell-secreted factors may also have contributed to those results.

It was critical to us to understand the biological significance of endothelial cell-induced activation of the STAT3/Akt/ERK pathways in squamous cell carcinomas. Surprisingly, we did not observe a significant effect in tumor cell proliferation when we blocked endothelial cell derived IL-6, CXCL8, or EGF. We attributed these negative results to the exceedingly high proliferation rate of OSCC3. We reasoned that these cells have such a high basal mitotic activity that blockade of one additional mitogenic pathway derived from the endothelial cells is not sufficient to cause a significant change in OSCC3 cell numbers. On the other hand, we observed that endothelial cell secreted factors significantly protected the tumor cells from anoikis. Notably, this protective effect was partially inhibited when we downregulated IL-6, CXCL8, or EGF in the endothelial cells. And finally, considering that IL-6, CXCL8, and EGF have been characterized as chemotactic factors, we studied their effect on tumor cell motility using migration assays. These experiments demonstrated that endothelial cells are capable of generating a chemotactic gradient that induces tumor cell motility towards the blood vessels. Such findings may help us to understand the mechanisms underlying the process of field cancerization, which involves the lateral spread of malignant disease and is commonly observed in patients with head and neck cancer (Ha and Califano, 2003; Warner et al., 2008). We hypothesize that endothelial cells play a key role in field cancerization by providing chemotactic signals that enhance the invasive phenotype of tumor cells, and by activating survival signals that protect the tumor cells against anoikis once these cells are displaced from their original microenvironment.

Collectively, these data led to a new model for crosstalk between endothelial and SCC cells. It is known that tumor cell-secreted VEGF binds to its cognate receptors in endothelial cells and induce expression of Bcl-2 (Kaneko et al., 2007; Nor et al., 1999). Bcl-2 enhances IL-6, CXCL8, and EGF synthesis and secretion by endothelial cells. The endothelial cell-secreted factors induce activation of the STAT3, Akt, and ERK signaling pathways in tumor cells. The biological outcome of this crosstalk is a significant increase in tumor cell survival and migration. We postulate that better understanding of the complexity of signal transduction processes between tumor cells and other cells from the tumor microenvironment may help to optimize the overall therapeutic benefit of molecularly targeted drugs. The fact that tumor-associated endothelial cells are readily accessible to drugs injected in the circulation makes them a particularly attractive therapeutic target. The work presented here demonstrated that blockade of specific pathways in endothelial cells may have a direct impact on tumor cell survival and migration, two critical components of the pathobiology of squamous cell carcinomas.

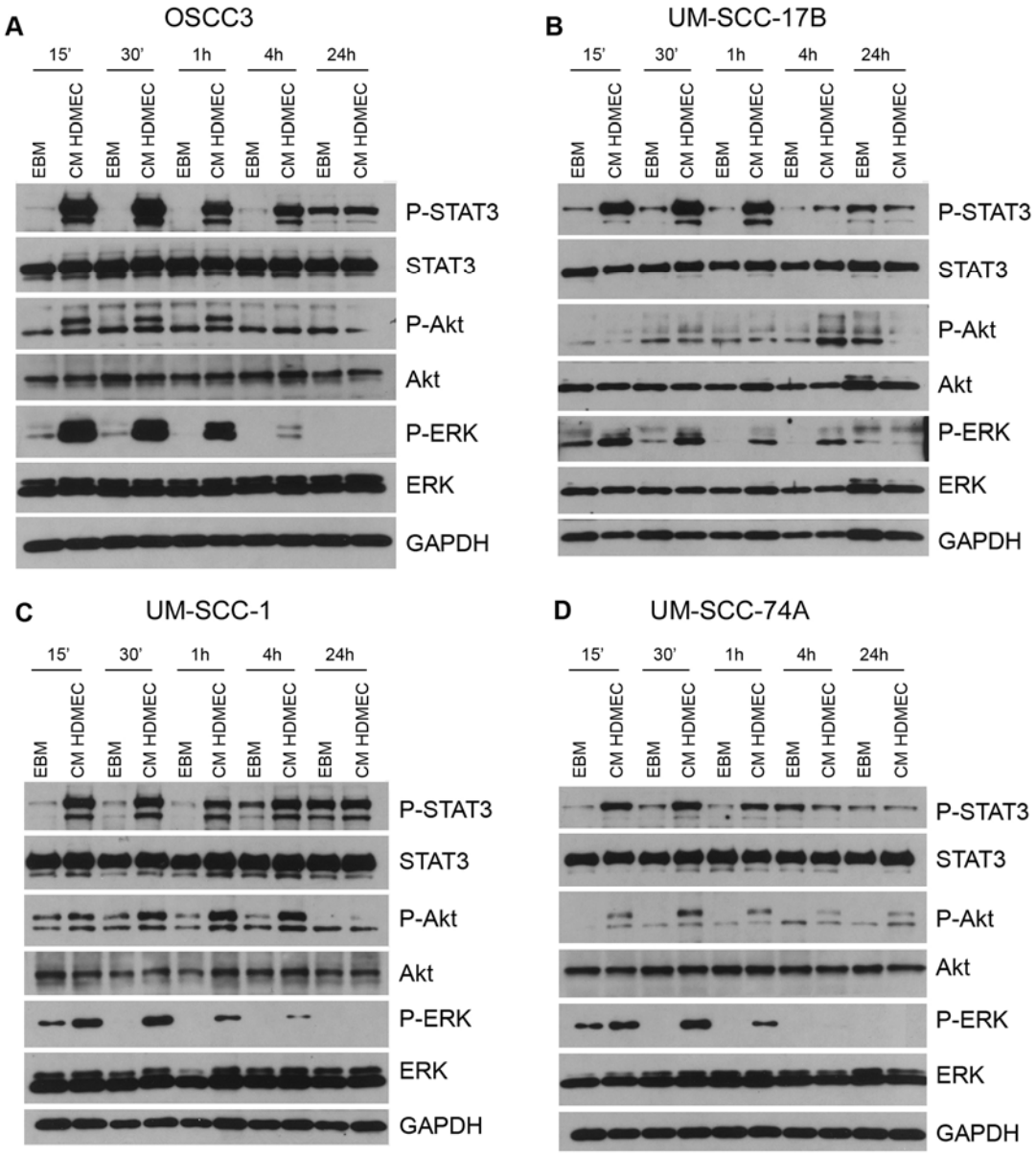

Figure 2.1. Endothelial cell–derived soluble factors activate STAT3, Akt, and ERK pathways in tumor cells. (A) OSCC3; (B) UM-SCC-17B; (C) UM-SCC-1; or (D) UM-SCC-74A were serum-starved overnight and exposed to HDMEC conditioned medium (CM) or control unconditioned medium (EBM) for the indicated time points. Phosphorylated and total STAT3, Akt, and ERK were detected by Western Blot.

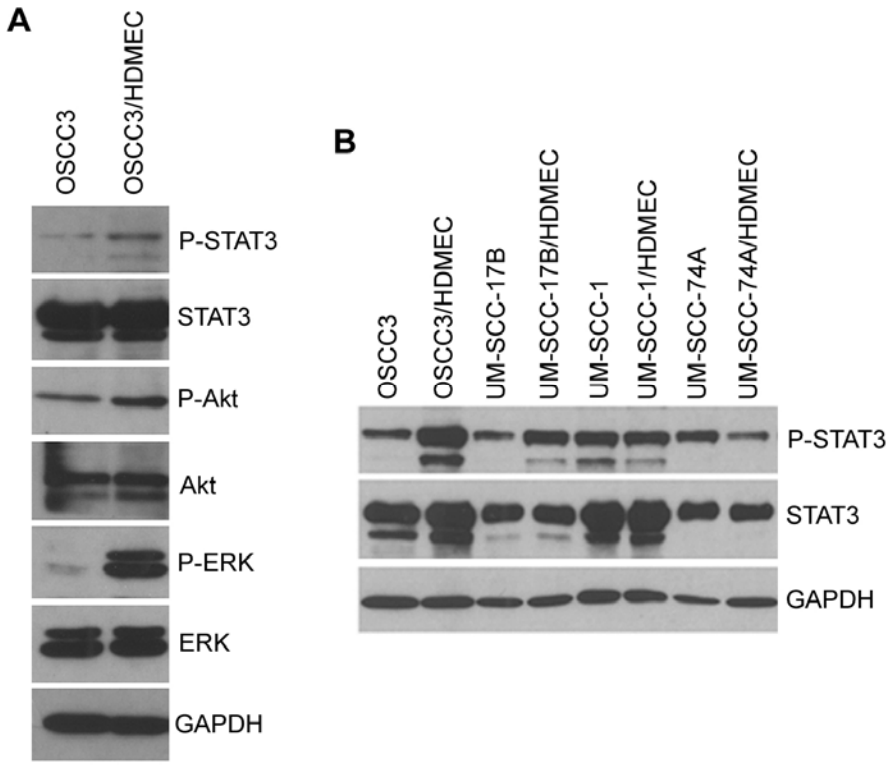

Figure 2.2. Tumor cells co-cultured with endothelial cells show higher phosphorylation of STAT3, Akt, and ERK compared to tumor cells cultured alone. (A) Western Blot for phosphorylated and total STAT3, Akt, and ERK in OSCC3 cultured alone or co-cultured with HDMEC for 1 hour. (B) Western Blot for phosphorylated and total STAT3 in OSCC3, UM-SCC-17B, UM-SCC-1, or UM-SCC-74A cultured alone or co-cultured with HDMEC for 24 hours.

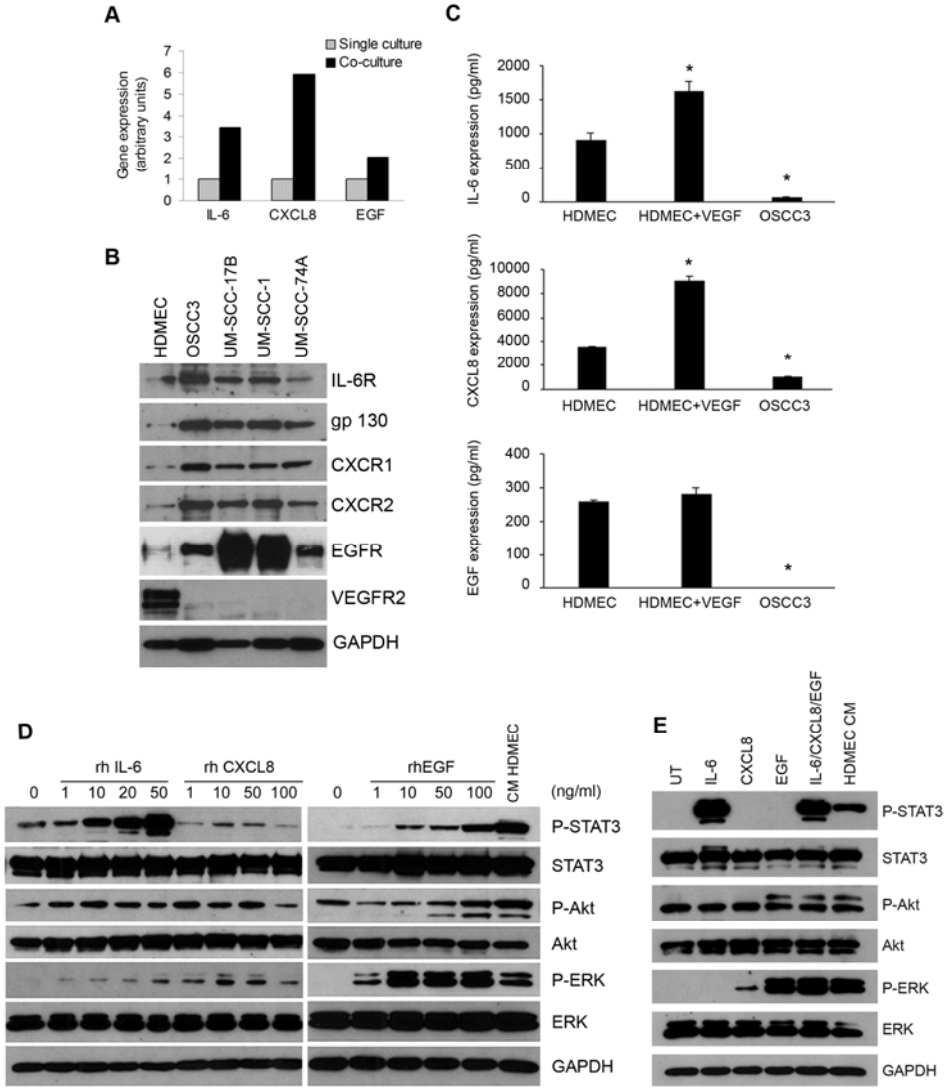

Figure 2.3. Endothelial cells show upregulated IL-6, CXCL8, and EGF expression when co-cultured with tumor cells. (A) Genome wide mRNA expression analysis was performed in HDMEC co-cultured with OSCC3, using HDMEC cultured alone (single culture) as controls. Black bars represent the fold-increase of IL-6, CXCL8, and EGF expression in HDMEC co-cultured with OSCC3 compared with HDMEC cultured alone (gray bars). (B) Western Blot for IL-6R, gp130, CXCR1, CXCR2, EGFR, and VEGFR2 expression in HDMEC, OSCC3, UM-SCC-17B, UM-SCC-1A, and UM-SCC-74A. (C) ELISA for IL-6, CXCL8, and EGF expression in HDMEC, OSCC3, or HDMEC exposed to 50 ng/ml $VEGF_{165}$. Asterisk depicts significant difference ($p<0.001$), as compared to HDMEC. (D) Western Blot for phosphorylated and total STAT3, Akt, and ERK in OSCC3 serum-starved overnight and exposed to 1-50 ng/ml rhIL-6, 1-100 ng/ml rhCXCL8, or 1-100 ng/ml rhEGF for 30 minutes. (E) Western Blot for phosphorylated and total STAT3, Akt, and ERK in OSCC3 serum-starved overnight and exposed for 30 minutes to 20 ng/ml rhIL-6, 50 ng/ml rhCXCL8, 50 ng/ml rhEGF, or the combination of these three recombinant proteins, compared to OSCC3 exposed to HDMEC CM.

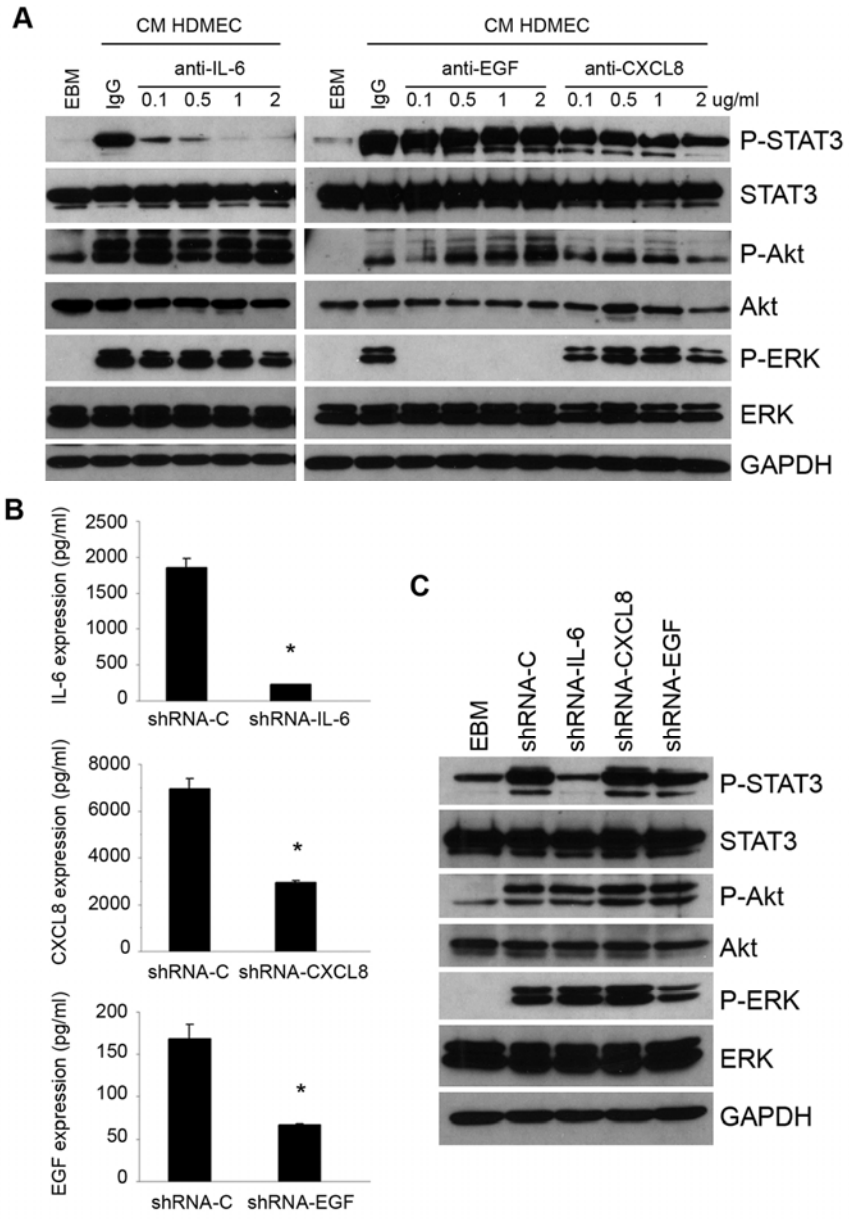

Figure 2.4. Effect of endothelial cell-secreted IL-6, CXCL8, and EGF on the phosphorylation of STAT3, Akt, and ERK in HNSCC cells. (A) Western Blot for phosphorylated and total STAT3, Akt, and ERK in OSCC3 serum-starved overnight and exposed to EBM; HDMEC conditioned medium (CM) containing 0-2 μg/ml anti-IL-6, anti-EGF, anti-CXCL8 neutralizing antibodies; or IgG control for 30 minutes. (B) ELISA for IL-6, CXCL8, or EGF expression in HDMEC transfected with shRNA-control (shRNA-C), shRNA-IL-6, shRNA-CXCL8, or shRNA-EGF. Asterisk depicts significant difference ($p<0.001$), as compared to shRNA-C. (C) Western Blot for phosphorylated and total STAT3, Akt, and ERK in OSCC3 serum-starved overnight and exposed to EBM, or to CM from HDMEC-shRNA-C, HDMEC-shRNA-IL-6, HDMEC-shRNA-CXCL8, or HDMEC-shRNA-EGF for 30 minutes.

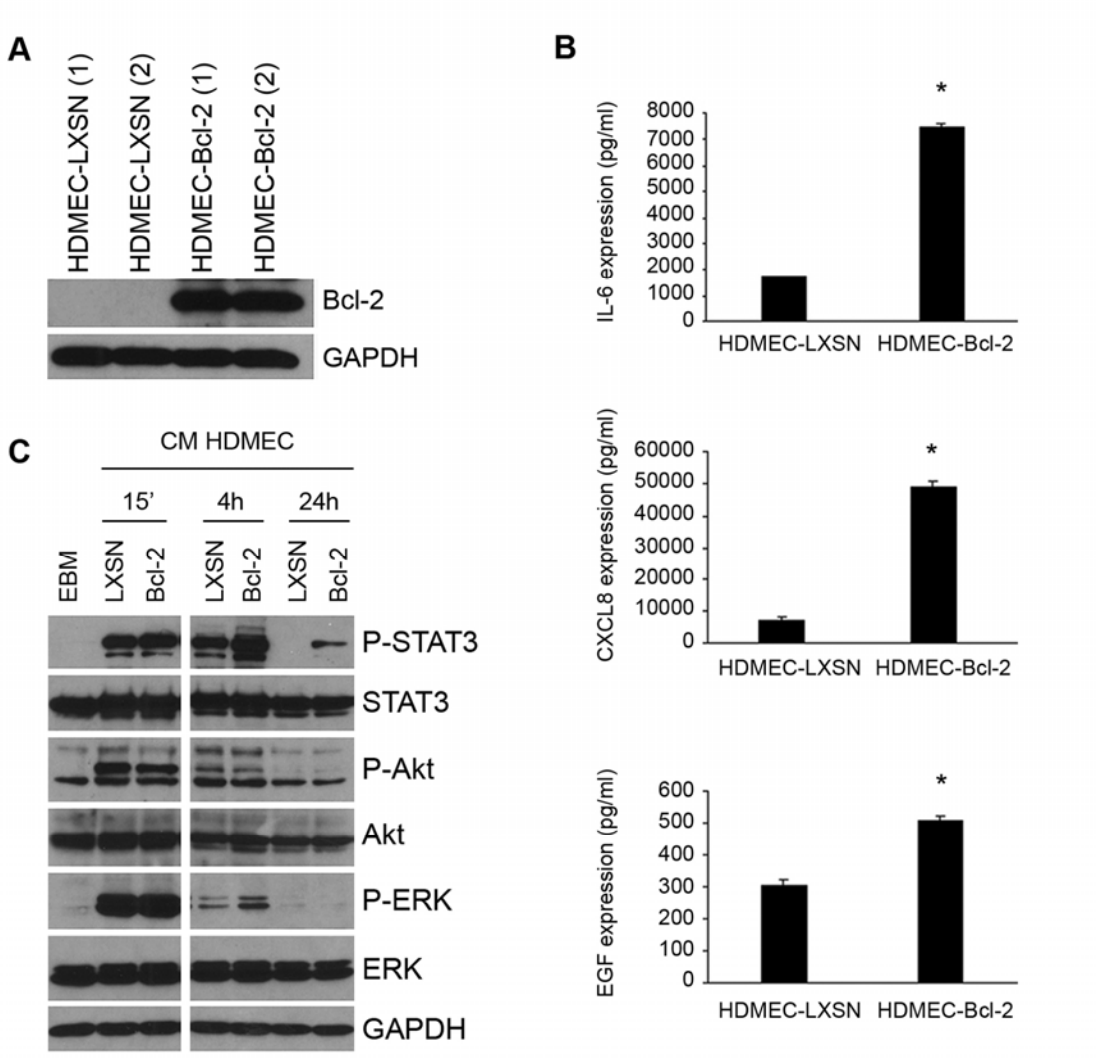

Figure 2.5. Bcl-2 induces IL-6, CXCL8, and EGF expression in endothelial cells. (A) Pools of endothelial cells overexpressing Bcl-2 (HDMEC-Bcl-2) were generated using retroviral vectors. Transgene expression was examined by Western Blot from HDMEC-LXSN (empty vector control) and HDMEC-Bcl-2. (B) ELISA for IL-6, CXCL8, or EGF expression in HDMEC-LXSN or HDMEC-Bcl-2. Asterisk depicts significant difference ($p<0.001$), as compared to control HDMEC-LXSN. (C) OSCC3 were serum-starved overnight and exposed to EBM, conditioned medium (CM) from HDMEC-LXSN, or from HDMEC-Bcl-2 at indicated time points. Western Blots were used to determine phosphorylated and total STAT3, Akt, and ERK in OSCC3.

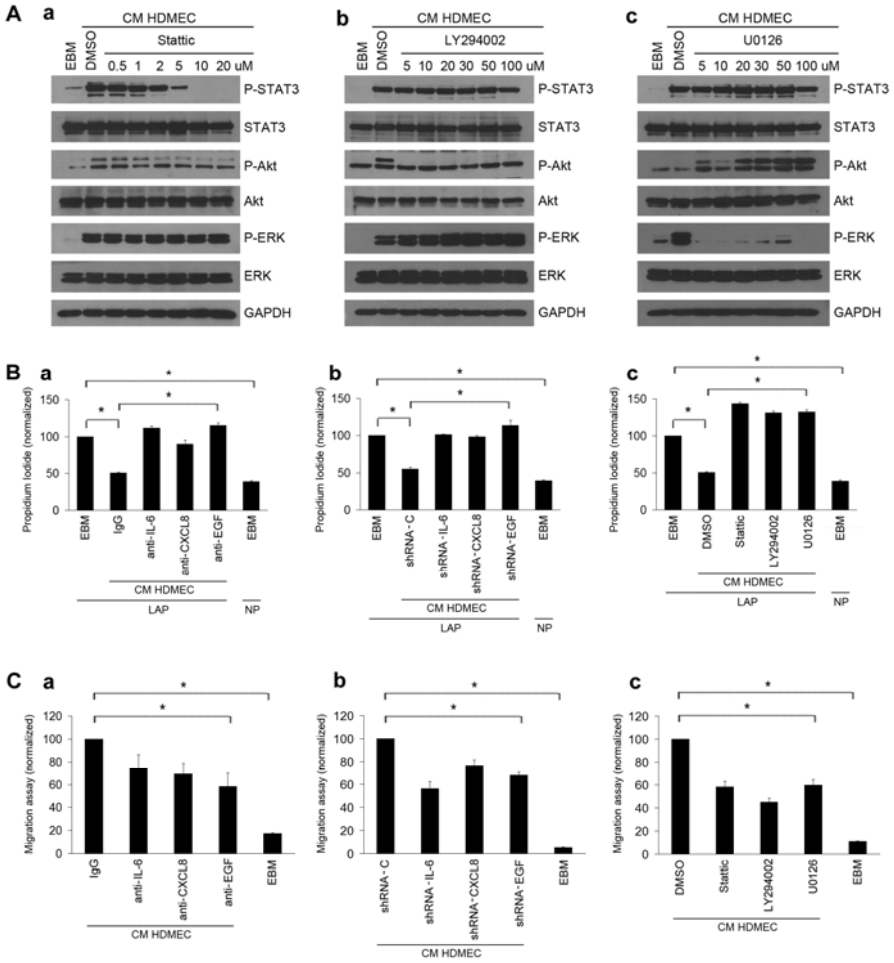

Figure 2.6. Endothelial cell-secreted IL-6, CXCL8, and EGF enhance tumor cell survival and migration via STAT3, Akt, and ERK pathways. (A) To optimize the dose of STAT3, Akt, and ERK inhibitors, OSCC3 were serum-starved overnight, pre-incubated for 1-2 hours with (a) 0-20 μM Stattic, (b) 0-100 μM LY294002, or (c) 0-100 μM U0126, and exposed to EBM or HDMEC conditioned medium (CM) for 30 minutes. Inhibition of STAT3, Akt, and ERK phosphorylation was determined by Western Blot. (B) To evaluate tumor cell survival, OSCC3 were maintained in low attachment plates (LAP) for 24 hours in EBM, (a) CM HDMEC containing 1 μg/ml anti-IL-6, anti-CXCL8, anti-EGF neutralizing antibodies, or IgG control; (b) CM from HDMEC-shRNA-IL-6, HDMEC-shRNA-CXCL8, HDMEC-shRNA-EGF, or HDMEC-shRNA-C; or (c) CM HDMEC containing 5 μM Stattic, 10 μM LY294002, 10 μM U0126, or DMSO vehicle control. OSCC3 cultured in regular plates (NP) with EBM were used as controls. (C) To evaluate tumor cell migration, OSCC3 were serum-starved overnight and allowed to migrate for 24 hours towards EBM, (a) HDMEC CM containing 1 μg/ml anti-IL-6, anti-CXCL8, anti-EGF, or IgG control; (b) CM from HDMEC-shRNA-IL-6, HDMEC-shRNA-CXCL8, HDMEC-shRNA-EGF, or HDMEC-shRNA-C; or (c) HDMEC CM containing 5 μM Stattic, 10 μM LY294002, 10 μM U0126, or DMSO vehicle control. Data presented here were normalized by control groups. Asterisk depicts significant difference ($p<0.001$), as compared to controls.

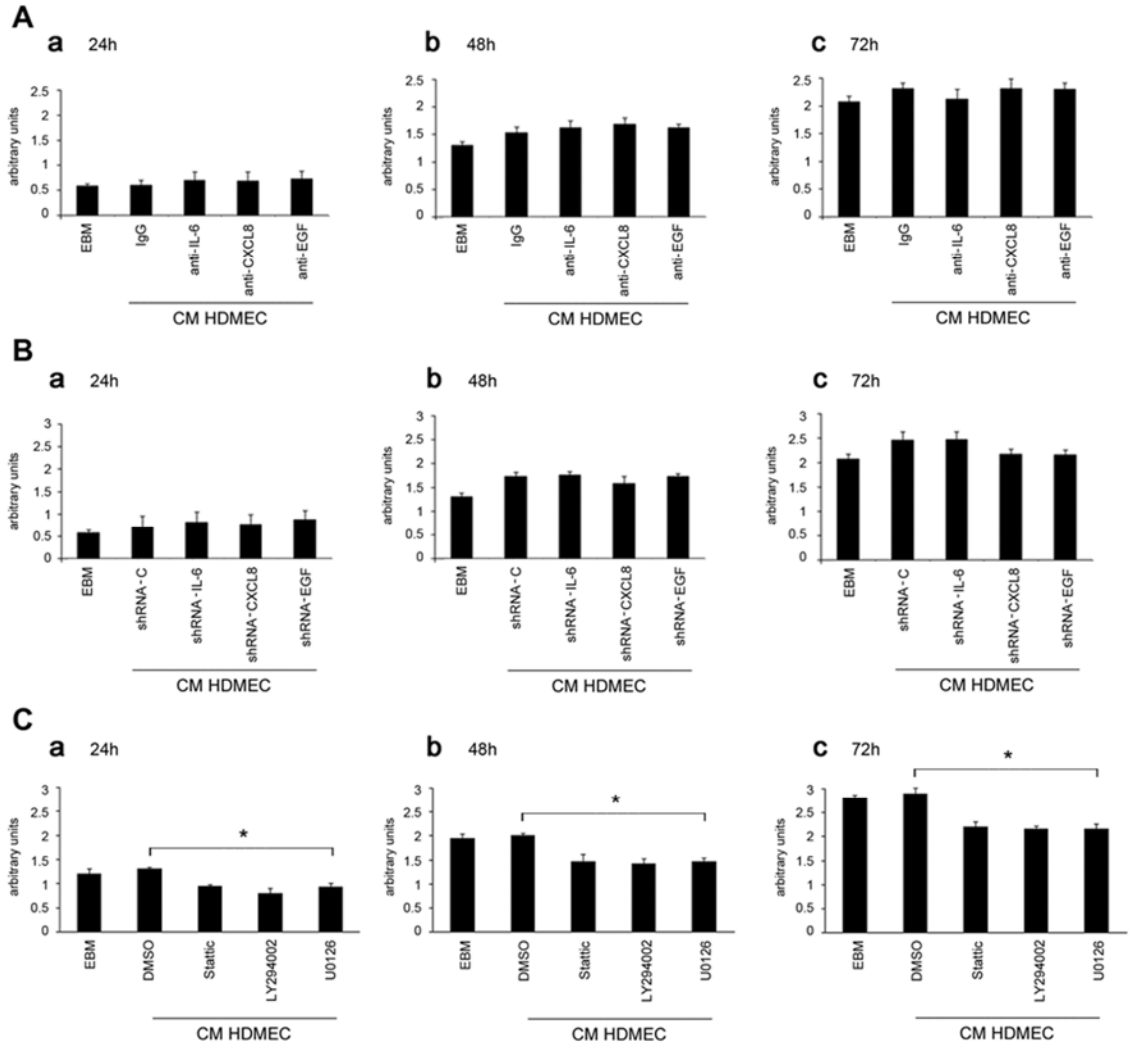

Figure 2.7. Endothelial cell-secreted IL-6, CXCL8, and EGF do not affect tumor cell proliferation. OSCC3 were exposed for 24 (a), 48 (b), or 72 (c) hours to EBM, (A) HDMEC conditioned medium (CM) containing 1 μg/ml anti-IL-6, anti-CXCL8, anti-EGF neutralizing antibodies, or IgG control; (B) CM from HDMEC-shRNA-IL-6, HDMEC-shRNA-CXCL8, HDMEC-shRNA-EGF, or HDMEC-shRNA-C; or (C) HDMEC CM containing 5 μM Stattic, 10 μM LY294002, 10 μM U0126, or DMSO vehicle control. Notably, direct inhibition of STAT3, Akt or ERK with chemical inhibitors slowed down tumor cell proliferation, as determined by SRB assays. Asterisk depicts significant difference ($p<0.05$), as compared to controls.

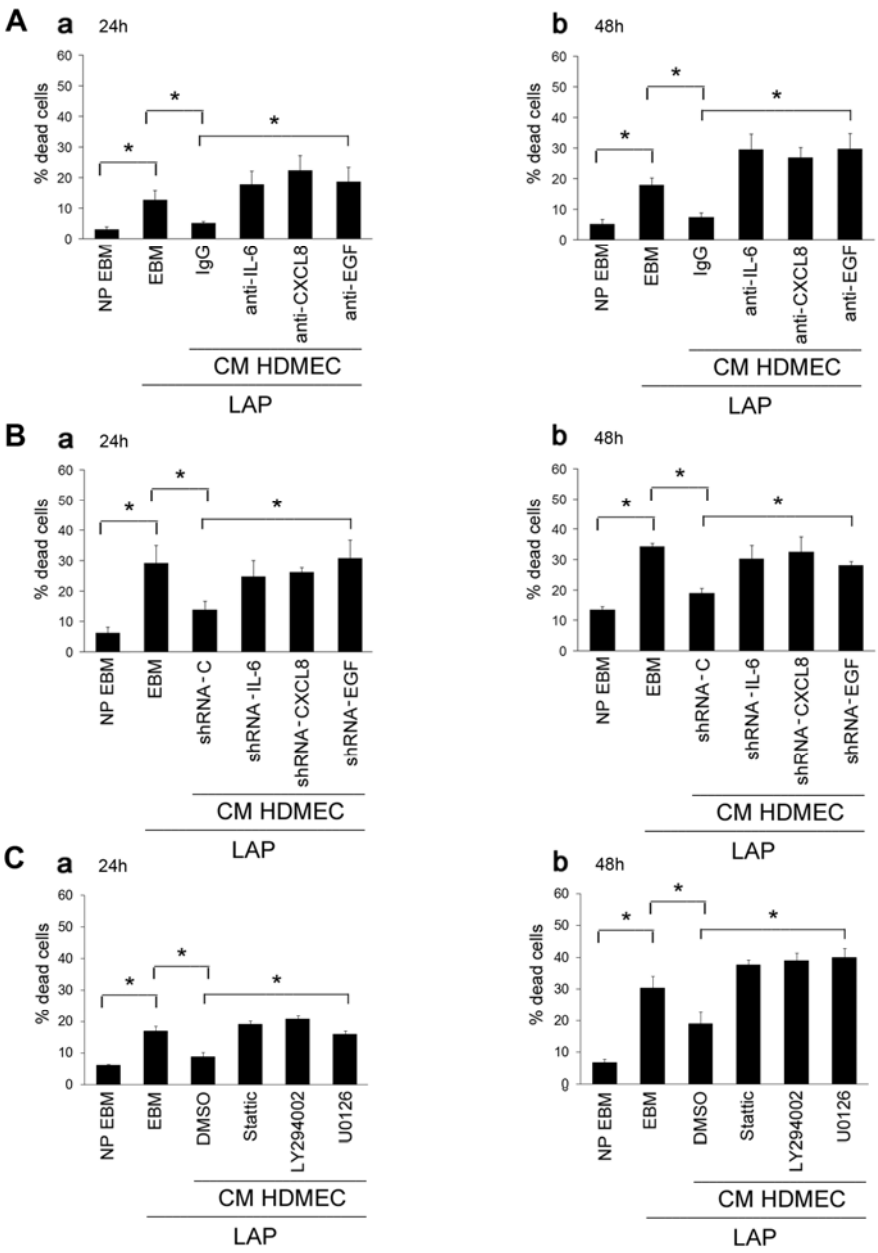

Figure 2.8. Endothelial cell-secreted IL-6, CXCL8, and EGF enhance tumor cell survival via STAT3, Akt, and ERK pathways. To evaluate tumor cell survival, OSCC3 were maintained in low attachment plates (LAP) for 24 hours (a) or 48 hours (b) in EBM, (A) CM HDMEC containing 1 μg/ml anti-IL-6, anti-CXCL8, anti-EGF neutralizing antibodies, or IgG control; (B) CM from HDMEC-shRNA-IL-6, HDMEC-shRNA-CXCL8, HDMEC-shRNA-EGF, or HDMEC-shRNA-C; or (C), CM HDMEC containing 5 μM Stattic, 10 μM LY294002, 10 μM U0126, or DMSO vehicle control. OSCC3 cultured in regular plates (NP) with EBM were used as controls. Percentage of dead cells was determined by the trypan blue exclusion method. Asterisk depicts significant difference ($p<0.001$), as compared to controls.

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CHAPTER III

ENDOTHELIAL CELL-DERIVED IL-6 MODULATES TUMOR GROWTH

Abstract

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer worldwide, with more than 500,000 new cases annually. Despite improvement in treatment strategies, the 50% five-year survival rate has not improved significantly over the past 30 years. The finding of novel molecular therapeutic targets may improve the treatment of HNSCC patients. Targeting endothelial cells rather than cancer cells is an attractive approach for cancer therapy, since the vascular endothelium is directly accessible to systemic drugs and less likely to acquire drug resistance. We have previously shown that endothelial cell-secreted factors activate STAT3, Akt, and ERK signaling pathways HNSCC cells. Here, we demonstrated that HNSCC cells adjacent to blood vessels showed increased phosphorylation of STAT3, Akt, and ERK in xenograft human tumors. Inhibition of endothelial cell-induced Akt signaling in HNSCC cells increased ERK phosphorylation, and inhibition of ERK pathway increased Akt activation, whereas STAT3 phosphorylation levels were not changed by blockade of endothelial cell-induced Akt or ERK pathways. Among these three pathways, STAT3

presented the higher phosphorylation levels, which is mainly induced by endothelial cell-secreted IL-6. Notably, downregulation of IL-6 in tumor-associated endothelial cells by shRNA inhibited the growth of xenograft human tumors and decreased intratumoral microvessel density. These results suggest that targeted inhibition of IL-6 secreted by tumor associated-endothelial cells might be beneficial for patients with HNSCC.

Introduction

Head and neck squamous cell carcinoma (HNSCC) includes malignant lesions arising in the oral cavity, larynx, and pharynx, and represents the sixth most common cancer worldwide, with more than half a million new cases annually (Jemal et al., 2008; Parkin et al., 2005). Despite improvement in treatment strategies involving surgery, radiation therapy, and chemotherapy, the prognosis of HNSCC patients with advanced stage of disease remains largely unsatisfactory as a consequence of loco-regional recurrence (Pignon et al., 2007; Scully and Bagan, 2008). The five-year survival rate after diagnosis of HNSCC is approximately 50%, and has not improved significantly over the past 30 years (Mao et al., 2004; Parkin et al., 2005). HNSCC development and progression involves the accumulation of genetic and epigenetic alterations, and the aberrant expression and/or function of regulators of cell signaling, growth, survival, motility, cell cycle, and angiogenesis (Forastiere et al., 2001; Haddad and Shin, 2008). The deregulation of signaling cascades including the transcription factor signal transducer and activator transcription 3 (STAT3), the phosphatidylinositol-3-kinase (PI3K)/Akt, and

the mitogen activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) pathways are implicated in the pathogenesis of HNSCC (Molinolo et al., 2009).

The recent development of molecular targeted therapies may lead to the rational selection of treatment modalities for HNSCC patients based on specific molecular mechanisms whose deregulated activity contributes to the initiation, development, and metastatic spread (Dietz et al., 2008; Glazer et al., 2009; Langer, 2008; Matta and Ralhan, 2009; Shirai and O'Brien, 2007). Several studies describe the targeted inhibition of signaling pathways, such as STAT3 (Aggarwal et al., 2006; Boehm et al., 2008; Fletcher et al., 2008; Grandis et al., 1998; Leeman et al., 2006), PI3K/Akt (Amornphimoltham et al., 2004; Moral and Paramio, 2008), and MAPK/ERK (Friday and Adjei, 2008; Roberts and Der, 2007) as potential therapeutic approaches for HNSCC. We have recently reported that factors secreted by primary human dermal microvascular endothelial cells (HDMEC) namely IL-6, CXCL8, and EGF activate STAT3, Akt, or ERK pathways in HNSCC (Neiva et al., 2009). We have also shown that inhibition of endothelial cell-secreted IL-6, CXCL8, and EGF decreases STAT3, Akt, and ERK phosphorylation respectively, and reduces tumor cell survival and migration *in vitro* (Neiva et al., 2009). However, we do not know whether downregulation of endothelial cell-secreted factors affects the activity of these signaling pathways *in vivo,* and whether endothelial cell-initiated signaling contributes to tumor growth.

The search for anticancer therapeutic agents that target tumor cells specifically and selectively with limited toxicity is one of the most important goals of current cancer research (Hajitou et al., 2006). Most chemotherapeutic drugs do not preferentially accumulate at the disease sites. In fact, the dose that reaches the tumor can be as low as 5

to 10% of the dose that accumulates in normal tissues (Bosslet et al., 1998). One approach in the development of more selective anticancer agents consists of targeted delivery of molecules to the tumor microenvironment by binding molecules specifically to tumor-associated markers (Borsi et al., 2002; Buchegger et al., 1983; Demartis et al., 2001; Mach et al., 1983). Tumor progression requires the formation of new blood vessels (Folkman, 1971). Therefore, several angiogenesis inhibitors have been developed to target endothelial cells and block tumor growth (Kerbel and Folkman, 2002). Targeting cells that support tumor growth, rather than cancer cells themselves, is an attractive approach for cancer therapy. The vascular endothelium is directly accessible to drugs injected in the circulation, is composed of genetically stable cells, and is less likely to accumulate mutations and acquire drug resistance (Kerbel and Folkman, 2002; Kolonin et al., 2001; St Croix et al., 2000). Although, recent studies in several experimental models suggested that both tumor and non-tumor (stromal) cells may be involved in the reduced responsiveness to the treatments, due to acquired resistance to antiangiogenic therapy (Shojaei and Ferrara, 2008).

Studies with antiangiogenic agents, including monoclonal antibodies that target vascular endothelial growth factor (VEGF) such as bevacizumab, and multitargeted receptor tyrosine kinase inhibitors such as sorafenib and sunitinib, have shown to improve overall survival in several solid malignancies (Le Tourneau and Siu, 2008). Bevacizumab was the first antiangiogenic drug to be approved by the FDA in 2004 (Hicklin and Ellis, 2005). It is currently used for treatment of metastatic colorectal cancer, non-small lung cancer, and advanced breast cancer (Kerbel, 2008). Sorafenib and sunitinib have also been approved by the FDA, and are used for the treatment of

metastatic renal cell cancer and hepatocellular carcinoma (Kerbel, 2008). The fact that HNSCC express high levels of VEGF and its receptors, and that VEGF expression is highly correlated with prognosis in patients with HNSCC (Shang et al., 2006; (Lothaire et al., 2006), make the study of antiangiogenic therapies especially attractive in the context of this malignancy. Although single-agent antiangiogenic drugs have not shown activity in HNSCC patients, promising results have been obtained with combination therapies involving antiangiogenic drugs in phase II trials (Fujita et al., 2007; Liang et al., 2008).

Despite all the advances in therapies targeting VEGF and other signaling molecules, several challenges still remain. The survival benefits of these treatments are relatively modest (Kerbel, 2008), the treatments are costly (Berenson, 2006; Schrag, 2004), and have significant side effects (Eskens and Verweij, 2006; Verheul and Pinedo, 2007). In addition, single-agent therapy that is effective initially may ultimately lead to drug resistance (Dietz et al., 2008; Le Tourneau and Siu, 2008), making the development of targeted therapies in HNSCC more challenging. The finding of novel molecular targets, and the inclusion of these agents in combined treatment regimes, is likely to increase therapeutic efficacy in HNSCC.

Materials and Methods

Cell culture

Oral squamous cell carcinoma (OSCC3, gift of M. Lingen, University of Chicago), were cultured in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin,

and 100 μg/ml streptomycin (Invitrogen). Tumor cells were serum-starved overnight before adding treatment. An immortalized human oral keratinocyte cell line (HOK-16B, gift of No-Hee Park, University of California, Los Angeles) was cultured in serum free medium OKM (ScienCell, Carlsbad, CA) containing 1% penicillin/streptomycin, and supplemented with 5 μg/ml BSA, 5 μg/ml transferring, 50 μg/ml bovine pituitary extract, 2.5 μg/ml insulin, 1 ng/ml FGF, 500 ng/ml epinephrine, 1 μg/ml hydrocortisone, 30 nM prostaglandin, and 40 μg/ml plant extract (OKGS, BulletKit, ScienCell). Cells were incubated overnight with OKM medium without supplements before adding treatment. Primary human dermal microvascular endothelial cells (HDMEC; Cambrex, Walkersville, MD) were cultured in endothelial growth medium-2 (EGM2-MV; Cambrex). Conditioned medium (CM) from HDMEC or OSCC3 were prepared in endothelial cell medium (EBM) without supplementation with growth factors or serum from 24-hour cultures.

Stable short hairpin RNA (shRNA) transduction

Lentiviruses expressing a short hairpin RNA (shRNA) construct for silencing IL-6 (Vector Core, University of Michigan) were generated in human embryonic kidney cells (293T) transfected by the calcium phosphate method, as described (Kaneko et al., 2007). A scrambled oligonucleotide sequence (shRNA-C) was used as control. Supernatants were collected 48 hours after transfection and used to infect HDMEC in 1:1 dilution medium containing 4 μg/ml polybrene (Sigma-Aldrich, St. Louis, MO). Cells were selected in EGM2-MV supplemented with 1 μg/ml puromycin (InvivoGen, San Diego,

CA). Downregulation of IL-6 was confirmed by ELISA and by expression of GFP under fluorescence microscopy.

Western blots

8 x 10^5 OSCC3 were plated in 60 mm dishes, starved overnight, and exposed to EBM, or conditioned medium (CM) collected from HDMEC or OSCC3 for the indicated time points. HDMEC CM and OSCC3 CM were normalized by total protein concentration. In addition, HOK-16B were exposed to HDMEC CM. Alternatively, tumor cells were exposed to rhIL-6 (BDP, NCI, Frederick, MD) for the indicated time points. Signaling pathways were blocked by pre-incubating tumor cells for 1-2 hours with 20 μM Stattic (STAT3 inhibitor V, Calbiochem, San Diego, CA), 20 μM LY294002 (PI3 kinase inhibitor, Cell Signaling Technology, Danvers, MA), or 20 μM U0126 (MEK1/2 inhibitor, Cell Signaling), as described (Neiva et al., 2009), and exposed to HDMEC CM or rhIL-6 for the indicated time points. Protein (30 μg) was electrophoresed on SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Primary antibodies were: mouse anti-human phospho-STAT3, rabbit anti-human STAT3, rabbit anti-human phospho-Akt, rabbit anti-human Akt, rabbit anti-human phospho-ERK1/2, mouse anti-human ERK1/2 (Cell Signaling); and mouse anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Chemicon, Millipore, Billerca, MA). Immunoreactive proteins were visualized by SuperSignal West Pico chemiluminescent substrate (Thermo Scientific, Rockford, IL).

Enzyme-linked immunosorbent assay (ELISA)

Supernatants of 24-hour endothelial or tumor cell cultures were collected and centrifuged to eliminate debris. IL-6 expression was determined using ELISA kits (Quantikine; R & D Systems, Minneapolis, MN) according to the manufacturer's instructions. Data were normalized by cell number.

Severe Combined Immunodeficient (SCID) Mouse Model of Human Tumor Angiogenesis

Xenograft human tumors vascularized with human blood vessels were generated under an UCUCA approved protocol, as described (Nor et al., 2001a; Nor et al., 2001b). Briefly, highly porous poly-L(lactic) acid (Boehringer Ingelheim, Ingelheim, Germany) scaffolds were seeded with 9 x 10^5 HDMEC and 1 x 10^5 OSCC3 in a 1:1 mixture of growth factor reduced Matrigel and EGM2-MV. Alternatively, tumors were generated using a line of human Kaposi's sarcoma cells (SLK, gift of G. Nuñez, University of Michigan). In addition, scaffolds were seeded with 9 x 10^5 HDMEC-shRNA-control or HDMEC-shRNA-IL-6 and 1 x 10^5 OSCC3. SCID mice (5-7-week-old male CB.17.SCID; Charles River Laboratories International, Inc., Wilmington, MA) were anesthetized with ketamine and xylazine, and 2 scaffolds were implanted in the subcutaneous space of the dorsal region of each mouse. Tumors were measured with a caliper every 2 days, starting at 14 days after implantation. Mice were euthanized after 28 days, implants were retrieved, photographed, measured, weighed on an electronic balance, fixed overnight in 10% buffered formalin at 4^0C, and embedded in paraffin following standard histological procedures.

Immunohistochemistry of tissue sections

Immunohistochemistry was performed in paraffin-embedded serial sections using phospho-STAT3 (Santa Cruz), STAT3, phospho-Akt, Akt, phospho-ERK, ERK (Cell Signaling), and Ki-67 (Biocare Medical, Concord, CA) antibodies, as described (Squarize et al., 2006).

Tumor microvessel density

Tumor microvessel density was determined following identification of blood vessels by immunohistochemistry with a polyclonal anti-human factor VIII antibody (Lab Vision, Fremont, CA), as previously described (Nor et al., 2001b). The number of stained microvessels was counted in 10 random fields per implant in a light microscope at 100X. Twelve implants were analyzed per condition.

Statistical analyses

T-tests or one-way analysis of variance was performed using the SigmaStat 2.0 software (SPSS; Chicago, IL). Statistical significance was determined at $p<0.05$.

Results

Endothelial cell-secreted factors, but not tumor cell-secreted factors, induce phosphorylation of STAT3, Akt, and ERK signaling pathways in oral squamous cell carcinoma cells

We have previously demonstrated that a crosstalk initiated by endothelial cells enhances tumor cell survival and migration *in vitro* via STAT3, Akt, and ERK signaling (Neiva et al., 2009). The overall hypothesis underlying this study is that the activation of signaling pathways in tumor cells induced by endothelial cell-secreted factors enhances tumor growth. To begin to address this hypothesis, we compared the effect of endothelial cell-secreted factors (paracrine pathway) with tumor cell-secreted factors (autocrine pathway) on the phosphorylation of STAT3, Akt, and ERK in HNSCC cells. We exposed OSCC3 to serum-free endothelial cell (HDMEC) conditioned medium (CM) or tumor cell (OSCC3) CM and analyzed phosphorylation events over time (Figure 3.1A). We observed that phosphorylation levels of STAT3, Akt, and ERK were higher in tumor cells exposed to endothelial cell CM than in tumor cells exposed to OSCC3 CM, or unconditioned medium (EBM). The induction of phosphorylation was observed primarily at early time points (15 to 30 minutes), decreasing at 1 hour (Figure 3.1A).

Endothelial cell-secreted factors activate STAT3, Akt, and ERK in oral squamous cell carcinoma cells and in oral keratinocytes

Trying to understand whether the effect of endothelial cell-secreted factors was specific to tumor cells (OSCC3), we also exposed human oral keratinocytes (HOK-16B) to endothelial cell CM and analyzed phosphorylation of STAT3, Akt, and ERK over time (Figure 3.1B). We observed that endothelial cell-secreted factors had a similar effect on STAT3 and ERK phosphorylation in keratinocytes, compared to tumor cells (higher phosphorylation levels at 15 to 30 minutes, and decreasing at 1 to 24 hours). STAT3 phosphorylation levels were very similar in both cell lines, whereas phosphorylation of

ERK was stronger in tumor cells compared with keratinocytes. Phosphorylation of Akt followed a different trend. Similarly to STAT3 and ERK activation, tumor cells phosphorylated Akt only in the presence of endothelial cell CM (15 minutes to 1 hour, decreasing at 24 hours). On the other hand, oral keratinocytes presented comparable Akt phosphorylation levels in the untreated (exposed to EBM unconditioned medium) and treated (exposed to endothelial cell CM) groups (Figure 3.1B).

Oral squamous cell carcinoma cells adjacent to blood vessels show increased phosphorylation of STAT3, Akt, and ERK *in vivo*.

To evaluate whether the trends of endothelial cell-induced phosphorylation of STAT3, Akt, and ERK in tumor cells *in vitro* translate into increased phosphorylation levels *in vivo*, we used the SCID mouse model of human tumor angiogenesis in which we engineer human tumors vascularized with human functional blood vessels that anastomize with the mouse vasculature (Nor et al., 2001a; Nor et al., 2001b; Warner et al., 2008). We implanted primary human endothelial cells (HDMEC) together with oral squamous cells carcinoma cells (OSCC3) in the subcutaneous of SCID mice and analyze the tissues by immunohistochemistry. We observed that levels of total STAT3, Akt, and ERK were uniform through the tissues with nuclear and cytoplasmic localization (Figure 3.1C, bottom panels). Interestingly, tumor cells adjacent to blood vessels showed phosphorylation of STAT3, Akt, and ERK, with nuclear localization (Figure 3.1C, top and middle panels). To validate these results, we coimplanted endothelial cells with a Kaposi's sarcoma cell line (SLK), and observed the same phosphorylation pattern (Figure 3.2).

STAT3 phosphorylation induced by endothelial cell-secreted factors is independent of Akt and ERK phosphorylation in oral squamous cell carcinoma cells

To explore the interdependence of molecular signaling events initiated by endothelial cells on tumor cells, we exposed OSCC3 to HDMEC CM in the presence of chemical inhibitors of STAT3, Akt, or ERK pathways and analyzed the interdependency of the phosphorylation events. To establish a baseline, we exposed OSCC3 to HDMEC CM and analyzed phosphorylation of STAT3, Akt, and ERK with a detailed time course up to 1 hour (Figure 3.3A). We observed that HDMEC CM induces first ERK phosphorylation (with strong activation as early as 1 minute, persisting until 15 minutes, and decreasing at 30 minutes), followed by STAT3 and Akt (increasing until 15 minutes, and maintaining activation for up to 1 hour) (Figure 3.3A). When we decreased STAT3 phosphorylation using the chemical inhibitor Stattic, we did not observe significant changes in the phosphorylation status of Akt or ERK (Figure 3.3B). However, when we inhibited Akt phosphorylation using the PI3K inhibitor LY294002 we observed an increase in ERK phosphorylation levels (maintaining strong phosphorylation for up to 1 hour), while phosphorylation levels of STAT3 did not change (Figure 3.3C). Similarly, when we inhibited ERK phosphorylation using the MEK1/2 inhibitor U0126 we observed increased Akt phosphorylation (maintaining strong phosphorylation for up to 1 hour), whereas phosphorylation levels of STAT3 remained unchanged (Figure 3.3D).

We then extended our time course experiments for 24 hours, and observed the same relationship between STAT3, Akt, and ERK phosphorylation in tumor cells induced by endothelial cell-secreted factors (Figure 3.4). STAT3, Akt, and ERK phosphorylation

were stronger at early time points (15 to 30 minutes), decreasing over time. Phosphorylation levels of STAT3 decreased at 1 hour and were maintained for up to 24 hours, phosphorylation of Akt decreased at 2 hours and disappeared at 4 to 24 hours, while phosphorylation of ERK decreased significantly at 1 hour and was absent at 3 to 24 hours (Figure 3.4A). Following the same trends as Figure 3.3, inhibition of STAT3 phosphorylation did not affect Akt or ERK phosphorylation levels (Figure 3.4B). On the other hand, inhibition of Akt phosphorylation increased activation of ERK (Figure 3.4C) at 1 to 24 hours (compared with Figure 3.4A). Likewise, inhibition of ERK phosphorylation increased Akt activation (Figure 3.4D), maintaining the phosphorylation state longer (compared with Figure 3.4A). No major effect was observed in STAT3 phosphorylation levels using Akt or ERK inhibitors. These results suggested that endothelial cell-induced Akt and ERK phosphorylation in OSCC3 have a compensatory effect, while the STAT3 pathway is activated independently.

IL-6 activates STAT3, Akt, and ERK signaling pathways in oral squamous cell carcinoma cells

We have previously demonstrated that IL-6, CXCL8, and EGF are involved in the endothelial cell-initiated STAT3, Akt, and ERK signaling in HNSCC cells, respectively. From these three endothelial cell-secreted factors, IL-6 was the cytokine that activated pathways in HNSCC more significantly, particularly STAT3 (Neiva et al., 2009). To understand HNSCC response to IL-6 stimulation, we performed a detailed time course analyzing the phosphorylation events in OSCC3 cells (Figure 3.5). We observed that when tumor cells were exposed to rhIL-6, the phosphorylation of STAT3, Akt, and ERK

followed similar patterns as when tumor cells were exposed to HDMEC CM (Figures 3.4A and 3.5A). We then exposed tumor cells to IL-6 in the presence of chemical inhibitors of STAT3, Akt, or ERK pathways and analyzed the phosphorylation response (Figures 3.5B-E). IL-6 strongly activated STAT3 pathway in OSCC3, and slightly activated Akt or ERK (Figure 3.5B). Blockade of STAT3 phosphorylation had no major effect on Akt, but increased ERK phosphorylation (Figure 3.5C). Blockade of Akt activation had no effect on STAT3, while increased ERK phosphorylation (Figure 3.5D). Lastly, inhibition of ERK phosphorylation had significant effect on STAT3 or Akt phosphorylation (Figure 3.5E). Collectively, these results demonstrated that the major effect of IL-6 in OSCC3 is the activation of the STAT3 pathway.

STAT3 phosphorylation in xenograft human oral squamous cell carcinomas correlates with tumor cell proliferation and presence of blood vessels

Our mechanistic experiments in which we inhibited endothelial cell- or IL-6-induced STAT3, Akt, or ERK pathways in tumor cells, suggested that STAT3 is activated independently of Akt and ERK activation. Furthermore, IL-6 activates mainly STAT3 signaling, while less robust effect on the phosphorylation of Akt and ERK was observed in OSCC3. These results led us to further explore the IL-6/STAT3 signaling *in vivo.* We used the SCID mouse model of human tumor angiogenesis, in which we implanted highly porous biodegradable scaffolds cointaining OSCC3 and endothelial cells, generating xenograft human oral squamous cell carcinomas. After 28 days, tumors were retrieved and tissues were analyzed by immunohistochemistry. Confirming our previous results (Figure 3.1C), we observed that while total STAT3 was present diffuse through the entire

tissue (Figure 3.6A) phosphorylated STAT3 had a tendency to concentrate adjacent blood vessels (Figure 3.6B). Interestingly, immunostaining for the cell proliferation marker Ki67 showed the same pattern as phosphorylated STAT3 (Figure 3.6C). These results suggested that phosphorylation of STAT3 in oral squamous cell carcinomas correlates with tumor cell proliferation and proximity to blood vessels.

Downregulation of IL-6 in tumor-associated endothelial cells inhibits tumor growth

We have previously demonstrated that downregulation of IL-6 in endothelial cells decreases tumor cell survival and migration *in vitro* (Neiva et al., 2009). These results led us to investigate the role of endothelial cell-secreted IL-6 on HNSCC. To investigate whether these *in vitro* trends have a biological effect *in vivo*, we used the SCID mouse model of human tumor angiogenesis and seeded OSCC3 together with endothelial cells secreting low levels of IL-6 (HDMEC-shRNA-IL-6), or endothelial cells secreting high levels of IL-6 (HDMEC-shRNA-control). Initially, we downregulated IL-6 expression in endothelial cells using lentivirus. The effectiveness of the knockdown was verified by ELISA (Figure 3.7A), and under fluorescence microscopy to confirm expression of GFP-positive cells (Figure 3.7B). We then implanted scaffolds seeded with OSCC3 and HDMEC-shRNA-IL-6 or HDMEC-shRNA-control in the subcutaneous of SCID mice, and observed tumor growth overtime. We observed a significant difference in tumor growth between the two groups. Tumors populated with HDMEC-shRNA-control grew faster and reached 2,000 mm^3 at 28 days after implantation, whereas tumors populated with endothelial cells secreting low levels of IL-6 presented approximately half of this size (Figure 3.7C). Tumors were macroscopically smaller in the group populated with

HDMEC-shRNA-IL-6 compared with the control group (Figure 3.7D, a). Mice were euthanized at 28 days post-implantation, tumors were removed, photographed (Figure 3.7D, b), measured (Figure 3.7E, a), and weighted (Figure 3.7E, b). These results demonstrated that downregulation of IL-6 in tumor-associated endothelial cells is sufficient to inhibit tumor growth.

Downregulation of IL-6 in tumor-associated endothelial cells inhibits tumor cell proliferation and decreases intratumoral microvessel density.

To explore the mechanisms involved in the inhibition of tumor growth mediated by downregulation of IL-6 in endothelial cells, we analyzed by immunohistochemistry tumor cell proliferation and intratumoral microvessel density. We observed that expression of the proliferation marker Ki67 was higher in tumors cells coimplanted with HDMEC-shRNA-control than in tumors cells coimplanted with HDMEC-shRNA-IL-6 (Figure 3.8A). Ki67 expression was quantified by blinded scoring the immunostained tissues (Figure 3.8B). Lastly, we analyzed whether downregulation of IL-6 in tumor-associated endothelial cells affect microvessel density. We observed a significant decrease in microvessel density in tumors populated with OSCC3 and HDMEC-shRNA-IL-6, compared to implants containing OSCC3 and HDMEC-shRNA-control (Figure 3.8C). Number of microvessels was quantified by counting vessels stained for von Willebrand factor (Factor VIII) (Figure 3.8D). Taken together, these results demonstrated that downregulation of IL-6 in tumor-associated endothelial cells reduces tumor cell proliferation and decreases microvessel density, leading to an inhibition of tumor growth.

Discussion

Recently, there has been increasing interest in the development of molecular-targeted agents that specifically modulate growth factors and signaling pathways that are deregulated in tumor cells. A better understanding of the molecular mechanisms underlying the development and progression of HNSCC may help to identify novel targets for pharmacological intervention in this devastating disease (Nagpal and Das, 2003; Squarize et al., 2006).

We have previously shown that factors secreted by endothelial cells, namely IL-6, CXCL8, and EGF, activate STAT3, Akt, or ERK signaling pathways in HNSCC, increasing tumor cell survival and migration *in vitro* (Neiva et al., 2009). In this study, we explore the biological consequences of these endothelial cell-initiated signaling events *in vivo.* In our previous report, we demonstrated that conditioned medium collected from endothelial cells stimulate phosphorylation of STAT3, Akt, and ERK in HNSCC. However, we did not know whether activation of these pathways was specific to endothelial cell-secreted factors. Several studies describe an autocrine effect of tumor cell-secreted factors on cancer progression (Gao et al., 2007; Kulbe et al., 2004; Lee et al., 2007a; Molinolo et al., 2009; Pries and Wollenberg, 2006; Sansone et al., 2007). Here, we demonstrated that tumor cells exposed to endothelial cell conditioned medium had higher levels of STAT3, Akt, and ERK phosphorylation that tumor cells exposed to conditioned medium collected from tumor cells. We recognize that tumor cells secrete a panel of growth factors and cytokines that stimulate a network signaling molecules. However, endothelial cells activated STAT3, Akt, and ERK in a significantly greater

extend than tumor cell-secreted factors in an autocrine manner. These results support our previous findings that endothelial cells secrete significantly higher levels of IL-6, CXCL8, and EGF than HNSCC cells (Neiva et al., 2009). We also explored whether these endothelial cell-initiated phosphorylation events were specific to advanced stage carcinoma cell lines. To address this question, we exposed a human oral keratinocyte cell line to endothelial cell conditioned medium and analyzed phosphorylation of STAT3, Akt and ERK. We observed that endothelial cells were also able to activate STAT3 and ERK pathways in keratinocytes, suggesting that factors secreted by endothelial cells may also play a role in the early events of HNSCC tumorigenesis. The fact that keratinocytes responded similarly to tumor cells, and more importantly, that Akt was phosphorylated even in untreated cells might be explained by the model of molecular cancer progression of HNSCC (Califano et al., 1996b). This model postulated that tissues with normal or benign appearance might already contain clonal genetic changes (Forastiere et al., 2001). In addition, several studies suggest that early genetic changes do not necessarily correlate with observable changes in morphology (Rosin et al., 2000).

To move our study forward, it was important for us to verify whether these *in vitro* findings would have a correlation with *in vivo* levels of STAT3, Akt, and ERK phosphorylation in tumor cells associated with the presence of endothelial cells. Using the SCID mouse model of human tumor angiogenesis (Nor et al., 2001a; Nor et al., 2001b; Warner et al., 2008), we co-implanted primary human endothelial cells with human oral squamous cells carcinoma cells and analyzed phosphorylation of STAT3, Akt, and ERK in the tumor tissues by immunohistochemistry. Notably, the levels of total STAT3, Akt, and ERK were uniformly distributed through the tumor mass, while tumor

cells expressing phosphorylated STAT3, Akt, and ERK were localized adjacent to blood vessels. We validated these results using a tumor cells line from mesenquimal origin (Kaposi's sarcoma), showing that the effect of endothelial cells on tumor cell phosphorylation of STAT3, Akt, and ERK was independent of tumor type.

Several studies have shown that deregulation of STAT3, Akt, and ERK is implicated in tumorigenesis (Chang et al., 2003; Friday and Adjei, 2008; Grandis et al., 1998; Leeman et al., 2006; Luo et al., 2003; Roberts and Der, 2007; Song and Grandis, 2000; Squarize et al., 2006; Vivanco and Sawyers, 2002; Zeng et al., 2002), suggesting that aberrant activity of a network of interrelated signaling pathways rather than a single deregulated route contributes to carcinogenesis (Molinolo et al., 2009). We have shown that endothelial cell-secreted factors activate STAT3, Akt, and ERK signaling in HNSCC (Neiva et al., 2009), however the crosstalk between these three pathways initiated by endothelial cells had not been explored. Trying to understand the relationship between these endothelial cell-initiated signaling events on tumor cells, we exposed OSCC3 to endothelial cell conditioned medium in the presence of chemical inhibitors of STAT3, Akt, and ERK pathways and analyzed the phosphorylation response. When we inhibited STAT3 phosphorylation, we observed no major changes in the activation of Akt or ERK. However, when we inhibited Akt phosphorylation, we observed an increase in ERK phosphorylation and vice-versa, whereas inhibition of Akt or ERK did not affect STAT3 phosphorylation levels. These results suggested that endothelial cell-induced Akt and ERK signaling have a compensatory effect, while STAT3 pathway is activated independently. These results are in accordance with accumulating evidence that Akt and ERK pathways may cooperate to promote the survival of transformed cells, and are

alternatively and/or coordinately expressed in several cancers, raising the possibility that a feedback loop might exist in this network (Carracedo et al., 2008; Gao et al., 2006; Grant, 2008; Kinkade et al., 2008; McCubrey et al., 2007; Uzgare and Isaacs, 2004). Our results showed that IL-6 secreted by endothelial cells strongly activated STAT3 in OSCC3. Several other studies have also demonstrated that IL-6 induces STAT3 in other cell types and diseases (Atreya and Neurath, 2008; Hirano et al., 2000; Hodge et al., 2005; Van Snick, 1990).

Next, we explored the role of endothelial cell-secreted IL-6 on tumor growth, using the SCID mouse model or human tumor angiogenesis. We observed a significant difference in tumor growth over time, tumor volume, and tumor weight when tumor-associated endothelial cells expressed low levels of IL-6. Early studies have shown that upregulation of Bcl-2 in microvascular endothelial cells accelerates tumor growth (Kaneko et al., 2007; Nor et al., 2001a). We have previously demonstrated that endothelial cells overexpressing Bcl-2 secrete higher levels of IL-6 than empty vector control cells (Neiva et al., 2009). These two findings support the results we presented here. We then explored the mechanisms involved in the inhibition of tumor growth by immunohistochemistry. Consistently with our previous results, tumor cells expressing phosphorylated STAT3 were located primarily adjacent to blood vessels and correlated with expression of the proliferation marker Ki67. Notably, expression of Ki67 was higher in tumors populated with OSCC3 and HDMEC-shRNA-control than in tumors populated with endothelial cells secreting low levels of IL-6. Lastly, we observed a significant decrease in microvessel density in tumors populated with OSCC3 and HDMEC-shRNA-IL-6, compared to controls. Collectively, these results demonstrated that downregulation

of IL-6 in tumor-associated endothelial cells reduces tumor cell proliferation and decreases microvessel density, leading to an inhibition of tumor growth. Our results are in accordance with several reports describing that STAT3 plays a crucial role in tumor cell proliferation and angiogenesis, and that IL-6 is the major activator of STAT3 pathway (Aggarwal et al., 2006; Hodge et al., 2005; Leeman et al., 2006).

In this study, we demonstrated that specific inhibition of endothelial cell-secreted IL-6 inhibits tumor growth. Targeting the vascular endothelium rather than the cancer cells has been an attractive therapeutic approach for the treatment of several malignancies (Kerbel and Folkman, 2002). Notably, endothelial cells are directly accessible to systemic drugs, are composed of genetically stable cells, and are less likely to accumulate mutations and acquire drug resistance (Kerbel and Folkman, 2002; Kolonin et al., 2001; St Croix et al., 2000). In addition, our results suggest that therapeutic blockade of signaling events initiated by endothelial cells might be beneficial for cancer patients even if this therapy does not result in the death of these endothelial cells.

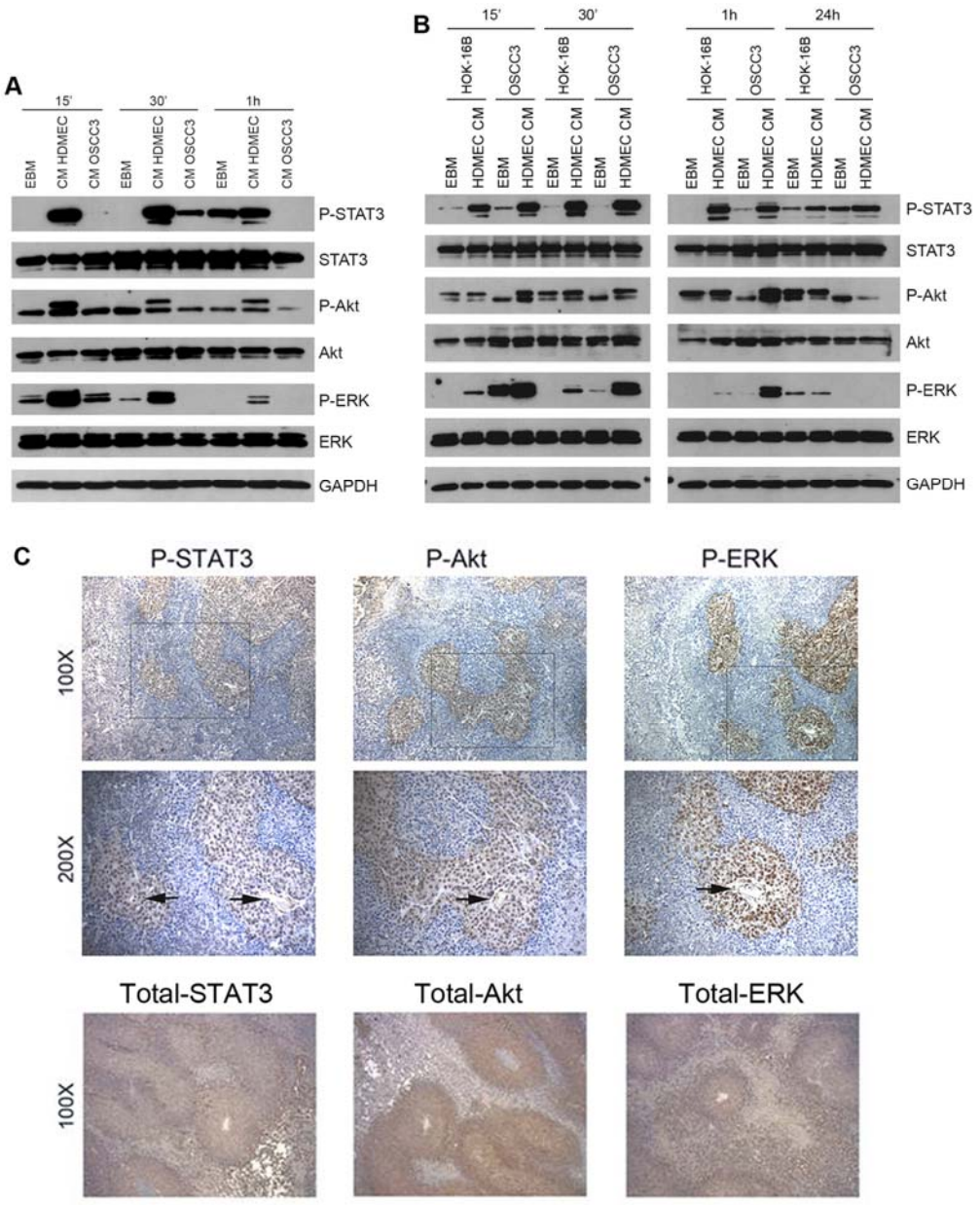

Figure 3.1. Endothelial cell-derived factors phosphorylate STAT3, Akt, and ERK in tumor cells *in vitro* and *in vivo*. (A) Western blot for phosphorylated and total STAT3, Akt, and ERK in OSCC3 serum-starved overnight and exposed to HDMEC conditioned medium (CM), OSCC3 CM, or control unconditioned medium (EBM) for the indicated time points. (B) Western blot for phosphorylated and total STAT3, Akt, and ERK in OSCC3 or HOK-16B serum-starved overnight and exposed to HDMEC CM or EBM for the indicated time points. (C) Immunohistochemical analysis for STAT3, Akt, and ERK in representative specimens from xenograft human squamous cell carcinomas. Top panels represent photomicrographs of phosphorylated STAT3, Akt, and ERK, with nuclear localization (100X). Middle panels represent higher magnification (200X). Arrows point to blood vessels. Bottom panels represent total STAT3, Akt, and ERK (100X) with nuclear and cytoplasmic localization.

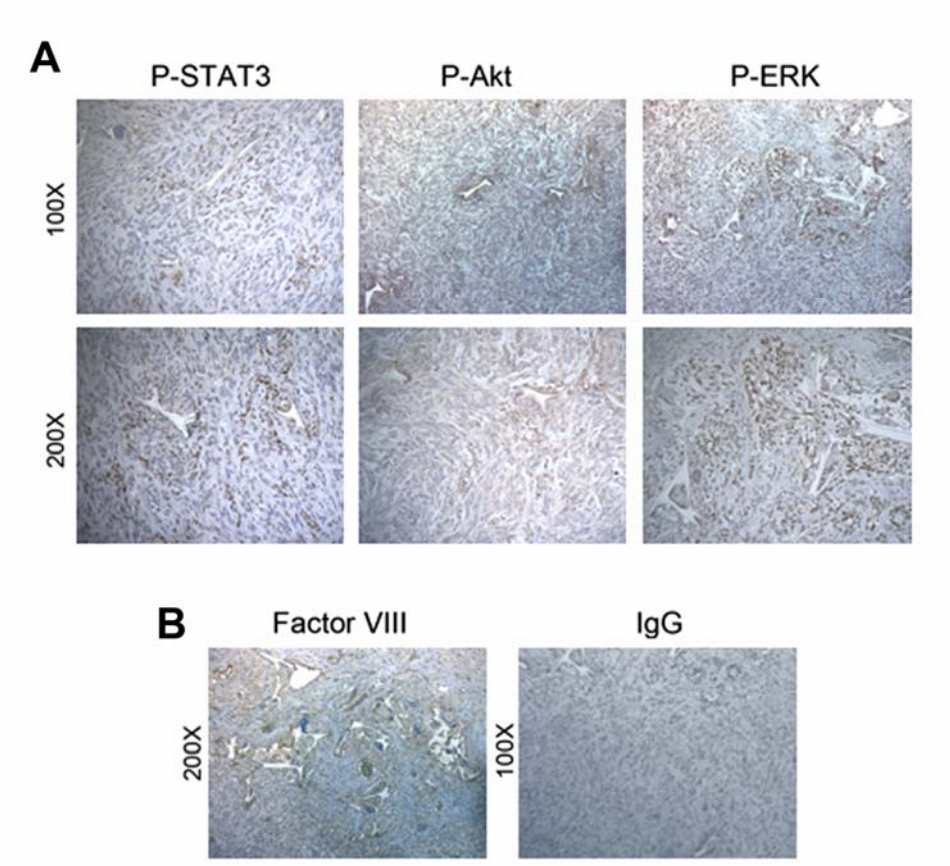

Figure 3.2. Tumor cells adjacent to blood vessels show increased phosphorylation of STAT3, Akt, and ERK. (A) Immunohistochemical analysis for phosphorylated STAT3, Akt, and ERK in representative specimens from xenografted human Kaposi's sarcomas. Top panels represent 100X and bottom panels represent 200X. (B) Immunostaining for von Willebrand factor (Factor VIII) showing blood vessels (200X), and IgG control (100X).

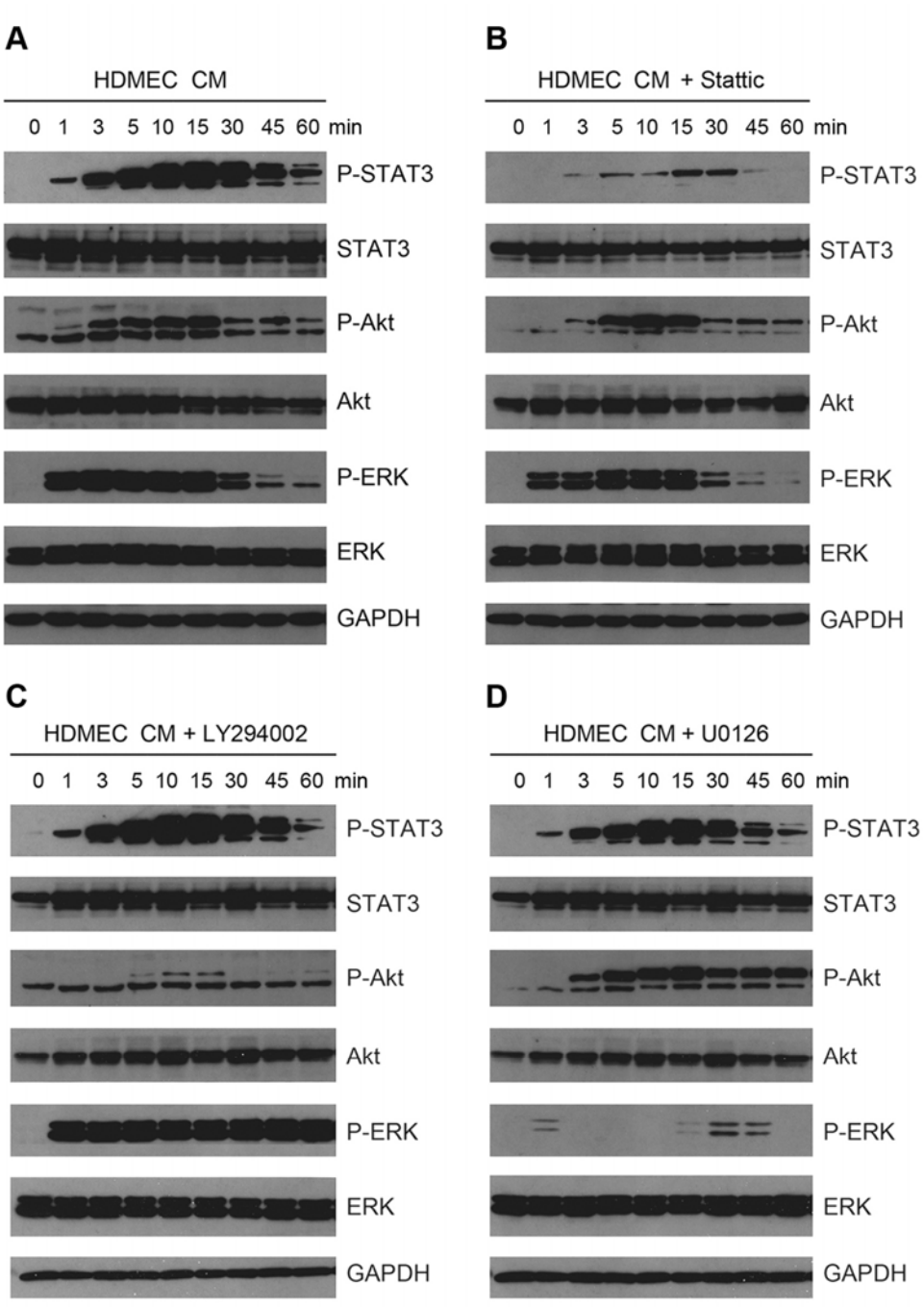

Figure 3.3. Blockade of endothelial cell-induced STAT3 phosphorylation in tumor cells does not affect Akt and ERK pathways, whereas inhibition of Akt or ERK have a compensatory mechanism and do not affect STAT3. OSCC3 were serum-starved overnight and exposed to (A) HDMEC conditioned medium (CM) or unconditioned medium (EBM) for the indicated time points. In addition, OSCC3 were pre-incubated for 1 to 2 hours with (B) 20 μM Stattic, (C) 20 μM LY294002, or (D) 20 μM U0126, and then exposed to HDMEC CM or EBM for the indicated time points. Phosphorylated and total STAT3, Akt, and ERK were determined by Western blot.

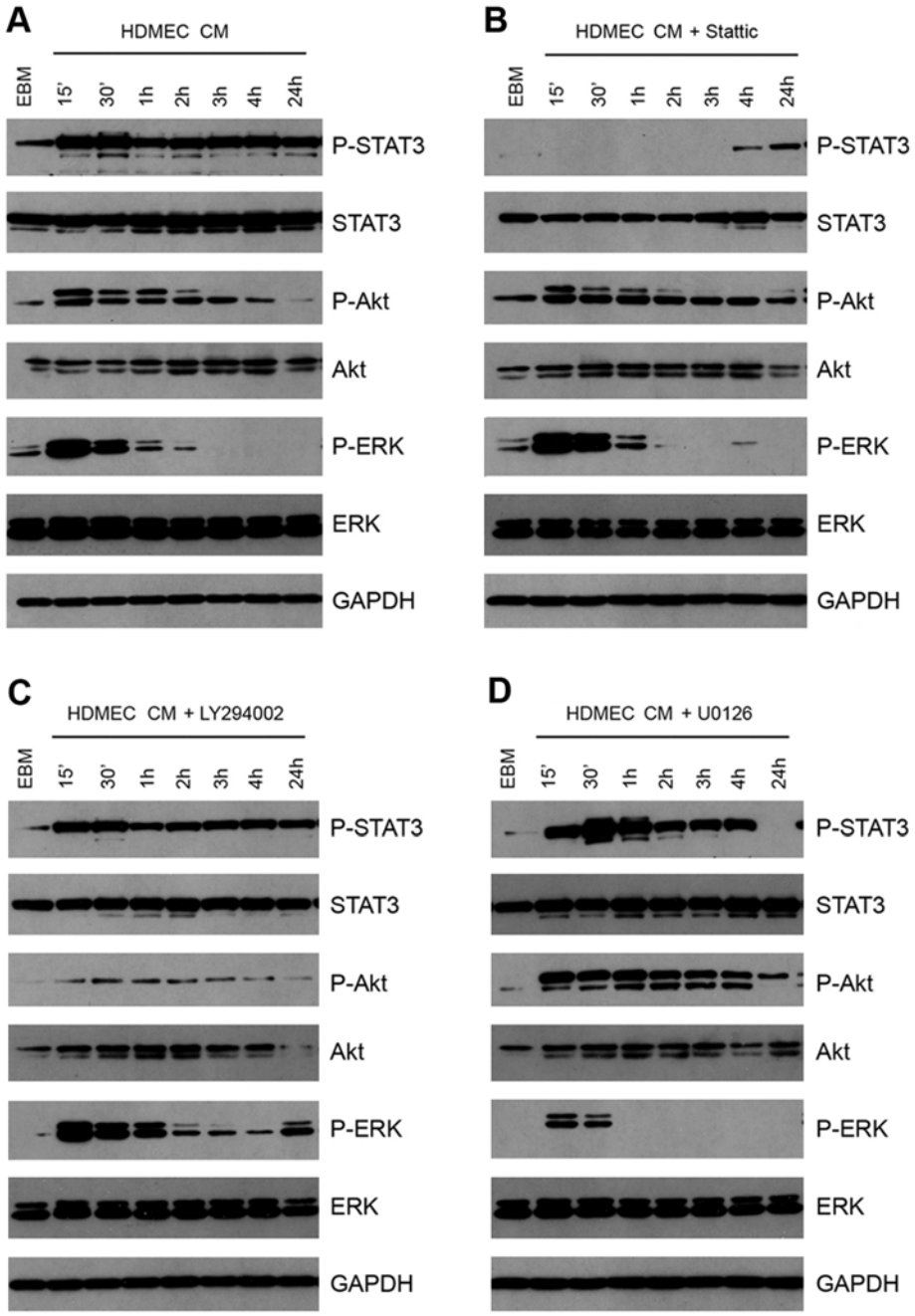

Figure 3.4. STAT3 phosphorylation induced by endothelial cell-secreted factors is independent of Akt and ERK phosphorylation. OSCC3 were serum-starved overnight and exposed to (A) HDMEC conditioned medium (CM) or unconditioned medium (EBM) for the indicated time points. In addition, OSCC3 were pre-incubated for 1 to 2 hours with (B) 20 μM Stattic, (C) 20 μM LY294002, or (D) 20 μM U0126, and then exposed to HDMEC CM or EBM for the indicated time points. Phosphorylated and total levels of STAT3, Akt, and ERK were determined by Western blot.

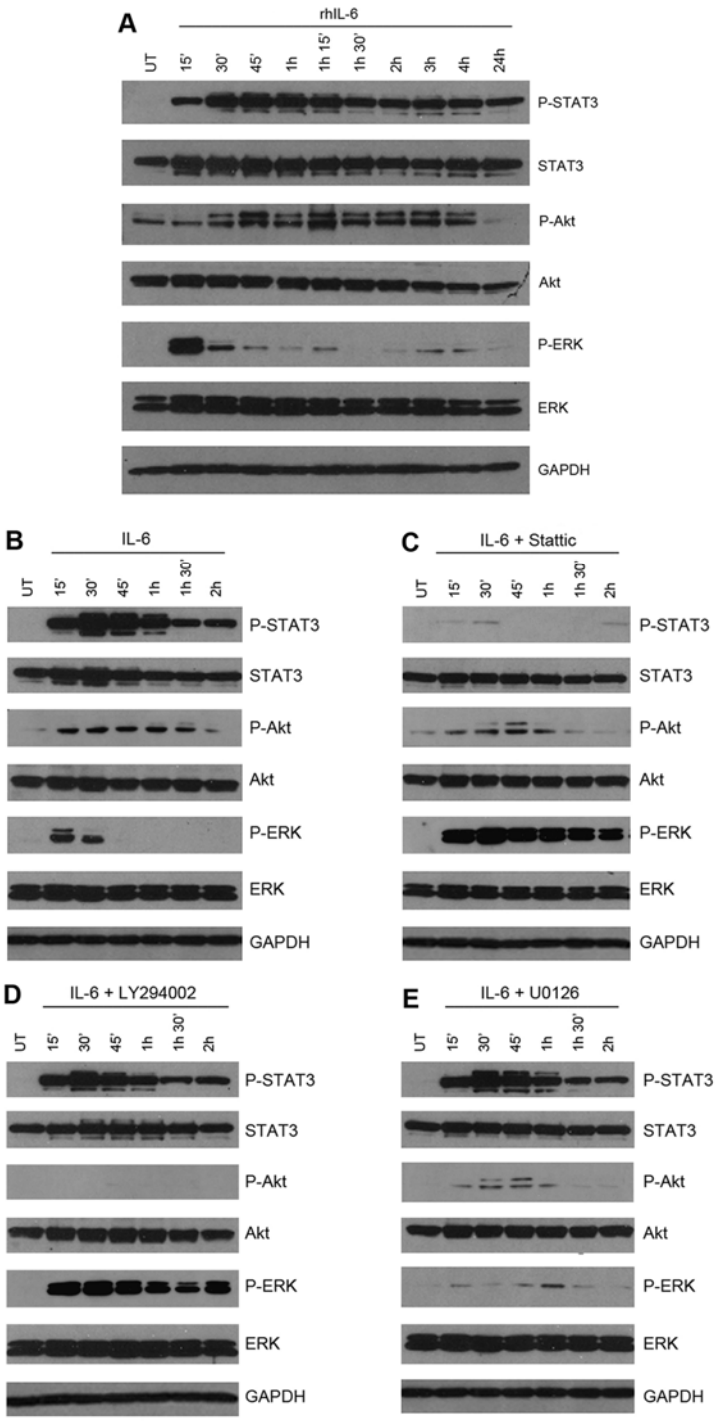

Figure 3.5. IL-6 potently activates STAT3 signaling in oral squamous cell carcinoma cells. (A-E) OSCC3 were serum-starved overnight and exposed to 20 ng/ml rhIL-6 for the indicated time points. Phosphorylated and total levels of STAT3, Akt, and ERK were determined by Western blots. (A) Extensive and detailed time course in OSCC3 exposed to rhIL-6. (B) Short time course in OSCC3 exposed to rhIL-6. In addition, OSCC3 were pre-incubated for 1 to 2 hours with (C) 20 μM Stattic, (D) 20 μM LY294002, or (E) 20 μM U0126, and then exposed to rhIL-6 for the indicated time points.

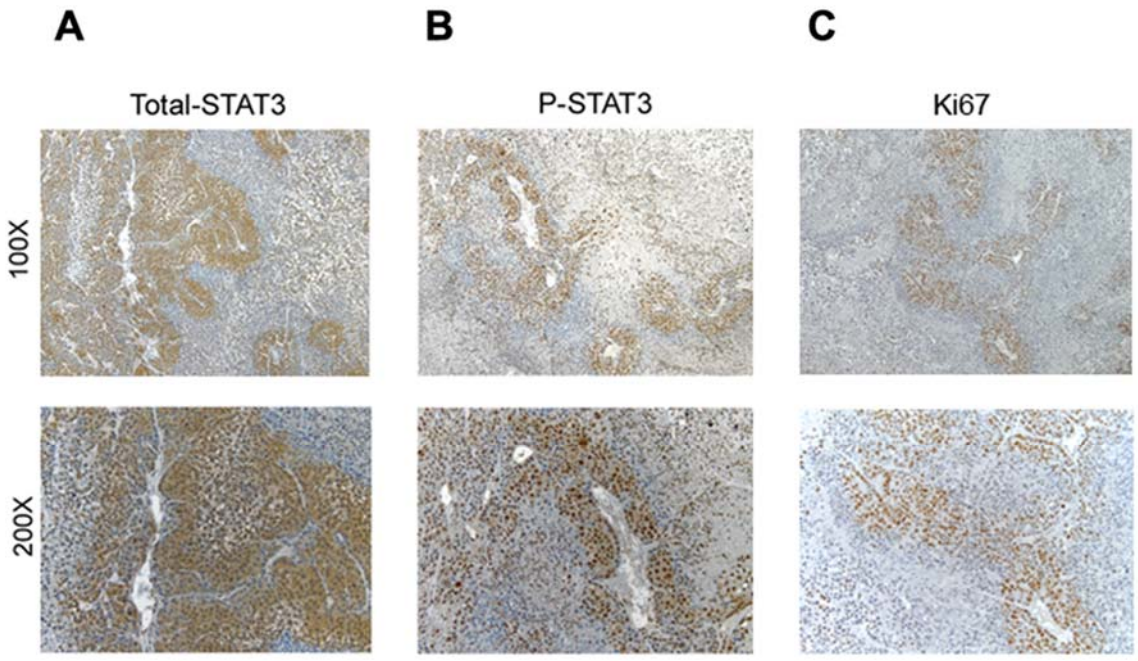

Figure 3.6. STAT3 phosphorylation in xenograft human oral squamous cell carcinomas correlates with tumor cell proliferation and presence of blood vessels. Xenograft human tumors were generated in SCID mice by coimplanting OSCC3 and HDMEC. Tumors were retrieved after 28 days, and tissues were analyzed by immunohistochemistry. Immunohistochemical analysis for (A) total STAT3 with nuclear and cytoplasmic localization, diffused through the tissue. (B) phosphorylated STAT3 with nuclear localization, concentrated in the proximity of blood vessels. (C) Ki67 with nuclear localization, concentrated adjacent to blood vessels. Top panels represent 100X and bottom panels represent 200X.

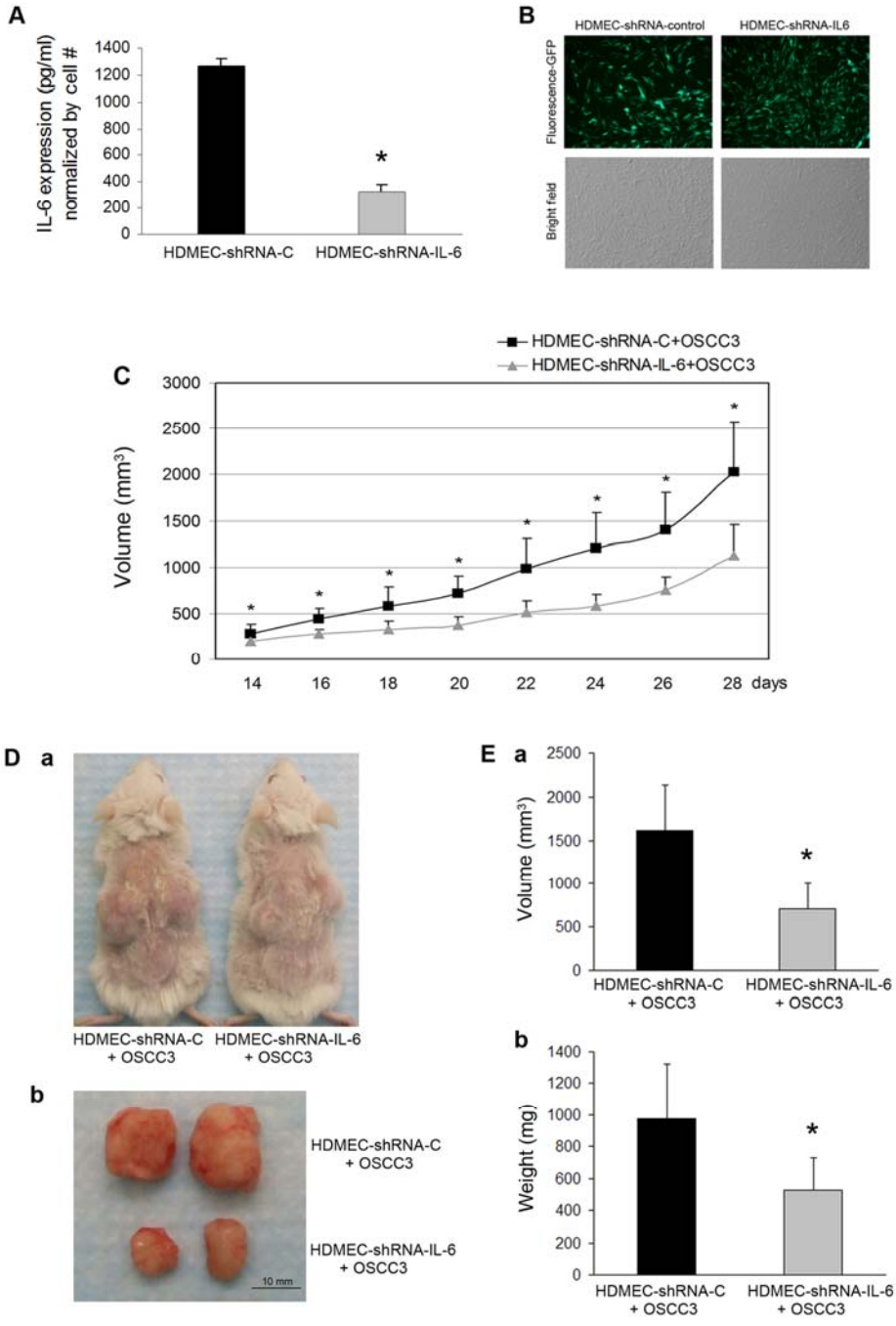

Figure 3.7. Downregulation of IL-6 in tumor-associated endothelial cells inhibits tumor growth. (A) ELISA for IL-6 expression in HDMEC transfected with shRNA-IL-6 or with a control scrambled oligonucleotide sequence (shRNA-C). (B) Fluorescence microscopy showing GFP expression in transfected-positive cells. (C) Tumor progression over time. (D) Macroscopic view of representative SCID mice bearing tumors (a), and the same tumors after retrieval (b). (E) Graphs depicting tumor volume (a) and tumor weight (b) after retrieval (28 days post-implantation). Asterisk depicts significant difference ($p<0.05$).

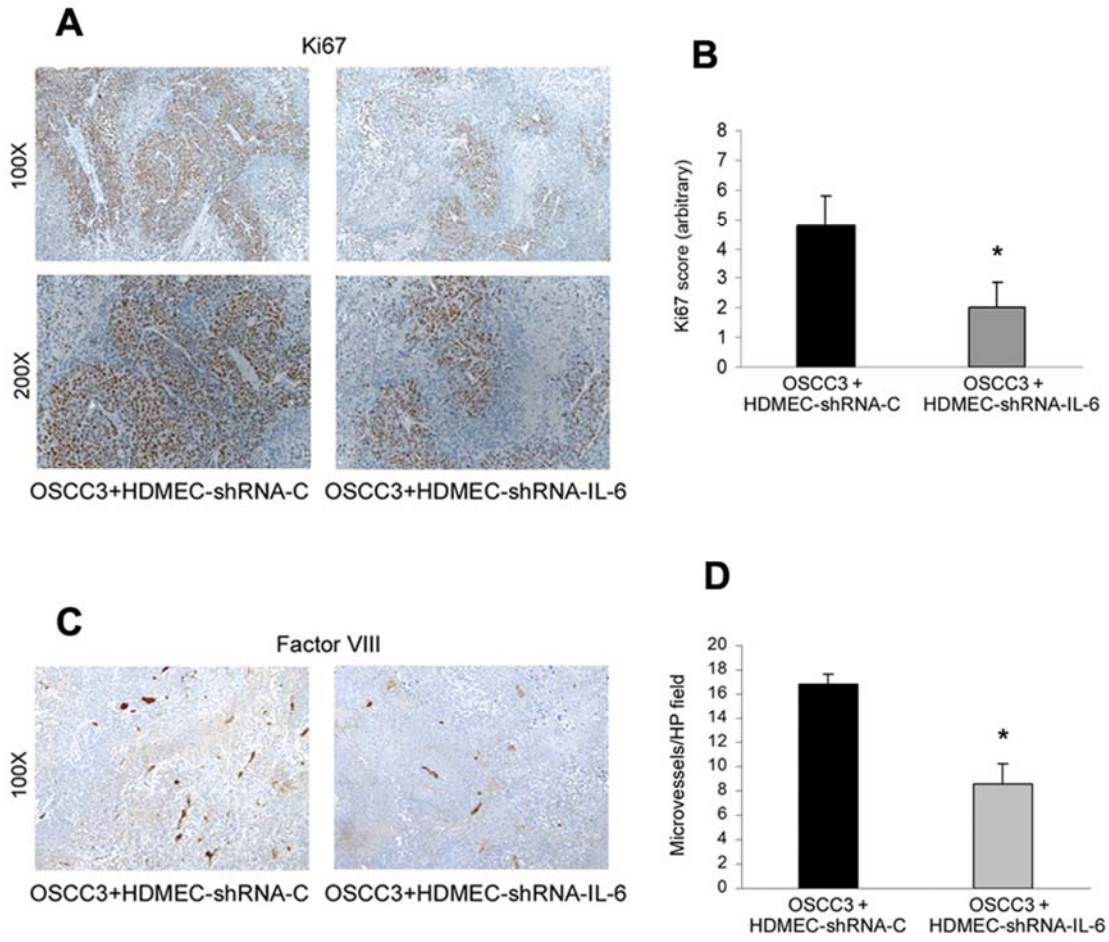

Figure 3.8. Downregulation of IL-6 in tumor-associated endothelial cells reduces tumor cell proliferation and decreases intratumoral microvessel density. (A) Immunohistochemical analysis for Ki67, indicating tumor cell proliferation in implants containing OSCC3 and HDMEC-shRNA-C, or OSCC3 and HDMEC-shRNA-IL-6 (top panels represent 100X and bottom panels represent 200X). (B) Quantification of tumor cell proliferation determined by scoring Ki67 immunostaining. Data represent mean values obtained in random microscopic fields (100X) from 12 tumors per condition. Asterisk depicts significant difference ($p<0.05$). (C) Immunohistochemistry for von Willebrand factor (Factor VIII) showing blood vessels (100X). Left panel shows a representative tumor populated with OSCC3 and HDMEC-shRNA-C, and right panel shows a tumor containing OSCC3 and HDMEC-shRNA-IL-6. (D) Quantification of microvessel density. Data represent mean values obtained in 10 random microscopic fields per implant (100X) from 12 tumors per condition. Asterisk depicts significant difference ($p<0.05$).

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CHAPTER IV

CONCLUSION

Our understanding of the process involving HNSCC development and progression has increased significantly over the past years. However, despite many advances in prevention and treatment of HNSCC, several challenges still remain. The poor prognosis for patients diagnosed with this devastating disease is in part a consequence of the limited knowledge of the signaling events underlying the malignant progression of HNSCC. Molecular targeted therapy is a promising approach to treat HNSCC and it has been shown to improve overall patient survival (Harari et al., 2009). With that in mind, we attempted to gain insights into the complex molecular events involving HNSCC cells and endothelial cells. The rationale to explore the crosstalk between endothelial cells and cancer cells is based on the knowledge that tumors are angiogenesis-dependent, and that this process of tumor angiogenesis requires active interaction between endothelial and tumor cells. The current paradigm in this field is that tumor cell-initiated signals are the dominant events in tumor angiogenesis and tumor growth, and little is known about the effect of endothelial cell-secreted factors on the phenotype and behavior of tumor cells. Here, we hypothesized that endothelial cells initiate signaling pathways in HNSCC cells that enhance tumor growth. The identification and characterization of the signaling events

initiated by tumor-associated endothelial cells may have important implications in cancer therapy.

In chapter II, we demonstrated that soluble mediators from primary human dermal microvascular endothelial cells activate three key intracellular molecules, namely STAT3, Akt, and ERK in a panel of HNSCC, suggesting that endothelial cells play an active role in the determination of tumor cell phenotype. Notably, we showed that endothelial cell-secreted factors were able to phosphorylate these signaling pathways in tumor cells without cell contact. One of the challenges was to find out which factors were mediating these effects in HNSCC cells. We then demonstrated that IL-6, CXCL8, and EGF were upregulated in endothelial cells co-cultured with HNSCC, and that blockade of endothelial cell-derived IL-6, CXCL8, or EGF inhibited the activation of STAT3, Akt, and ERK in tumor cells, respectively.

Trying to understand the mechanisms involved in this upregulation of IL-6, CXCL8, and EGF in endothelial cells, we explored a potential role for Bcl-2, based on previous reports. Previous studies have shown that tumor cell-secreted VEGF induces Bcl-2 expression in endothelial cells, and that upregulation of Bcl-2 in microvascular endothelial cells is sufficient to enhance tumor progression (Nor et al., 1999; Nor et al., 2001a). Notably, Bcl-2 is significantly upregulated in the endothelial cells of head and neck tumor microvessels in human patients (Kaneko et al., 2007). In addition, Bcl-2 induces CXCL1 and CXCL8 expression in endothelial cells (Karl et al., 2005). We observed that Bcl-2 signaling also induces expression of IL-6, CXCL8, and EGF, providing a mechanism for the upregulation of these cytokines in tumor-associated

endothelial cells. Moreover, Bcl-2 expression in endothelial cells had a significant impact on the phosphorylation status of STAT3 and ERK in HNSCC cells.

At this point, it was critical to us to expand our studies to better understand the biological significance of endothelial cell-induced activation of the STAT3, Akt, and ERK pathways in HNSCC. We demonstrated that endothelial cell-secreted factors significantly protected HNSCC cells from anoikis, and this protective effect was partially inhibited when we downregulated IL-6, CXCL8, or EGF in the endothelial cells. And finally, considering that IL-6, CXCL8, and EGF have been characterized as chemotactic factors, we explored their effect on tumor cell motility. Indeed, we observed that endothelial cells were able to generate a chemotactic gradient that induces tumor cell migration towards the endothelial cells. Based on these results, we speculated that endothelial cells play a key role in field cancerization by providing chemotactic signals that enhance the invasive phenotype of tumor cells, and by activating survival signals that protect the tumor cells against anoikis once these cells are displaced from their original microenvironment.

In chapter III, we explored the crosstalk between the endothelial cell-induced signaling pathways in HNSCC, with an emphasis on IL-6-mediated events. Here, we demonstrated that HNSCC cells exposed to endothelial cell conditioned medium presented higher levels of STAT3, Akt, and ERK phosphorylation than HNSCC cells exposed to conditioned medium collected from tumor cells. Furthermore, we observed that endothelial cell-secreted factors were also able to induce STAT3 and ERK phosphorylation in oral keratinocytes, suggesting that microvascular endothelial cells may also play a role in the early events of HNSCC tumorigenesis.

At this stage, it was important to verify whether these *in vitro* findings would have a correlation with *in vivo* levels of STAT3, Akt, and ERK phosphorylation in tumor cells associated with the presence of endothelial cells. Using the SCID mouse model of human tumor angiogenesis (Nor et al., 2001a; Nor et al., 2001b; Warner et al., 2008), we demonstrated that tumor cells adjacent to blood vessels express high levels of phosphorylates STAT3, Akt, and ERK, whereas total protein was expressed through the entire tissues.

Several studies suggest that aberrant activity of a network of interrelated signaling pathways rather than a single deregulated route contributes to carcinogenesis. Therefore, we explore the crosstalk between STAT3, Akt, and ERK signaling initiated by endothelial cells. Using chemical inhibitors for these pathways, we observed that endothelial cell-induced Akt and ERK signaling have a compensatory effect, while STAT3 pathway is activated independently. These results suggested that Akt and ERK cooperate in the progression of HNSCC, and that a possible feedback loop might exist in this network. On the other hand, STAT3 activation was independent of Akt and ERK phosphorylation status.

Due to the fact that STAT3 was an independent, consistent, and potent pathway activated by endothelial cells in HNSCC, we decided to further explore the effect of endothelial cell-secreted IL-6 *in vivo*. Using the SCID mouse model or human tumor angiogenesis, we observed a significant inhibition of tumor growth over time, tumor volume, and tumor weight when tumor-associated endothelial cells expressed low levels of IL-6. In addition, downregulation of IL-6 in tumor-associated endothelial cells reduced phosphorylation of STAT3 in tumor cells, inhibited tumor cell proliferation, and

decreased intratumoral microvessel density. Targeting the vascular endothelium rather than the cancer cells has been an attractive therapeutic approach for the treatment of several malignancies, since endothelial cells are directly accessible to systemic drugs, genetically stable, and less likely to acquire drug resistance (Kerbel and Folkman, 2002). Our results suggest that therapeutic blockade of signaling events initiated by endothelial cells might help to optimize the overall therapeutic benefit of molecular targeted drugs. The work presented here demonstrated that blockade of specific pathways in tumor associated-endothelial cells may have a direct impact on tumor cell survival, migration, and overall tumor growth.

Figure 4.1 proposes a model that incorporates the findings presented in this dissertation. We propose this new model for the crosstalk between endothelial and HNSCC cells. It is known that tumor cell-secreted VEGF binds to its cognate receptors in endothelial cells and induce expression of Bcl-2. Here, we demonstrated that Bcl-2 enhances IL-6, CXCL8, and EGF synthesis and secretion by endothelial cells. These endothelial cell-secreted factors activates STAT3, Akt, and ERK signaling pathways in tumor cells. The biological outcome of this crosstalk is a significant increase in tumor cell survival and migration, which will ultimately lead to an increase in tumor growth.

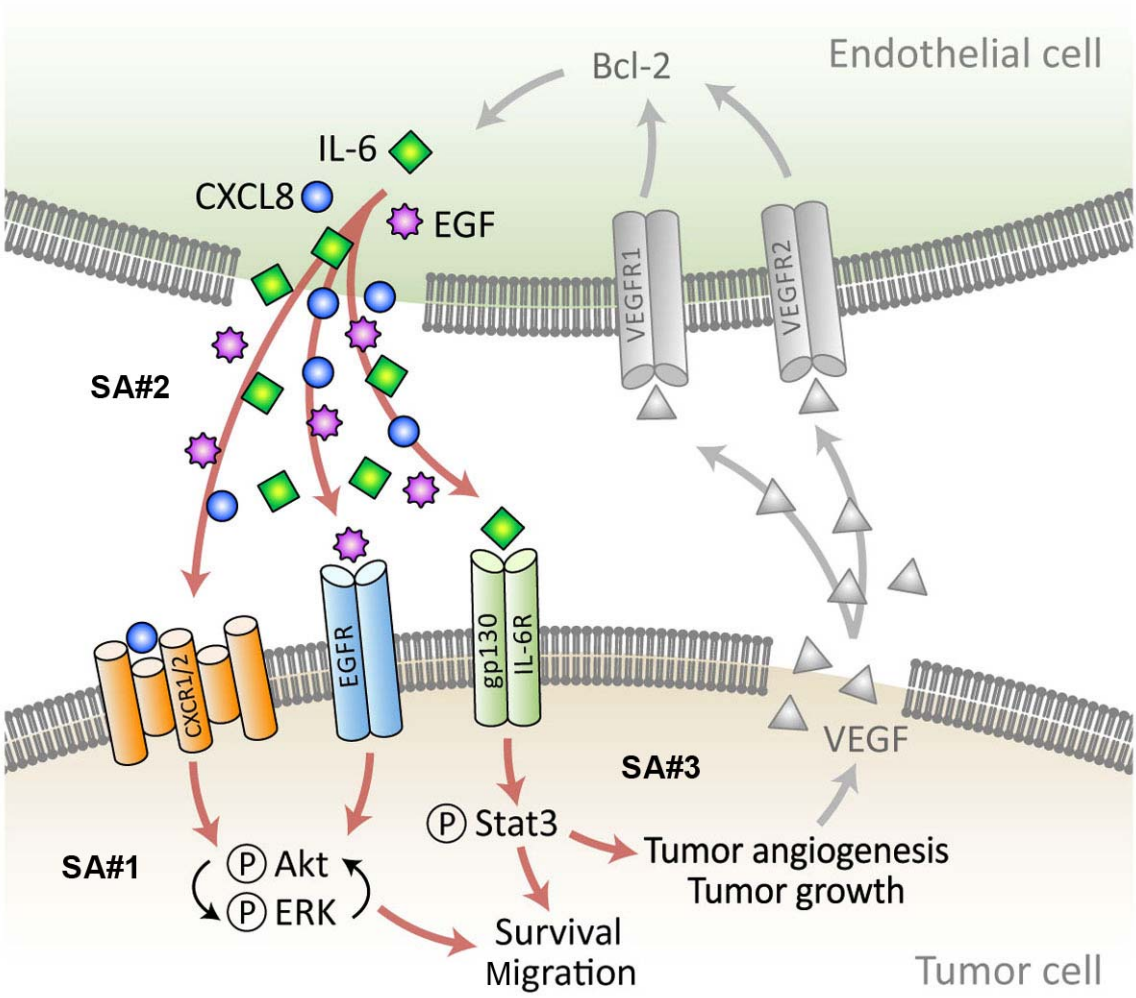

Figure 4.1. Diagram proposing a model for the endothelial cell-initiated crosstalk with tumor cells that is described in this dissertation. Endothelial cells secrete IL-6, CXCL8, and EGF that bind to their cognate receptors in tumor cells and induce phosphorylation of STAT3, Akt, and ERK. These phosphorylation events enhance tumor cell survival and migration. A positive feedback loop can be generated by STAT3 phosphorylation in tumor cells. It is known that STAT3 signal upregulation of VEGF expression in HNSCC and that tumor cell-secreted VEGF induces Bcl-2 expression in endothelial cells. Here, we showed that Bcl-2 signaling induces IL-6 secretion by endothelial cells, which in turn activate STAT3 in cancer cells, promote tumor angiogenesis, tumor growth, and maintain this feedback loop.

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