THE ROLE OF TRISTETRAPROLIN IN HEAD AND NECK SQUAMOUS CELL CARCINOMA

by

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DEDICATION

To my husband and my family-

For continuously supporting my academic endeavors through the years!

I owe it all to you.

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ABSTRACT

Head and neck cancer (HNSCC) is the sixth most common cancer in the world but its treatment has not significantly improved in several decades. Pro-inflammatory mediators such as interleukin-6 (IL-6), vascular endothelial growth factor (VEGF), prostaglandin 2 (PGE_2) and matrix metalloproteinases (MMPs) promote tumor invasion and angiogenesis. However, targeted inhibition of individual pro-inflammatory mediators or their receptors had limited success in the treatment of HNSCC. This is likely due to redundancy in function between pro-inflammatory mediators. Therefore, an attractive treatment target would be a common mechanism that regulates multiple pro-inflammatory mediators in HNSCC. The objective of this dissertation is to identify molecular mechanisms such as Tristetraprolin (TTP), an RNA-binding protein that mediates the decay of multiple pro-inflammatory mediators in macrophages. We determined that TTP expression was reduced in HNSCC and inversely correlated with the secretion of IL-6, VEGF, and PGE_2. Immunohistochemical staining of tissue microarrays for IL-6 demonstrated that staining intensity is prognostic for poor disease-specific survival, tumor recurrence and development of second primary tumors, and poor overall survival. Moreover, low TTP and high IL-6 are prognostic for poor disease specific survival in HNSCC. Upstream regulators of TTP were identified using complementary approaches. Rap1B, a small GTPase with a critical role in HNSCC, induces p38 MAPK-mediated phosphorylation (i.e. suppression) of TTP. Loss of TTP promotes mRNA

stability and secretion of IL-6, MMP-9, and MMP-2. Using *in vitro* and *in vivo* strategies, we demonstrate that suppression of TTP increases invasion of HNSCC via IL-6, MMP-9 or MMP-2. Moreover, high IL-6 and high MMP-9 are prognostic for poor clinical outcomes in HNSCC patients. In exploring the role of tumor derived ligands and angiogenesis, we determined that galanin activates p38 MAPK. siRNA-mediated knockdown of p38 MAPK decreases VEGF and PGE_2 secretion in HNSCC. p38 MAPK knockdown also decreases the length and number of endothelial cell sprouts, whereas TTP knockdown has the reverse effect. Knockdown of IL-6 in HNSCC cells transduced with shTTP reduces angiogenesis. In conclusion, targeting upstream regulators of pro-inflammaotry mediators, such as p38 and TTP, may improve treatment response of HNSCC by inhibiting multiple pro-inflammatory mediators that promote invasion and angiogenesis.

CHAPTER I

INTRODUCTION

PROBLEM STATEMENT

Globally, head and neck squamous cell carcinoma (HNSCC) affects 600,000 patients annually and 50% of these patients will succumb to the disease within five years of diagnosis[1, 2]. This survival rate is worse than cervical cancer, melanoma and lymphoma [3]. HNSCC secretes multiple pro-inflammatory mediators which likely contribute to their invasive capability [4]. However, targeted therapy against individual pro-inflammatory mediators had limited success in improving survival but treating patients with a cocktail of inhibitors is impractical. This underscores the need to understand the mechanisms that promote tumor progression in HNSCC so that novel therapeutic strategies can be developed to facilitate personalized medicine and ultimately to improve patient survival.

One novel approach to reduce pro-inflammatory secretion is to promote the degradation of pro-inflammatory mediator mRNA by shortening its half-life. Cytokines, at term that for the purposes of this dissertation will be used interchangeably with pro-inflammatory mediators, are highly regulated post-transcriptionally. Therefore identifying proteins that regulate cytokine mRNA stability could provide a unique mechanism to reduce their production in HNSCC, which could limit the aggressiveness

of the cancer. Tristetraprolin (TTP) is an RNA-binding protein (RNA-BP) that induces decay of cytokine mRNA by binding to the 3' untranslated region (UTR) of TNF-α, GM-CSF, COX-2 and IL-6 mRNA transcripts [5-8]. Very little is known about the role of TTP or its regulation in promoting tumor progression in any cancer. Moreover, neither TTP expression nor its function has been investigated in HNSCC. Given that TTP promotes the decay of multiple cytokines, loss of its function could be responsible for increasing cytokine secretion and facilitating tumor progression.

In this dissertation work, in the first chapter we will elucidate the role of TTP in modulating cytokine secretion in HNSCC. We demonstrate that TTP is downregulated in HNSCC and its expression is inversely correlated with cytokine secretion by altering mRNA stability. In the second chapter, we demonstrate how loss of TTP promotes tumor invasion and tumor progression via upregulation of IL-6, MMP-9 or MMP-2. We delineate the mechanism of TTP inactivation and show that rap1B activates p38 MAPK to phosphorylate (inactivate) TTP. Finally, we establish that the suppression of p38 MAPK decreases angiogenesis while loss of TTP has the reverse effect via upregulation of IL-6. Ultimately, due to its role in modulating secretion of multiple cytokines, alteration of TTP expression and function shows promise as a therapeutic strategy in the treatment of HNSCC.

GENERAL HYPOTHESIS

Downregulated Tristetraprolin (TTP) in HNSCC promotes the secretion of pro-inflammatory mediators that facilitate tumor progression.

Our general hypothesis will be tested by addressing the following specific aims:

Aim 1: To investigate the extent to which Tristetraprolin regulates pro-inflammatory mediators VEGF, IL-6 and PGE_2 in HNSCC (Chapter II).

Aim 2: To explore the mechanisms that regulate TTP function and determine how low TTP promotes invasion in HNSCC (Chapter III).

Aim 3: To determine the role of TTP in the promotion of angiogenesis in HNSCC (Chapter IV).

BACKGROUND AND SIGNIFICANCE

Head and neck squamous cell carcinoma – statistics, treatment and outcomes

Head and neck cancer (HNSCC) is the sixth most common cancer in the world[1]. Annually there are almost 600,000 people diagnosed with HNSCC and accounts for nearly 300,000 deaths each year[1, 9, 10]. With a five- year survival rate of approximately 50%, this disease has a poorer prognosis than cervical cancer, lymphoma and melanoma[3]. Unfortunately treatment has not improved greatly in forty years and is primarily based on the tumor stage using the TNM classification (tumor size, lymph node involvement and metastasis). Tumors that are less than 1 cm in diameter are treated with surgery alone, whereas late stage tumors are treated with a combination of surgery, radiation and chemotherapy. About 80% of all early stage HNSCC lesions are effectively treated with surgery; however, there is a subset of patients whose tumors recur and behave aggressively [11, 12]. Consequently, a significant number of patients with early stage

HNSCC who ultimately die of disease would likely have benefited from a more tailored treatment. Prognosis for patients with a recurrent primary tumor is very poor[13, 14]. In contrast, some advanced stage cancers are not as aggressive and would have benefited from less invasive treatment [15]. Thus, treatment selection based on tumor stage is not an optimal choice. This underscores the need for better therapeutic approaches in the treatment of HNSCC. Understanding the mechanistic basis for HNSCC tumor progression in the context of invasion and angiogenesis would facilitate the identification of treatment targets usable for personalized therapy and for the development of effective therapeutic strategies.

Development and progression of HNSCC

HNSCC is a lesion of surface epithelial origin and represents over 90% of all head and neck cancers[16]. During the progression towards malignancy, the surface epithelium undergoes cellular and architectural changes. When these changes are restricted to the surface epithelium, the pre-cancerous condition is termed epithelial dysplasia. Invasion of these cells beyond the basement membrane leads to the transformation to cancer. Mutations in cancer cells promote uncontrolled proliferation, invasion, survival, angiogenesis, evading growth suppressors, and replicative immortality which are the six hallmarks of cancer[17]. This dissertation will focus on mechanisms that promote invasion and angiogenesis in HNSCC.

Many of the mutations that facilitate malignant transformation occur in tumor suppressor genes and oncogenes. Tumor suppressor genes act like brakes in normal cell

function by turning off physiologic processes such as proliferation, invasion, survival, etc., when these processes are not warranted. The loss of tumor suppressor gene function facilitates pro-tumorigenic phenotypes. Alternatively, oncogenic mutations lead to overexpression of oncoproteins that enhance oncogenic phenotypes. Once normal epithelial cells develop a critical number of mutations, typically six or more [17], cells undergo malignant transformation; the basement membrane is degraded and cells invade the underlying connective tissue, a condition known as squamous cell carcinoma. While therapeutic strategies targeting oncogenes have had some success in treating certain cancers, targeting tumor suppressor genes is a challenge because the gene has to be replaced or activated.

Although a quarter of all diagnoses still have no known risk factors, the most common risk factors have been identified in the development of HNSCC. Most cases are due to tobacco use (smoking and/or chewing), alcohol consumption, positive Human Papillomavirus p16 (HPV16) status, genetic predisposition, environmental exposures, and nutrient consumption[18, 19]. Tobacco use is the dominant risk factor in HNSCC development making it 5 to 35 times more likely to develop when compared to non-smokers[18]. While alcohol consumption increases HNSCC risk 3 to 5 times compared to non-drinkers[20], when tobacco and alcohol consumption are combined the risk is magnified 100 times [21]. Recently, positive HPV16 status has been implicated in the development of HNSCC, primarily in the oropharyngeal region [22]. While triggers for HNSCC have been identified, the molecular basis for disease progression has not been completely elucidated. Moreover, since multiple factors can contribute to HNSCC development, it can lead to tumors that are very heterogenic from one another and it is

difficult to identify a single molecular target that would improve treatment for HNSCC patients.

Inflammation, metastasis and invasion in cancer

Nearly 25% of all cancers are associated with chronic inflammation. Alterations in host immune response, angiogenesis and metabolism are prominent features of HNSCC and many other cancers [23]. Tumor-induced immune cells respond to the tumor microenvironment to facilitate growth and survival through the production of pro-inflammatory, pro-angiogenic, and immunoregulatory cytokines that are produced by HNSCC and other cancers that contribute towards the pathogenesis of tumor progression [4, 23-25]. Immune cells themselves can promote tumor progression as well. The interaction between cancer cells and immune cells is critical to understand.

Innate immunity and chronic inflammation play major roles in cancer progression [26]. Chronic inflammation plays a large role in colon cancer and other cancers. High levels of pro-inflammatory mediators, such as IL-6, promote cellular proliferation and enhance mutation rates [27]. COX-2 is also involved in colon cancer progression; preventing COX-2 expression via COX-2 inhibitors limits cell cycle growth and progression of colon cancer. Thus, targeting inflammatory pathways can be beneficial to cancer treatment.

Pro-inflammatory mediators derived from immune cells associated with the tumor matrix can also support tumor growth and progression. Immune cells including natural killer (NK) cells and tissue associated macrophages (TAMs) and many others can be found within the tumor matrix and directly associated with the tumor [28]. NK cells normally target virally infected cells and cells that are undergoing stress for death. By

secreting enzymes, they can induce adjacent cells to undergo cell death by apoptosis or osmotic cell lysis. TAMs play an important role in degrading the apoptotic tumor cells, however they are thought to be the largest contributor to chronic inflammation in tumor cells that promotes tumor growth [28]. In HNSCC, TAMs [29] can secrete IL-6 and these macrophages can promote a paracrine loop with cancer cells to promote tumor progression [30]. It is not clear how these cells completely promote tumor progression, or how to effectively target these cells to inhibit tumor growth. Despite the fact that the immune cells can promote tumor progression, for the purposes of this thesis, pro-inflammatory mediators will be investigated in the context of the HNSCC itself.

Multiple cancers such as head and neck, breast and colon cancers, are associated with increased secretion of pro-inflammatory mediators[25]. In particular, HNSCC cells produce high levels of cytokines such as IL-4, IL-6, IL-8, IL-10, GM-CSF, VEGF, PGE_2, as well as bFGF [4, 23, 24]. These inflammatory cytokines have a critical role in malignant transformation including tumor growth, survival, invasion, angiogenesis and metastasis (spread)[31, 32]. One of the most critical events in HNSCC tumor progression is invasion. The presence of metastasis at the time of diagnosis decreases the prognosis even more[13]. Invasive cells are highly adept at surviving in different local environments and become highly motile. The disease may progress further to spread to regional lymph nodes or distant sites via the lymphatics. However it is unclear what molecular mechanisms promote tumor invasion, particularly in HNSCC.

In this dissertation, several pro-inflammatory mediators will be discussed in the context of tumor progression: IL-6, VEGF, PGE_2, MMP-2, and MMP-9.

IL-6 has many functions in homeostatic regulation, including a role in the immune system, in induction of inflammation, in bone resorption and production, and in various other cellular processes [33]. Tumor-secreted IL-6 induces tumor cell proliferation via autocrine and paracrine loops [34] and suppression of IL-6 decreases invasion in HNSCC[35]. High IL-6 in the serum is predictive of tumor recurrence and poor survival[36]. Moreover, studies have shown that IL-6 may promote invasion via feedback loops in colon cancer but neither the role of IL-6 in invasion nor its mechanism of regulation has been fully defined. [37]

Invasion and local spread of the tumor is one of the most important risk factors for poor prognosis. Matrix metalloproteinases (MMPs) have a critical role in promoting invasion and enhancing motility by remodeling the extracellular matrix[38]. Nearly 20 different forms of these MMP enzymes exist. Typically MMPs are secreted in an inactive state (latent form) and are then kept inactive by the maintenance of a cysteine residue in the pro-domain. Proteolytic cleavage of the pro-domain by MMPs unmasks the catalytic domain that contains a zinc ion [39]. Activation of MMPs leads to the degradation of a variety of components in the basement membrane and facilitates the movement of the tumor into adjacent areas. In HNSCC, MMPs such as MMP-2 and MMP-9 are collagenases/gelatinases and have a critical role in tumor progression [40]. Both MMP-2 and MMP-9 are prognostic for poor survival in HNSCC[40].

Tumor growth would not be possible without the continuous support of a blood supply. Angiogenesis is the production of new vasculature from existing vessels and supports tumor growth and invasion. Factors such as VEGF and PGE_2 promote angiogenesis. VEGF is the mostly widely studied inducer of angiogenesis and can be

both tumor-derived and endothelial-derived. It supports a feed forward loop that promotes angiogenesis[41]. PGE_2 is highly secreted in HNSCC[42] and promotes endothelial cell migration and proliferation[43]. In human tumors, PGE_2 is associated with VEGF localization and angiogenesis[44]. *In vivo* studies with a selective COX-2 inhibitor (a surrogate for PGE_2) decreased tumor growth of HNSCC cells in mice because of inhibition of angiogenesis [45].

Identifying biomarkers in HNSCC would improve survival by enabling development of novel therapeutic targets. Since HNSCC is very heterogeneous, it is likely that a panel rather than a single biomarker will be required to predict which tumors will behave aggressively. Given that HNSCC is associated with high levels of pro-inflammatory mediators, it was hypothesized that drugs targeting these pro-inflammatory mediators would be successful. For instance, *in vitro* studies have shown that inhibitors targeting endothelial growth factor receptors (EGFRs) were very successful in decreasing cell growth, invasion and metastasis[46]. However, EGF inhibitors, the only FDA approved adjuvant therapy in HNSCC[47], were not as successful as predicted in human clinical applications [48]. Patients that received the EGF inhibitor, Cetuximab®, saw only marginal improvement in survival when compared with patients given a placebo[49] and only 13% of patients responded to treatment[50]. Multiple other inhibitors targeting overexpressed pro-inflammatory mediators in cancers have been evaluated with similar results. As an additional example, *in vitro* studies with COX-2 inhibitors demonstrated decreased cell growth and/or invasion [51, 52], however clinical trials with COX-2 inhibitors in breast cancer patients who had progressed after initial treatment with chemotherapy saw no improved effect of treatment with the COX-2 inhibitors in combination with additional

chemotherapy [53]. Moreover, a trial with ovarian cancer was stopped due to increased cardiovascular risks[54]. In fact tail-vein injections of breast cancer cells treated for short periods of time with VEGF inhibitors were more aggressive and metastatic due to possible 'metastatic conditioning" [55]. The failure of these inhibitors targeting a single pro-inflammatory mediator may be primarily due to multiple pro-inflammatory mediators that have redundant functions and can compensate for one another. Treating patients with multiple drugs simultaneously is impractical due to drug toxicity. Therefore the identification of factors that regulate multiple pro-inflammatory mediators concurrently is likely an optimal option for improving treatment and survival.

Regulation of pro-inflammatory mediators

Pro-inflammatory mediators are essential to an intact and robust immune system. They are stringently regulated to ensure properly timed expression. They are highly regulated at many levels: transcriptionally, post-transcriptionally, and post-translationally [56, 57]. In the case of cancers that secrete pro-inflammatory mediators, the regulatory check-points have been overridden through mutations, epigenetic changes and gene amplifications. These genetic alterations lead to the overproduction of pro-inflammatory mediators that are detrimental for patient survival because they promote tumor proliferation, cell survival and invasion. Given that HNSCC as well as colon, breast and prostate cancers secrete copious amounts of pro-inflammatory mediators, identifying mechanisms that regulate post-transcriptional mRNA stability of pro-inflammatory mediators may be a promising therapeutic strategy. Recent studies have focused on

RNA-binding proteins as an area of interest because of their intrinsic ability to modulate pro-inflammatory mediator production by altering mRNA stability.

RNA-binding proteins

RNA-binding proteins (RNA-BPs) bind to the adenine and uridine rich elements (AREs) in the 3'-untranslated region (UTR) of mRNAs. Once bound to the mRNA they either promote stability or induce decay. For instance, RNA-BPs like human related antigen protein (HuR) bind to the AREs and induce mRNA stability and increase translation[6, 58]. In contrast, other RNA-BPs such as Tristetraprolin (TTP) induce mRNA decay. This is important for cancer research because AREs are located at the distal 3'-UTR of mRNA transcripts of many short-lived cytokines (e.g. granulocyte macrophage colony-stimulating factor [GM-CSF], TNF-α, IL-2, IL-3, and IL-6), and other pro-inflammatory factors (cyclooxygenase 2 [COX-2] and matrix metalloproteinase 13 [MMP-13])[7, 8, 59, 60]**.** The high evolutionary and inter-species conservation of AREs is evidence of the important regulatory role of these elements in cell survival and function. For example, the 3'-UTR of mouse and human IL-3 mRNA has 93% sequence homology compared to the 45% homologous open-reading frame [61]. This makes the study of RNA-BPs crucial for tumors that are highly inflammatory because they may be a method to target multiple pro-inflammatory mediators simultaneously.

Tristetraprolin ((TTP), also known as TIS11b, ZFP36 and located on chromosome 19) is a zinc finger binding protein that binds to the AREs in the 3'-UTR of mRNA and promotes decay by either 5'-3' degradation in exosomes or 3'-5' degradation in processing-bodies[59, 60]. The role of TTP has been extensively studied in the context of inflammation in macrophages due to the development of a *Zfp36* (TTP) knockout mouse. $TTP^{-/-}$ mice have excessive levels of circulating TNF-α and to a lesser extent GM-CSF [62].

Because of high circulating levels of TNF-α, by six months of age these mice are suffering from a severe arthritis-like syndrome including cachexia, dermatitis, myeloid hyperplasia and conjunctivitis[62]. Macrophages from these mice and wild type littermates have helped identify targets of TTP. Overexpression studies of TTP have also been employed to identify molecular targets and have shown that TTP overexpression reduces pro-inflammatory mediator secretion in macrophages [63].

The role of TTP in regulating multiple pro-inflammatory mediators in HNSCC is unknown. In fact, there are very few studies about the role of TTP in cancer. In breast cancer, low TTP expression correlates with high levels of VEGF, while in colon cancer low TTP promotes tumor survival via the upregulation of VEGF and COX-2. Furthermore, TTP acted as a "tumor suppressor" when transfected in to PB-3c (murine mast cell line) cells transfected with v-H-ras[64]. Additionally TTP overexpression in Colo320 cells decreased tumor growth in nude mice [65]. In fact one study found that TTP, located on chromosome 19q13.1, expression may be reduced due to CpG island methylation upstream to the promoter region [66], however it is not clear how TTP is downregulated in different epithelial derived cancers. Moreover, the role of low TTP in HNSCC progression via upregulation of multiple pro-inflammatory mediators has not been investigated.

TTP regulation

Mitogen-activated protein kinases (MAPKs) are critical signaling molecules in almost all eukaryotic cell types [67]. These kinases have crucial roles in the innate immune response in dendritic and other immune cells [68, 69]. The role of the stress-response p38

MAPK in initiation of inflammatory cytokine mRNA transcription is well known [70]. In contrast, the role of p38 MAPK in post-transcriptional regulation of mRNA of pro-inflammatory mediators is relatively unknown, especially in cancer [71, 72]. Upon p38 MAPK activation, signal transduction events occur, some of which mediate mRNA stability. For example, constitutive p38 MAPK activity increases cytokine expression, including COX-2, in colorectal cancer, and IL-6 is increased in multiple myeloma – both of which contribute to tumor proliferation [73, 74]. Traditionally, p38 MAPK pathway stimulation in cancer occurs through the interleukin IL-1β receptor (IL-1βR).

Excess p38 MAPK activation in some pathological conditions contributes to deregulated cytokine expression [75-77]. Consistent with this observation, p38 inhibitors used to treat IL-6-dependent multiple myeloma cells *in vitro* prevent proliferation and induce apoptosis [78]. In fact, clinical studies with p38 inhibitors in inflammatory diseases have shown promise in altering disease progression, but the mechanisms by which this occurs are presently unknown [79, 80]. Thus, mechanistic knowledge regarding how p38 MAPK influences pro-inflammatory mediator mRNA stability via phosphorylation of TTP could drive the discovery of specific therapeutic approaches to treat cancers associated with pro-inflammatory mediators.

TTP itself can be negatively regulated in inflammatory cells. In macrophages, TTP is inactivated via p38 MAPK-mediated phosphorylation. Upon phosphorylation at two serine sites[81, 82], TTP can no longer bind to the 3'-UTR of mRNA and is sequestered in processing bodies in the cytoplasm or is degraded [83, 84]. This leads to increased mRNA stability and subsequent translation. Inactivation of TTP is important during the inflammatory response since there is a need for the production of pro-inflammatory

mediators to promote healing. The effect of active p38 on mRNA stability has been explored through mitogen-activated protein kinase 2 (MK2). Once activated, MK2 is released from the nucleus and phosphorylates RNA-BPs such as tristetraprolin (TTP), resulting in activation or inactivation [85]. This change in downstream target protein function due to phosphorylated p38 and subsequent MK2 phosphorylation alters mRNA stability in macrophages[86-90]. While TTP may be inactivated directly via MK2, we focused our studies on p38-mediated phosphorylation of TTP in HNSCC.

While the mechanism of TTP phosphorylation has been elucidated in macrophages, the regulatory mechanisms of TTP inactivation are unknown in any cancer, especially from the perspective of tumor progression.

Although p38 MAPK has been shown to promote tumor progression in HNSCC [91], the upstream regulatory mechanism of p38 MAPK activation requires further investigation. Rap1B is a small guanosine triphosphatase (GTPase) that is a critical signaling molecule in HNSCC[40, 92-95]. Rap1B regulates multiple functions in the cells including differentiation, migration and proliferation. Rap1B shuttles between inactive guanosine diphosphate (GDP) and active GTP-bound forms. Guanine nucleotide exchange factors (GEFs) activate rap1B while the inactivation of rap1B is mediated by rap1GTPase activating proteins (rap1GAPs) [96]. Activation of rap1B stimulates multiple signaling pathways such as extracellular signal-regulated kinase (ERK) and phosphatidylinositol 3-kinase AKT to promote growth, differentiation, adhesion and apoptosis. In rap1B knockout mice, p38 MAPK activity is reduced[97]. Additionally endothelial cell migration and sprouting are reduced in these animals. This may be due to decreased secretion of VEGF and bFGF secretion in the rap1B null mice[97]. Rap1B is

activated and overexpressed in HNSCC [95], however its role in activating p38 MAPK in human cancer is unknown.

To summarize, pro-inflammatory mediators promote progression of HNSCC, a heterogeneous cancer of epithelial origin. Treatments that target multiple pro-inflammatory mediators simultaneously have not been identified. It is plausible that TTP, which is an RNA-binding protein that degrades multiple mRNAs, could be a potential treatment target. Moreover, upstream regulators of TTP have not been identified in any cancer. Since TTP can be inactivated by upstream kinases such as p38 MAPK in macrophages, p38 inhibitors thereby may decrease the secretion of multiple pro-inflammatory mediators simultaneously. The use of p38 inhibitors may be an adjuvant therapy for HNSCC patients and may potentially improve patient survival. The purpose of this study is to explore the role of TTP in HNSCC tumor progression. Figure 1.1 is the proposed signaling mechanism for TTP in HNSCC tumor progression.

ORGANIZATION OF DISSERTATION CONTENTS

In **Chapter II,** we establish the role of Tristetraprolin (TTP) in HNSCC in promoting the secretion of multiple pro-inflammatory mediators. Studies will focus on how TTP expression alters pro-inflammatory mediator secretion and the mechanism by which this occurs. Correlative studies in human HNSCC will evaluate TTP and IL-6 expression relative to patient survival.

Chapter III will investigate the mechanism by which low TTP promotes invasion and tumor progression both *in vitro* and *in vivo*. Then we will evaluate the interaction of

MMP-9 and IL-6 in terms of poor outcomes in HNSCC and lastly, we will identify upstream regulators of TTP. In **Chapter IV**, the role of TTP and tumor progression is investigated by identifying what factors promote angiogenesis in HNSCC and how upstream regulators of p38 promote angiogenesis. **Chapter V** presents a summary of the major findings and future directions of this dissertation work.

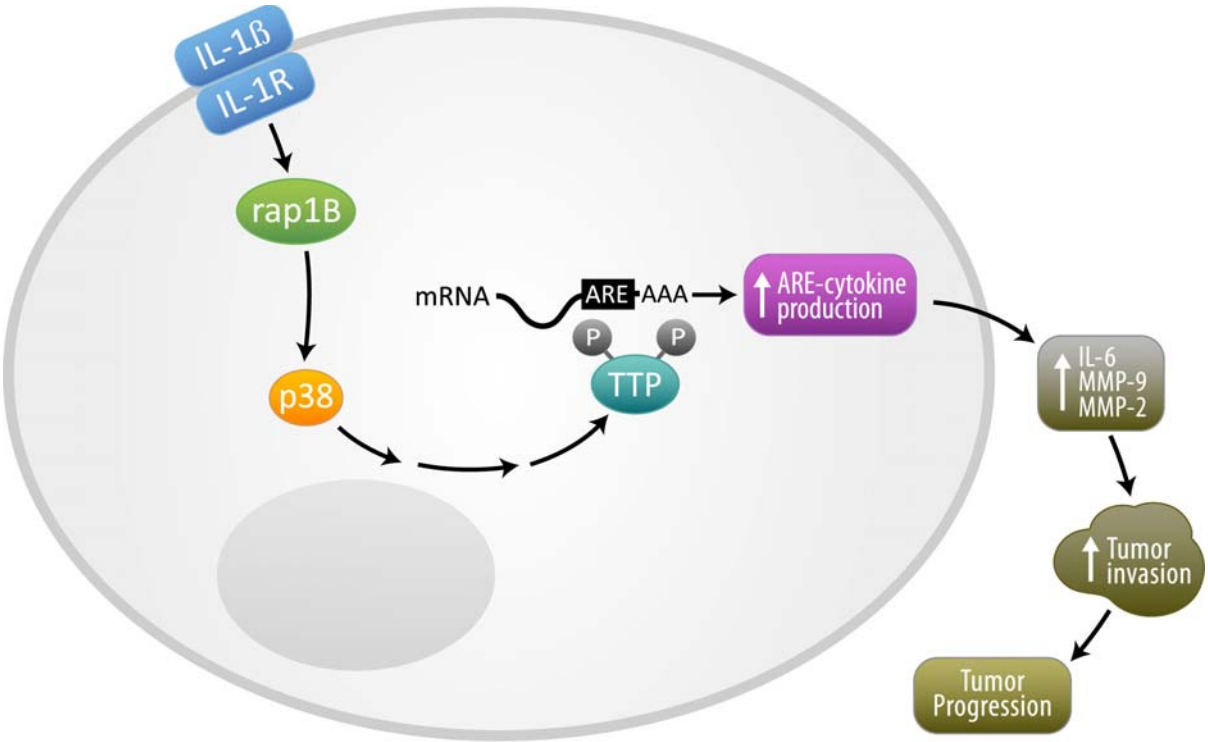

Figure 1.1: Proposed signaling mechanism of TTP in HNSCC tumor progression. Rap1B activates p38 MAPK that subsequently mediates TTP phosphorylation (inactivation). Loss of TTP function or expression leads to enhanced mRNA stability of multiple pro-inflammatory mediators (i.e. cytokines) which facilitate tumor progression.

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CHAPTER II

TRISTETRAPROLIN REGULATES INTERLEUKIN-6, WHICH IS CORRELATED WITH TUMOR PROGRESSION IN PATIENTS WITH HEAD AND NECK SQUAMPOUS CELLS CARCINOMA

ABSTRACT

Tumor-derived cytokines play a significant role in the progression of head and neck squamous cell carcinoma (HNSCC). Targeting proteins, such as tristetraprolin (TTP), that regulate multiple inflammatory cytokines may inhibit the progression of HNSCC. However, TTP's role in cancer is poorly understood. The goal of the current study was to determine whether TTP regulates inflammatory cytokines in patients with HNSCC. METHODS: TTP messenger RNA (mRNA) and protein expression were determined by quantitative real-time–polymerase chain reaction (Q-RT-PCR) and Western blot analysis, respectively. mRNA stability and cytokine secretion were evaluated by quantitative RT-PCR and enzyme-linked immunoadsorbent assay, respectively, after overexpression or knockdown of TTP in HNSCC. HNSCC tissue microarrays were immunostained for interleukin-6 (IL-6) and TTP. RESULTS: TTP expression in HNSCC cell lines was found to be inversely correlated with the secretion of IL-6, vascular endothelial growth factor (VEGF), and prostaglandin E_2 (PGE_2). Knockdown of TTP increased mRNA stability and the secretion of cytokines. Conversely, overexpression of TTP in HNSCC cells led to decreased secretion of IL-6, VEGF, and PGE_2. Immunohistochemical staining of tissue microarrays for IL-6 demonstrated that staining intensity is prognostic for poor

disease-specific survival (P = .023), tumor recurrence and development of second primary tumors (P = .014), and poor overall survival (P = .019). CONCLUSIONS: The results of the current study demonstrated that down-regulation of TTP in HNSCC enhances mRNA stability and promotes secretion of IL-6, VEGF, and PGE_2. Furthermore, high IL-6 secretion in HNSCC tissue is a biomarker for poor prognosis. In as much as enhanced cytokine secretion is associated with poor prognosis, TTP may be a therapeutic target to reduce multiple cytokines concurrently in patients with HNSCC.

INTRODUCTION

Cytokines have a significant role in cancer, including roles in malignant transformation, tumor growth, survival, invasion, angiogenesis, and metastasis.1-3 Several cytokines and pro-inflammatory factors, including interleukin-1 (IL-1), IL-4, IL-6, IL-8, IL-10, granulocyte-macrophage colony-stimulating factor (GM-CSF), vascular endothelial growth factor (VEGF), prostaglandin E2 (PGE_2), and cyclooxygenase-2 (COX-2), are up-regulated in cancer.[1,4-6] Some of these molecules mediate the role of inflammation in cancer. By promoting cell proliferation, invasion, and survival, cytokines facilitate tumor progression in multiple cancers, including colorectal, breast, head and neck, and non-small cell lung cancers.[3,7,8] Because cytokines play a critical role in tumor progression, their expression is highly regulated at many levels: messenger RNA (mRNA) transcription, post-transcription, and the initiation of protein translation. Post-transcriptional regulation of cytokines occurs at different stages such as nuclear export, cytoplasmic localization, stability/degradation, and translation of RNA into protein.[9] RNA-binding proteins (RNA-BPs) are trans-acting factors that regulate these events by

binding cis elements on target mRNA. Cis elements such as adenine and uridine (AU)-rich elements (AREs) are located transcripts. Because cytokine transcripts contain AREs in their 3-UTR, proteins that promote degradation or stability of transcripts by binding to this region have therapeutic potential.

Expression of RNA-BPs that regulate cytokine mRNA stability may be altered in cancer.[10,11] In breast cancer, overexpression of human-related antigen R (HuR), an RNA-BP that stabilizes mRNA, contributes to poor prognosis and the increased secretion of VEGF.12 Conversely, in colon cancer, tristetraprolin (TTP), which decreases mRNA stability, is down-regulated, correlating with increased COX-2 expression.[13] These changes in RNA-BPs underscore their significance in cancer progression.

HNSCC is the sixth most common cancer, annually affecting approximately 500,000 individuals world-wide.[14] The 5-year survival rate is approximately 50%, primarily because of late detection. HNSCCs secrete multiple cytokines including VEGF, IL-6, and COX-2.15-18 Because COX-2 and IL-6 inhibitors decrease cell proliferation and invasion in HNSCC in vitro, cancer therapy targeted toward tumor-secreted inflammatory cytokines may inhibit tumor progression.[3,17-19] Treating HNSCC with agents directed at a single cytokine is unrealistic because of the secretion of multiple cytokines with over- lapping functions.20-22 Given the importance of cytokines such as IL-6, PGE_2, tumor necrosis factor- α (TNF-α), VEGF, and epidermal growth factor in HNSCC progression,[3,5,6] down-regulation of multiple cytokines simultaneously by targeting a common upstream protein may inhibit HNSCC progression. However, to our knowledge, the role of TTP in the regulation of multiple cytokines has not been investigated in HNSCC. Further- more, the relation between TTP expression and

cytokine secretion in HNSCC progression has not been established. In the current study, we investigated whether TTP regulates multiple inflammatory cytokines in HNSCC and the significance of the overexpression of one of these cytokines on tumor progression.

MATERIALS AND METHODS

Cell Culture. The human HNSCC cell lines UM-SCC-1, UM-SCC-5, UM-SCC -11A, UM-SCC-14A, UM-SCC-17B, UM- SCC-22B, UM-SCC-74A, and UM-SCC-81B and OSCC3 cells were cultured as described previously.[23,24] UM-SCC-1, UM-SCC-5, UM-SCC-11A, UM-SCC-14A,UM-SCC-17B, UM-SCC-22B, UM-SCC-74A, and UM- SCC-81B were validated by genotyping. OSCC3 cells were obtained from Dr. Peter Polverini at the University of Michigan and were genotyped in the University of Michigan DNA Sequencing Core. The human papillomavirus type 16 (HPV-16)-immortalized human oral keratinocyte cell line (HOK-16B, a generous gift from Dr. No-Hee Park, University of California at Los Angeles) was maintained in low-calcium (Ca^{2+}) keratinocyte growth medium (Cascade Biologics, Portland, Ore).[25] Primary human oral keratinocytes (NHK) were cultured in oral keratinocyte growth medium (ScienCell, Carlsbad, Calif).

Western Blot Analysis. Whole-cell lysates were prepared by sonicating cell suspensions on ice, and Western blot analysis was performed as described.[24] Membranes were blocked and incubated in the primary antibody overnight at 4°C or for 1 hour at room temperature. Antibody concentrations were as follows: rabbit anti-TTP (Abcam, Cambridge, UK) at a dilution of 1:2000, actin (Cell Signaling Technology, Inc, Danvers, Mass) at a dilution of 1:3000, and horseradish peroxidase-conjugated donkey anti-rabbit

immunoglobulin (Ig) G and goat anti-mouse secondary antibodies (dilutions of 1:2000 to 1:5000; Jackson ImmunoResearch Laboratories, Inc, West Grove, Pa). Immunoreactive proteins were observed using the SuperSignal West Pico Chemiluminescent Substrate system (Pierce, Rockford, Ill) and exposed to x-ray film.

Quantitative-Real-Time-Polymerase Chain Reaction. Total RNA was isolated from cells with QIAzol (Qiagen, Valencia, Calif). Complementary DNA (cDNA) was synthesized using SuperScript II (Invitrogen, Carlsbad, Calif). Quantitative real-time polymerase chain reaction (Q-RT-PCR) was performed with SYBR Green Master Mix on an Applied Biosystems ABI 7500 RealTime PCR System (Applied Biosystems, Foster City, Calif). Data were analyzed using the delta-delta cycle threshold method with normalization to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and then expressed relative to NHK.

Adenoviral Infection and IL-1β Induction. A DL3 replication-deficient serotype-5 adenovirus (Ad-5) containing cytomegalovirus (CMV)-β–galactosidase (gal) and human TTP were generated through the University of Michigan Vector Core as described.[26] The cell lines UM-SCC-11A and UM-SCC-17B and OSCC3 cells ($35X10^4$) were transduced with 1000 multiplicity of infection (MOI) Ad-5–β-gal or Ad-5–TTP in serum-free media. Three hours after transduction, 10% fetal bovine serum (FBS) was added. Twenty-one hours later, HNSCC cell lines were serum starved for 4 hours and incubated with 1 ng/mL of IL-1β or phosphate-buffered saline (PBS) in serum-free media. Conditioned media were collected after 24 hours.

Enzyme-Linked Immunoadsorbent Assay for IL-6, VEGF, and PGE_2. Conditioned medium from NHK, HOK-16B, OSCC3, and HNSCC cell lines (UM-SCC-1, UM-SCC-11A, UM-SCC-14A, UM-SCC-17B, UM-SCC-22B, UM- SCC-74A, and UM-SCC-81B) was processed as described.[27] The total cell number was measured with a trypan blue enumeration assay. IL-6 and VEGF secreted into the medium were measured by a noncompetitive enzyme-linked immunoadsorbent assay (ELISA) whereas PGE_2 was measured using a competitive ELISA (R&D Systems, Minneapolis, Minn).

Transient TTP Knockdown. The UM-SCC-1, UM-SCC-22B, and UM-SCC-81B cell lines were transfected with ON-TARGET plus TTP (*Zfp36*) SMART Pool (Dharmacon Inc, Lafayette, Colo) small interfering RNA (siRNA). The UM-SCC-1 cell line was transfected with Nucleofectin (Amaxa Biosystems, Gaithersburg, Md) as described.[23,28] The UM-SCC-22B and UM-SCC-81B cell lines ($25X10^4$ in 6 well dishes) were transfected with 1 μg of siRNA using Lipofectomine RNAimax (Invitrogen), as described.[29] Four hours after transfection, 10% FBS was added. Conditioned media and whole-cell lysates were collected as described earlier.

Stable TTP Knockdown and mRNA Decay. For stable knockdown of TTP, the UM-SCC-1 and UM- SCC-22B cell lines were infected with short hairpin RNA (shTTP and sh vesicular stomatitis virus glycoprotein [VSVG] control) in lentiviral particles in a VSVG back- bone (University of Michigan Vector Core). Stable cell lines were selected in 10 μg/mL of puromycin (Sigma Chemical Company, St. Louis, Mo). For mRNA decay

experiments, $35X10^4$ cells were plated in 60-mm dishes. The next day, the cells were serum starved for 4 hours and treated with 10 nM of actinomycin D for 0, 1, 2, and 3 hours (Sigma Chemical Company). After incubation, cells were washed with PBS and RNA was harvested.

University of Michigan Oral Cavity/ Oropharyngeal Cancer Organ Preservation Trial. This randomized clinical trial of patients with stage III/IV HNSCC (TNM staging system) compared patients treated concurrently with chemotherapy and radiotherapy versus those treated with surgery and radiotherapy after induction chemotherapy.[30] A tissue microarray (TMA) constructed from pretreatment specimens from this clinical trial was used for immunhistochemical studies after Institutional Review Board approval.

Immunohistochemistry. Immunodetection in tissue sections was performed as described[24] with affinity-purified anti-IL–6 (R&D Systems; 25 μg/mL), affinity-purified TTP antibody (Abcam; 3 μg/mL), or the corresponding IgG controls at the same concentrations.

Statistical Analysis. Statistical analysis of in vitro assays was performed using a Student t test or 1-way analysis of variance (ANOVA). A P value <.05 was considered to be statistically significant. For analysis of TMA data, interpretation and scoring were performed by a board certified pathologist, as described.[23,28] The covariates of interest were T classification and N classification, which were analyzed as ordinal data. The outcomes of interest were overall survival, disease-specific survival (DSS), time to

indication of surgery at the primary tumor site, and time to disease recurrence or the development of second primary tumors. The Spear- man correlation coefficient was used to evaluate univariate associations between markers and numerical and ordinal variables of interest. The Cox proportional hazards model was used to relate time-to-event outcomes to marker levels and other numerical and ordinal covariates. Statistical analyses of TMA data were performed using SAS statistical software (version 9.0; SAS Institute Inc, Cary, NC). A 2-tailed P value significant of .05 was considered to be statistically significant.

RESULTS

TTP Is Down-Regulated in HNSCC. TTP expression was evaluated by quantitative real-time– PCR and normalized to GAPDH. The TTP transcript was lower in 6 HNSCC cell lines compared with NHK (Fig. 2.1A) ($P < .05$, Student t test). The UM-SCC-11A and UM-SCC-22B cell lines had significantly more TTP mRNA than NHK ($P < .05$, Student t test). Whole-cell lysates from 9 HNSCC cell lines and from 2 nonmalignant cell lines NHK and immortalized keratinocytes (HOK-16B, designated as IHOK) were immunoblotted with anti-TTP antibody. As shown in Figure 2.1B (immunoblot and densitometric analyses), TTP expression was higher in NHK and IHOK compared with HNSCC cell lines when normalized to actin as a loading control.

OSCC3 cells expressed the least amount of TTP com- pared with NHK (83% reduction), whereas the UM- SCC-81B cell line demonstrated the least reduction in TTP expression compared with NHK. There was an over- all decrease in TTP expression of 62% (standard deviation [SD], 24.6%) in HNSCC cell lines compared with NHK.

IL-6, VEGF, and PGE_2 were quantified by ELISA in conditioned media from normal and IHOK and HNSCC cells to correlate TTP expression with cytokine secretion. In 7 of the 11 HNSCC cell lines, IL-6 secretion was found to be significantly up-regulated (*indicates $P < .001$, 1-way ANOVA for all cell lines noted) in HNSCC cell lines compared with NHK. VEGF and PGE_2, the other 2 cytokines evaluated, demonstrated a similar trend. The increase in VEGF secretion was significant in 8 of 9 HNSCC cell lines (*indicates $P < .001$, 1-way ANOVA). The increase in PGE_2 secretion was significant in 5 of 9 HNSCC cell lines ($P < .001$, 1-way ANOVA). Of the cell lines evaluated, NHK secreted the least VEGF and PGE_2 (Figs. 2.1D and 2.1E, respectively). IHOK demonstrated increased VEGF and PGE_2 secretion compared with normal cells, but less than that observed in the HNSCC cell lines. The UM-SCC-81B cell line secreted high levels of all 3 cytokines evaluated, despite high TTP expression in these cell lines (Fig. 2.1B). However, in the majority of cell lines (>65%), TTP expression was found to be inversely correlated with cytokine secretion.

Overexpression of TTP Reduces Cytokine Secretion in HNSCC. Three HNSCC cell lines with approximately 50% reduced endogenous TTP expression compared with NHK (Fig. 2.1B) were used for overexpression studies. To verify TTP overexpression, whole-cell lysates were generated from HNSCC cell lines transduced with Ad-5 that contained either TTP or β-gal (control) and were immunoblotted for TTP. As shown in Figure 2.2A, TTP was overexpressed in the UM-SCC-11A cell line UM-SCC- 17B cell line, and OSCC3 cells transduced with Ad-5– TTP compared with cells transduced with control vector.

IL-1β is secreted by inflammatory cells adjacent to a tumor and activates the p38 mitogen-activated protein kinase (MAPK) pathway within tumor cells.[31] Mitogen-activated protein kinase-activated protein kinase 2 (MK2), the downstream target of p38 MAPK, inactivates TTP by phosphorylation.[32,33] Therefore, we investigated the effect of TTP on cytokine secretion in the presence and absence of IL-1β. In mock-transduced cells, IL-1β increased cytokine secretion in all 3 cell lines, which is consistent with inactivation of residual TTP. However, overexpression of TTP led to a significant reduction in IL-6, VEGF, and PGE_2 secretion in all 3 cell lines (Figs. 2.2B, 2.2C, and 2.2D) (*indicates $P < .001$) when compared with cells transfected with control vector, even in the presence of IL-1β.

In a complementary approach, TTP expression was down-regulated by RNA interference (RNAi) in 3 HNSCC cell lines representing a spectrum of TTP expression. Knockdown of TTP, verified by immunoblot analysis, led to increased secretion of IL-6, VEGF, and PGE_2 (Fig. 2.3). In the UM-SCC-1 cell line, small interfering TTP (siTTP) mediated a significant increase in all 3 cytokines evaluated (Fig. 2.3A) ($P < .05$). IL-6 secretion was increased more than PGE_2 secretion in the UM-SCC- 22B cell line ($P < .05$). VEGF secretion demonstrated a similar trend (Fig. 2.3B). In the UM-SCC-81B cell line, which strongly expresses TTP (Fig. 2.1A), siTTP also induced a significant increase in the secretion of IL-6, VEGF, and PGE_2 (Fig. 2.3C) ($P < .05$). Collectively, over-expression and knockdown data suggest that TTP inversely regulates IL-6, VEGF, and PGE_2 secretion in HNSCC.

TTP Regulates Cytokine mRNA Stability in HNSCC. To determine whether TTP

targets cytokine mRNA for degradation in HNSCC, cells were treated with actinomycin D, which inhibits DNA polymerase. Stable cell lines with shTTP or shVSVG in the UM-SCC-1 and UM- SCC-22B cell lines were generated. Knockdown of TTP, confirmed by immunoblot analysis (Fig. 2.4A), increased cytokine mRNA stability. For the UM-SCC-1 cell line, mRNA transcripts of IL-6 and COX-2 in shTTP transduced cells remained between 90% and 100% stable over a 3-hour period. In contrast, in cells transduced with shVSVG, mRNA transcripts degraded to nearly 50% at 3 hours (Fig. 2.4B) ($P < .05$). Similarly, in the UM-SCC- 22B cell line, IL-6 and COX-2 mRNAs were more stable in cells transduced with shTTP (approximately 100% after 3 hours) compared with control (shVSVG) cells, in which IL-6 and COX-2 mRNA were degraded in 3 hours (Fig. 2.4C) ($P < .05$).

Increased IL-6 Secretion Is Correlated With Human HNSCC Progression. Immunohistochemical studies were performed on normal epithelial and HNSCC tumor tissue and a TMA of pretreatment tissue specimens from HNSCC. Normal epithelial tissue was found to demonstrate strong TTP staining in the basal one-third of the epithelium (Fig. 2.5A Left, indicated by arrows), whereas the invasive epithelial islands in the HNSCC tissue did not demonstrate strong TTP staining (Fig. 2.5A Right, inset). IgG controls were found to be negative (data not shown). Figure 2.5B (Top and Bottom) demonstrates high IL-6 and low TTP staining within the same HNSCC core. IgG controls were appropriately negative (data not shown). Low TTP and high IL-6 intensity were found to be correlated with poor DSS, ($P = .043$) (Fig. 2.5C). In addition, TMA findings revealed that high IL-6 intensity is prognostic for bad patient outcomes, specifically poor

DSS (P = 0.023) (Fig. 2.5D), tumor recurrence or the development of a second primary tumor (P= 0.014) (Fig. 2.5E), and poor overall survival (P = 0.019) (data not shown). Thus, these findings based on a small sample size indicate that IL-6 and TTP expression are inversely correlated and that IL-6 is associated with poor patient outcome.

DISCUSSION

Changes in oncogenic mRNA stability can alter the production of inflammatory cytokines. For example, up-regulation of HuR, an RNA-BP that stabilizes mRNA, enhances COX-2 and VEGF production.[34,35] This increase in cytokine production is detrimental because tumor-derived cytokines facilitate oncogenic phenotypes such as invasion, proliferation, and survival, thereby promoting tumor growth.[1,3,7,13,36] Consistent with this theory, increased COX-2 production in colon cancer increases tumor invasion.[8] The results of the current study demonstrate that down-regulation of TTP in HNSCC stabilizes transcripts and promotes the secretion of multiple cytokines, including IL-6, VEGF, and COX-2. Furthermore, IL-6 and TTP expression are inversely correlated in human HNSCC tissue. High IL-6 expression is predictive of poor DSS, tumor recurrence, and the development of second primary tumors. On the basis of the findings in this study performed in a small group of patients, patients with HNSCC tumors with high IL-6 staining intensity appear to have a much poorer prognosis than those patients with tumors expressing IL-6 at low levels.

HNSCC is a disease with a poor prognosis, with 5- year survival rates of approximately 50%.[37] HNSCCs secrete multiple growth, inflammatory, and angiogenic factors, including VEGF, IL-6, IL-8, and GM-CSF, which promote tumor progression.[3,4,5]

IL-6 promotes tumor growth in mice by promoting the survival and proliferation of HNSCC cells.[2] Other cytokines, such as VEGF, promote angiogenesis and metastasis.[37,38]

The critical role of cytokines in the progression of many cancers makes them an attractive treatment target.[3,4,36,39] In a murine model, IL-6 monoclonal antibodies decreased the size of prostate cancer xenografts significantly.[40] However, targeting individual cytokines is unlikely to be of therapeutic benefit in humans. For example, results from a clinical trial in patients with multiple myeloma with antibodies against IL-6 indicated that there was no improvement in clinical outcome for these patients.[39] This suggests that cytokines secreted can compensate for one another and therefore this limits the effect of an inhibitor specific for each cytokine. Targeting a common regulatory mechanism for multiple cytokines such as IL-6, VEGF, and PGE_2 in HNSCC may decrease tumor progression and improve response to treatment.

Cytokine mRNA expression is tightly regulated in resting cells through continuously active mRNA decay mechanisms. Induction of mRNA decay via inflammatory signals facilitates rapid changes in the cellular production of cytokines through alterations in binding of RNA-BP.[41] RNA-BPs regulate multiple cytokines by inducing decay or inhibiting translation. RNA-BPs may bind to multiple AREs, possibly contributing to competition for binding. At least 20 different proteins that can bind to ARE segments have been identified to date, including TTP, HuR, butyrate response factor (BRF)-1 and BRF-2, ARE/ poly(U)-binding/degradation factor-1 (AUF-1), T cell intracellular antigen-1 (TIA-1) and T-cell-restricted intracellular antigen-related protein (TIAR). However, only a subset of RNA-BPs has been shown to influence the stability or translational efficiency of target mRNAs.

One of the most well-studied RNA-BPs is TTP, which targets mRNA for rapid degradation by binding to the AREs in the 3-UTR.[42] Studies with knockout mice suggest that TTP is crucial in regulating TNF- α and IL- 6.[43] Because TTP regulates mRNAs of multiple inflammatory cytokines by increasing turnover and inducing decay, loss of TTP may lead to the increased production of multiple cytokines, which may be associated with tumor progression. In some cancers, such as colon cancer, loss of TTP contributes to tumor growth by increasing COX-2, VEGF, or matrix metalloproteinase-1 (MMP-1) production.[12,13,44,45] However, these studies did not evaluate how TTP overexpression influences the production of multiple cytokines. In the current study, we demonstrated that knockdown of TTP in HNSCC stabilizes the transcripts of multiple cytokines and enhances invasion and migration.

Because of the host-tumor microenvironment, inflammatory mediators, such as IL-1β, produced by inflammatory cells that are adjacent to the tumor may activate the p38 MAPK pathway within the tumor cells.[31] Therefore, in the current study, we investigated the effect of TTP on cytokine secretion in the presence or absence of IL-1β. However, TTP overexpression was found to decrease the secretion of cytokines regardless of stimulation with IL-1β. Although the absolute value of the reduction (ie, pg/mL/million cells) was greater with the addition of IL-1β, the fold-decrease in cytokine secretion after TTP overexpression was greater in unstimulated compared with IL-1β–treated cells, which is consistent with inactivation of part of the overexpressed TTP by IL-1β.

TTP expression was found to be reduced in all HNSCC cell lines compared with NHK. We observed that there was a 62% (SD, 24.6%) reduction in TTP expression in HNSCC compared with NHK. However, reduced TTP expression was not found to

correlate 100% with increased cytokine secretion in HNSCC cells, which may be because of phosphorylation-mediated inactivation of TTP. MK2, the downstream target of p38 MAPK, phosphorylates TTP at serine sites 52 and 178 and renders the protein inactive by inducing binding of TTP to 14-3- 3 protein.[32,33] We observed that TTP expression was inversely correlated with cytokine secretion except for the UM-SCC-81B cell line, which was found to strongly express TTP but secreted high levels of cytokines. This may be the result of TTP phosphorylation because active p38 MAPK, which inactivates TTP by phosphorylation, is up-regulated in HNSCC. We were unable to immunoprecipitate TTP using available antibodies, but p38 MAPK is active in this cell line. Collectively, these data suggest that loss or inactivation of TTP contributes to the production of multiple cytokines in HNSCC. Because p38 MAPK can be constitutively active in HNSCC,[15] the results of the current study suggest that inhibition of p38 MAPK in HNSCC would decrease cytokine secretion by preventing inactivation of TTP.

We also observed that TTP knockdown increased the secretion of multiple cytokines, but the effects on individual cytokines varied. This suggests that other pathways or multiple RNA-BPs are involved in the regulation of cytokines and the final effect on secretion may be determined by a balance among these factors. For example, TTP overexpression in mouse embryonic fibroblasts isolated from TTP knockout mice decreased VEGF but increased proliferation, whereas TTP overexpression in HeLA cells decreased cellular proliferation,[12] suggesting that other proteins may influence proliferation in different cell types. These studies underscore the importance of delineating how cytokines are regulated in the context of a particular cell or tissue.

IL-6 is a pleiotropic cytokine that functions during inflammation, immunity, bone

metabolism, neural development, reproduction, and hematopoiesis.[46] Although epithelial malignancies may have elevated IL-6, to the best of our knowledge, the presence of tumor-derived IL-6 in HNSCC has not been investigated in the context of tumor progression.[47] The identification of biomarkers that are prognostic of poor survival will allow for the selection of tumors for more aggressive treatment. The current study findings, derived from a small group of patients, suggest that HNSCCs with low TTP and high IL-6 expression have poorer DSS than HNSCCs with high TTP and low IL-6 expression. Similarly, in breast cancer, down-regulation of TTP is a negative prognostic indicator associated with increased tumor grade and mortality.[12] However, TTP expression may not be the actual prognostic indicator in HNSCC. Our findings with IL-6, independent of TTP expression, support this theory. HNSCCs with high IL-6 expression have poor outcomes, including poor DSS, tumor recurrence, and the development of second primary tumors. Consistent with these observations, high serum IL-6 in HNSCC patients is correlated with poor overall survival and an increase in IL-6 during treatment is associated with tumor recurrence.[37,48] Furthermore, high serum IL-6 is associated with resistance to radiotherapy.[49]

Given the importance of cytokines in invasion and proliferation, both of which contribute to HNSCC progression, disruption of cytokine secretion is an attractive treatment strategy. However, targeting cancers with a cocktail of inhibitors is impractical and, as mentioned earlier, targeting individual cytokines, such as monoclonal antibodies against IL-6 in patients with multiple myeloma, is ineffective in improving patient survival.[36] Therefore, up-regulation of a protein, such as TTP, that inhibits multiple cytokines is an attractive treatment strategy, particularly if it can be combined with p38

inhibitors, because p38, which inactivates TTP, is constitutively active in HNSCC.[15] However, because of the importance of cytokines in the immune response, the potential for systemic immunosuppression as a side effect of p38 inhibitors would be a challenge, particularly because it may promote tumor progression.[50, 51] This may be overcome by strategies that specifically target the treatment to tumor cells. Future studies will explore the mechanisms by which TTP-mediated cytokine secretion regulates tumor progression and the feasibility of targeting this protein to control tumor growth.

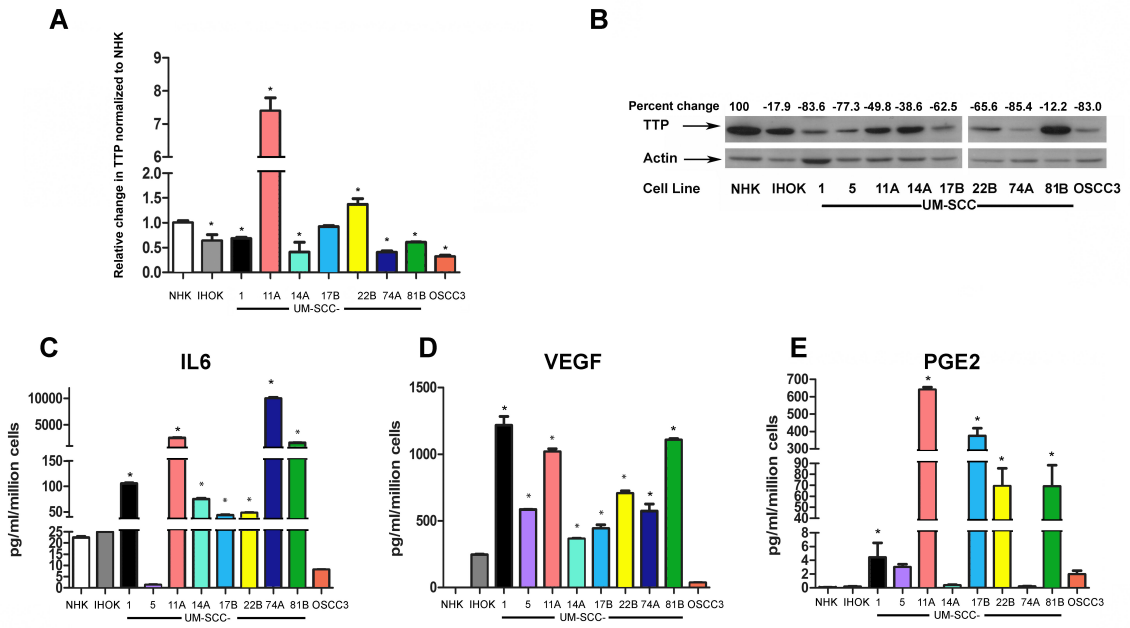

Figure 2.1: Tristetraprolin (TTP) is down-regulated in head and neck squamous cell carcinoma (HNSCC) cell lines. (A) Total RNA was isolated from normal human keratinocytes (NHK), HOK-16B immortalized keratinocytes (IHOK), and HNSCC cell lines. Complementary DNAs (cDNAs) were prepared and quantitative real-time–polymerase chain reaction was performed using SYBR Green Master Mix (Applied Biosystems, Foster City, Calif). (B) Whole-cell lysates from NHK, IHOK, and HNSCC cell lines were electrophoresed and immunoblotted with TTP antibody. Actin was used as a loading control. TTP expression was quantified by densitometry, normalized to actin and then to NHK, and expressed as the percentage change. (C, D, and E) Conditioned media from NHK, IHOK, and HNSCC cell lines was collected at 24 hours (*indicates $P < .001$ compared with NHK). Enzyme-linked immunoadsorbent assay experiments were performed in triplicate with similar results.

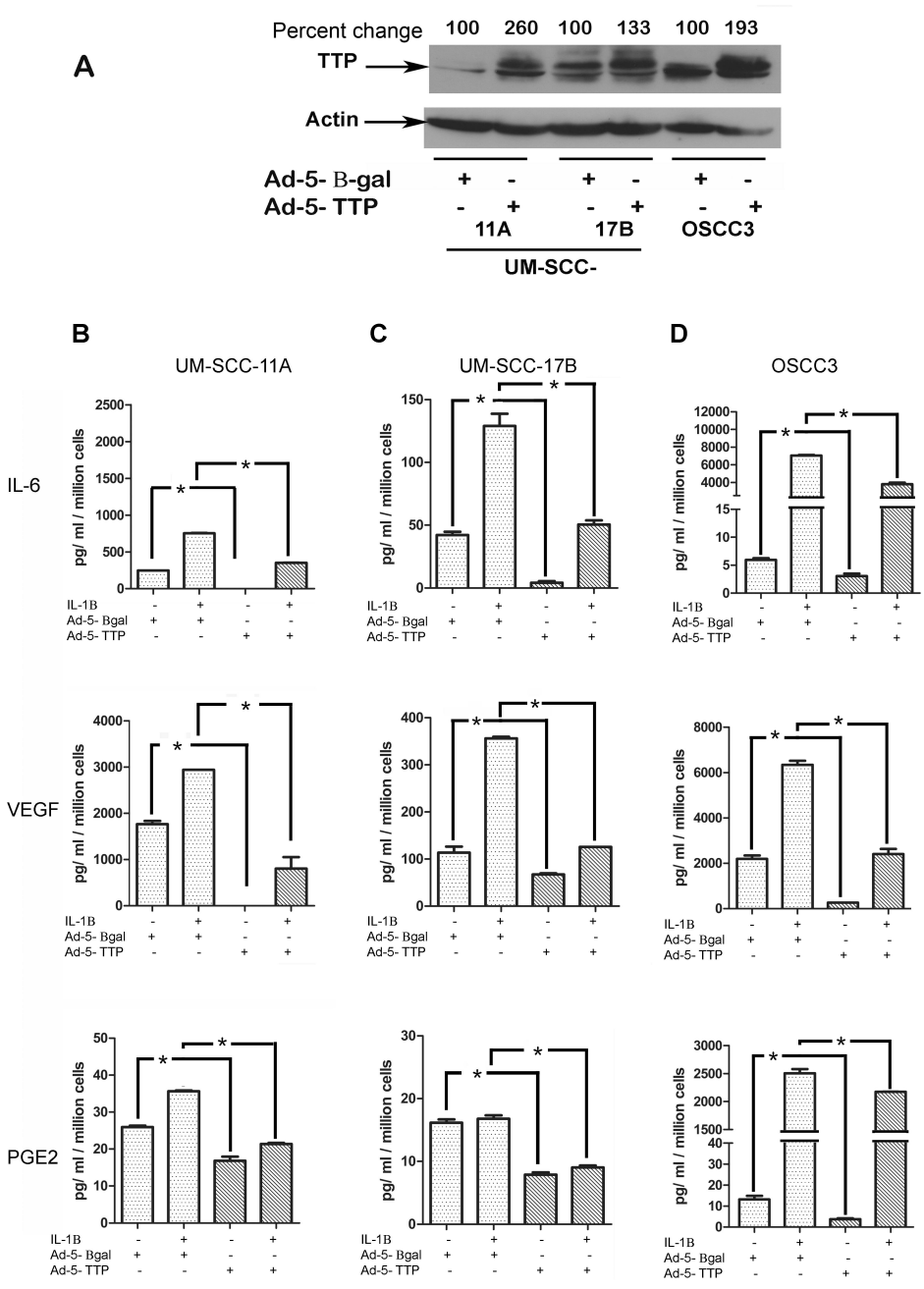

Figure 2.2: Overexpression of tristetraprolin (TTP) in head and neck squamous cell carcinoma (HNSCC) cell lines decreases cytokine secretion. (A) The UM-SCC-11A cell line, (B) UM-SCC-17B cell line, and (C) OSCC3 cells were transduced with serotype-5 adenovirus containing β-galactosidase (β-gal) (control) or TTP. Cell lysates and conditioned media were harvested. Cell lysates were electrophoresed and immunoblotted with TTP antibody. Conditioned media was used to quantify interleukin-6 (IL-6), vascular endothelial growth factor (VEGF), and prostaglandin E2 (PGE_2) secretion by enzyme-linked immunoadsorbent assay. All experiments were performed in triplicate (*indicates $P < .001$). Standard error bars are present in all graphs. + indicates positive; -, negative.

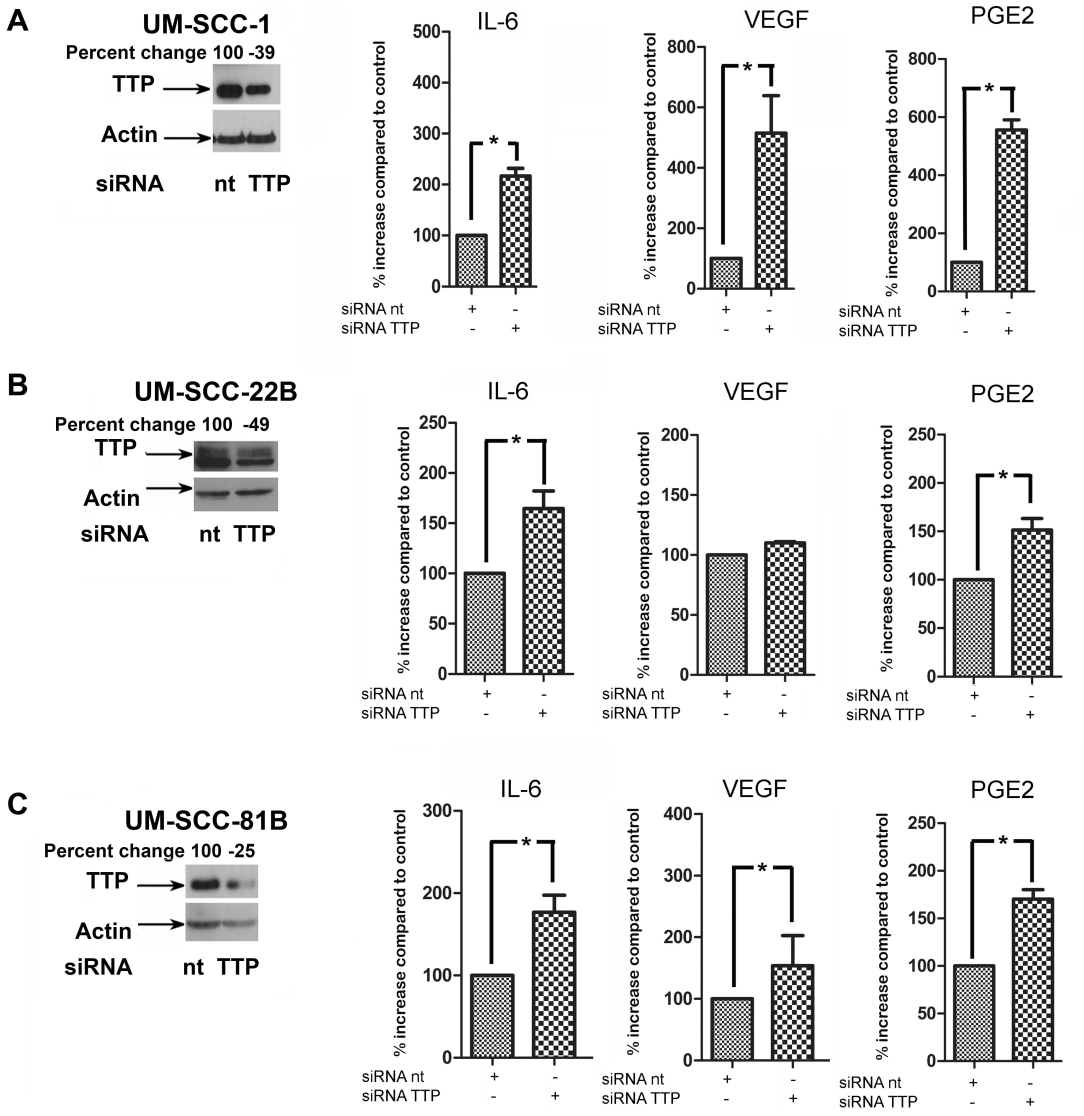

Figure 2.3: Tristetraprolin (TTP) knockdown induces cytokine secretion in head and neck squamous cell carcinoma cells. (A) The UM-SCC-1, (B) UM-SCC-22B, and (C) UM-SCC-81B cell lines were transfected with small interfering RNA (siRNA) against TTP or nontarget (nt) siRNA. Whole-cell lysates were electrophoresed and immunoblotted with TTP antibody. Actin was used as loading control. Conditioned media was assayed in triplicate for interleukin-6 (IL-6), vascular endothelial growth factor (VEGF), and prostaglandin E2 (PGE_2) by enzyme-linked immunoadsorbent assay. All experiments were performed in triplicate. Data are representative of the mean and standard deviation of 3 replicates within an experiment (*indicates $P < .05$). + indicates positive; -, negative.

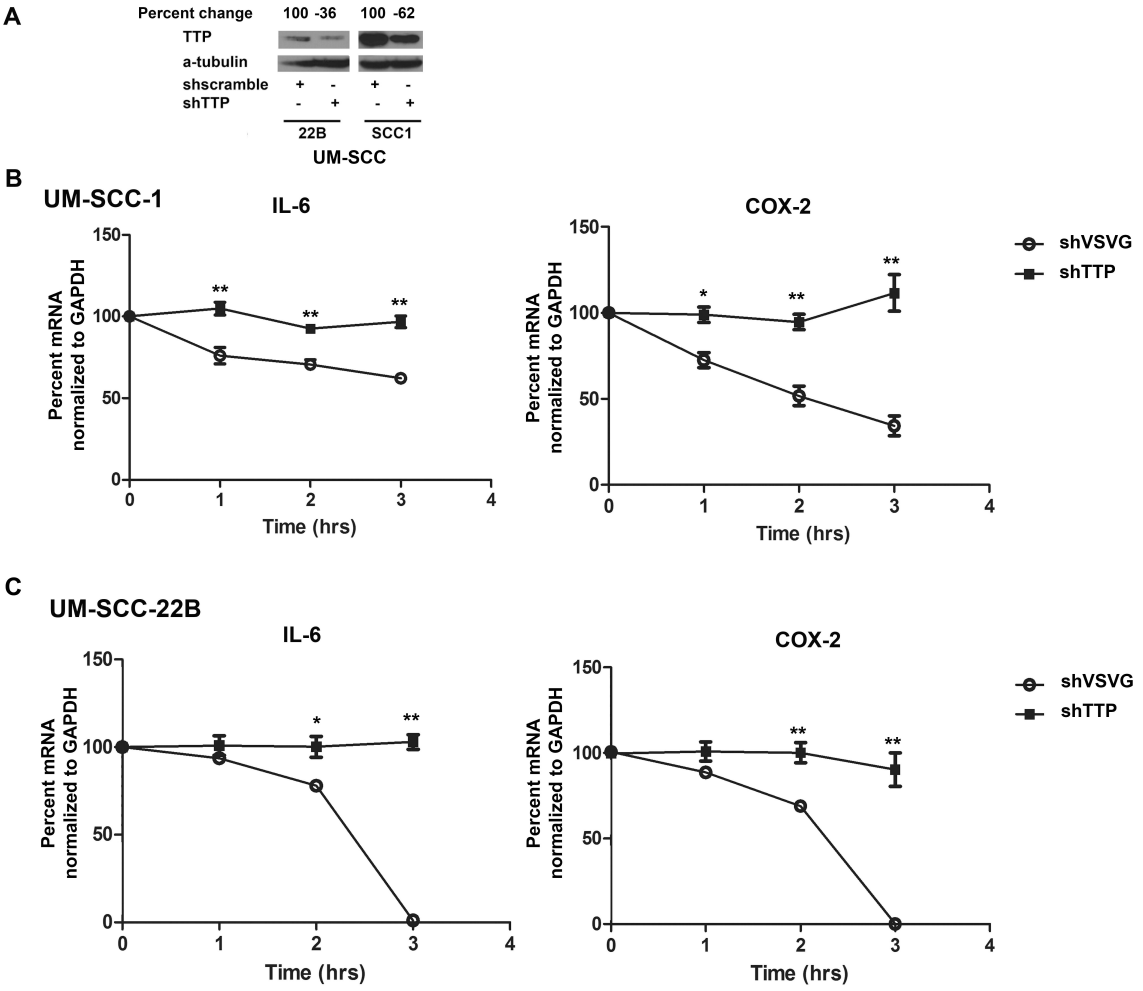

Figure 2.4: Tristetraprolin (TTP) knockdown increases cytokine messenger RNA (mRNA) half-life. (A) Whole-cell lysates from the UM-SCC-1 and UM-SCC-22B cell lines that stably expressed short hairpin vesicular stomatitis virus glycoprotein (shVSVG) or short hairpin TTP (shTTP) were immunoblotted. (B and C) RNA was purified, complementary DNAs (cDNAs) were prepared, and quantitative real-time–polymerase chain reaction was performed using SYBR Green Master Mix (Applied Biosystems, Foster City, Calif). Cycle threshold values for interleukin-6 (IL-6) and cyclooxygenase-2 (COX-2) were normalized to glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) and expressed as a percentage of time 0. Data are representative of the mean and standard deviation of 3 individual experiments (*indicates P < . 05; **, P < .001).

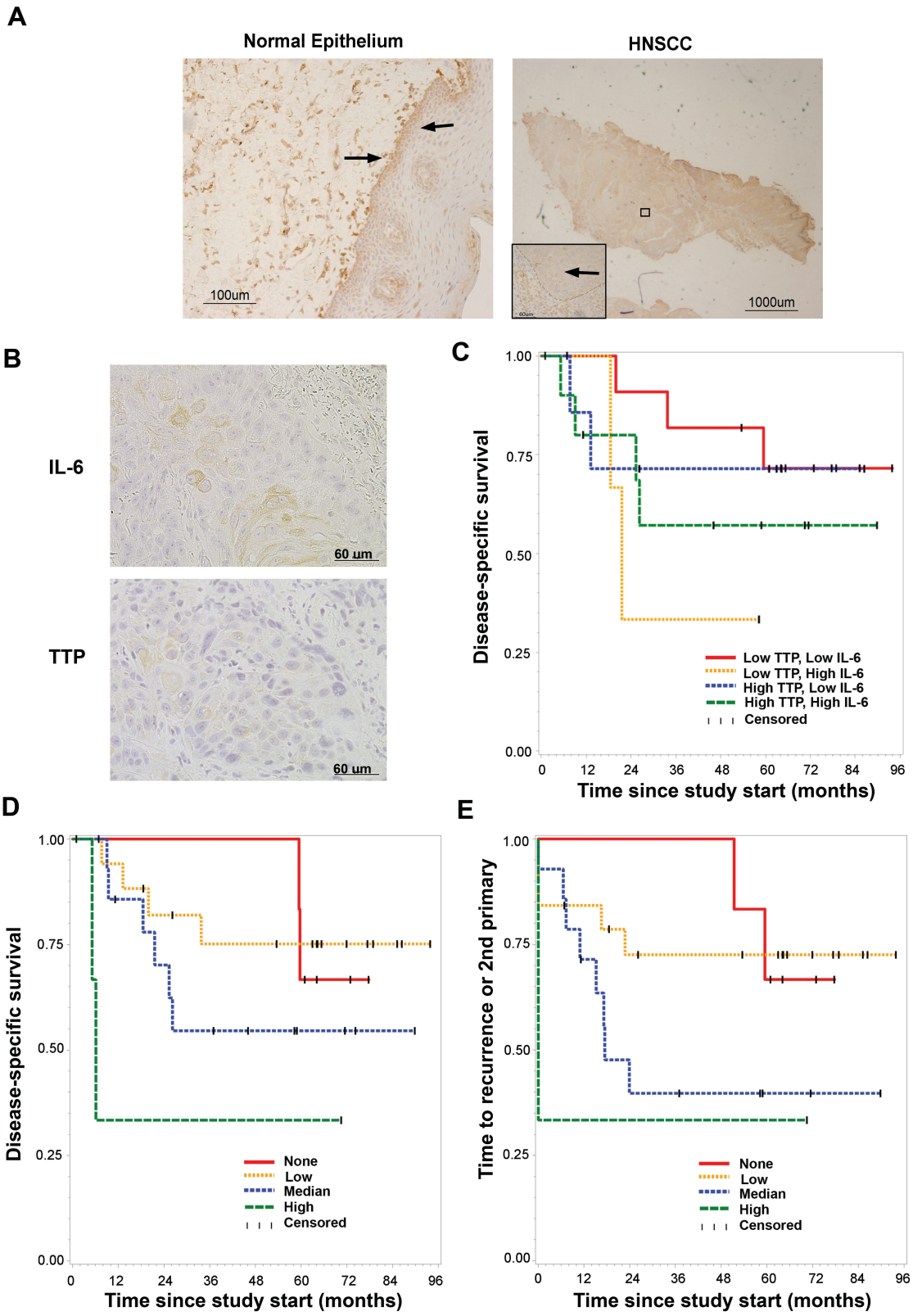

Figure 2.5: Interleukin-6 (IL-6) intensity is prognostic for disease-specific survival and tumor recurrence in patients with head and neck squamous cell carcinoma (HNSCC). (A) Immunohistochemistry was performed on tissue sections of normal

human epithelium (Left; bar = 100 μm) and human HNSCC (Right; bar =1000 μm) with tristetraprolin (TTP) antibody. Arrows indicate staining in the basal one-third of normal epithelium. Inset: Higher magnification of the outlined box demonstrating invasive epithelium is shown (bar = 60 μm). Arrow indicates invasive epithelium. (B) Immunohistochemistry was performed on tissue sections of a human HNSCC tissue microarray with the IL-6 and TTP antibodies. The slides were counterstained with hematoxylin. The top and bottom panels represent high IL-6 and low TTP staining, respectively, in the same HNSCC tissue section (bars = 100 μm). (C) Low TTP and high IL-6 are correlated with poor disease-specific survival. Patient groups are indicated by colored lines. Red line indicates low TTP and low IL-6 (n = 9 patients); yellow line, low TTP and high IL-6 (n = 3 patients); blue line, high TTP and low IL-6 (n =11 patients); green line, high TTP and high IL-6 (n = 10 patients). Events (indicated by a drop in the graph lines) were deaths from HNSCC. Subjects who did not experience the events, such as patients who died of unrelated causes, were censored. (D and E) High IL-6 intensity is prognostic for (D) poor disease-specific survival and (E) the development of tumor recurrence and second primary tumors. Patient groups are indicated by colored lines. Red line indicates no IL-6 (n = 6 patients); yellow line, low IL-6 (n = 19 patients); blue line, medium IL-6 (n = 14 patients); green line, high IL-6 (n = 3 patients). IL-6 intensity is also prognostic for poor overall survival (data not shown).

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CHAPTER III

SUPPRESSION OF TRISTETRAPROLIN OR INACTIVATION VIA RAP1-INDUCED p38 PROMOTES INVASION IN HEAD AND NECK SQUAMOUS CELL CARCINOMA BY STABILIZING mRNA AND SECRETION OF IL-6, MMP-9 and MMP-2

ABSTRACT

Loco-regional tumor invasion and metastasis lead to poor survival in squamous cell carcinoma of the head and neck (HNSCC). Pro-inflammatory mediators such as interleukin-6 (IL-6) and matrix metalloproteinases (MMPs) promote tumor invasion and aggressive behavior. However, targeted inhibition of individual pro-inflammatory mediators or their receptors had limited success in the treatment of HNSCC. This is likely due to redundancy in activities between pro-inflammatory mediators. Therefore, identification of a mechanism that regulates multiple pro-inflammatory mediators, will facilitate identification of a novel treatment target. We previously showed that tristetraprolin (TTP), an RNA binding protein, induces the decay of multiple pro-inflammatory mediators including IL-6 and PGE_2 and is downregulated in HNSCC. The mechanism by which TTP and its functional effects are regulated has not been investigated in HNSCC, and is the focus of this study. Using complementary approaches, we show that rap1B, a small GTPase with a critical role in HNSCC, induces p38 MAPK-mediated phosphorylation (suppression) of TTP. Suppression of TTP enhances mRNA stability and secretion of IL-6, MMP-9 and MMP-2. Active TTP induces mRNA turnover

of IL-6, MMP-9 and MMP-2 via the 3'-UTRs. Using *in vitro* and *in vivo* strategies, we demonstrate that low expression or p38-mediated inactivation of TTP promotes secretion of multiple pro-inflammatory mediators that induce tumor invasion. Moreover, suppression of TTP increases invasion of HNSCC via IL-6, MMP-9 or MMP-2. Importantly, high IL-6 and high MMP-9 are prognostic for poor clinical outcomes in patients with HNSCC. Thus, TTP and its upstream regulators may represent novel targets to suppress multiple pro-inflammatory mediators concurrently with the potential to improve patient survival in HNSCC.

INTRODUCTION

Squamous cell carcinoma of the head and neck (HNSCC) is the sixth most common cancer in the world [1]. Approximately 600,000 people are diagnosed with HNSCC and the disease accounts for nearly 350,000 deaths annually [2]. At about 40-50%, the five year survival rate is poorer than cervical cancer, lymphoma and melanoma [2, 3]. The dismal survival rate is attributed to tumor invasion and metastasis at the time of diagnosis [2]. New inhibitors must be identified since current treatment regimens have only marginally improved survival in over four decades.

In pre-cancerous lesions, invasion and migration of transformed epithelial cells into the underlying tissues, leads to malignant transformation and spread to other sites. Thus, invasion is an essential phenotype of cancer progression [4]. In order for tumor cells to invade, they need to degrade the basement membrane and become motile. Tumor-derived pro-inflammatory mediators such as matrix metalloproteinase-9 (MMP-9), MMP-2 and

interleukin-6 (IL-6), lead to cellular motility and tumor invasion [5, 6]. MMP-9 and MMP-2 are gelatinases that degrade type IV collagen in the basement membrane and are activated by other MMPs in the tumor matrix [7]. MMP-9 and MMP-2 are associated with poor prognosis in multiple cancers including HNSCC [8, 9]. In HNSCC patients, we and others have shown that high IL-6 is a biomarker for poor disease-specific survival [10, 11]. The simultaneous upregulation of multiple cytokines and pro-inflammatory mediators, each correlated with poor prognosis, may explain why targeted therapy against single growth factors or their receptors had limited success in HNSCC [12]. However, targeting MMP-9, MMP-2 and IL-6 simultaneously with a cocktail of drugs is impractical, underscoring the importance of identifying a common mechanism that regulates multiple cytokines and pro-inflammatory mediators.

Pro-inflammatory mediators are modulated during transcription, post-transcription and post-translation stages. RNA-binding proteins (RNA-BPs) that impact the stability of RNA transcripts have a significant role in tumor progression. RNA-BPs regulate mRNA post-transcriptionally by binding the adenylate-uridylate (AU)-rich elements in the 3' untranslated region (3'-UTR) of mRNA thereby inducing decay or stabilization of the transcript [13, 14]. Tristetraprolin (TTP) is an RNA binding protein that induces decay of mRNA of IL-6, vascular endothelial growth factor (VEGF), IL-8, prostaglandin-E2 (PGE_2) and other pro-inflammatory mediators in macrophages and some cancers [10, 15, 16]. Other RNA-BPs, such as human antigen R (HuR), have the opposite effect and stabilize mRNA. Since RNA-BPs regulate multiple pro-inflammatory mediators that influence tumor progression and invasion, RNA-BPs may represent a potential treatment target.

TTP (also known as TISS11, ZFP36 and Nup475), a zinc finger RNA binding protein, induces mRNA turnover of pro-inflammatory mediators by presenting mRNA to the RNA decay machinery [14, 17]. We and others demonstrated that TTP expression is reduced in cancer including head and neck, breast, glioma and colon cancers [10, 15, 18, 19]. In HNSCC, downregulation of TTP induces secretion of pro-inflammatory mediators [10]. Furthermore, we showed that TTP expression is inversely correlated with invasion [10], but the mechanism by which this occurs is unknown. Active TTP binds to the 3'-UTR of mRNA transcripts [16] and induces degradation in mouse embryonic fibroblasts [20]. In macrophages, TTP is inactivated by phosphorylation [21]. It is unclear if these mechanisms are conserved in cancer.

In the current study, we investigate the mechanism by which TTP mediates invasion in HNSCC. We demonstrate that suppression of TTP leads to an invasive phenotype *in vitro* and *in vivo* due to increased secretion of IL-6, MMP-9 and MMP-2. Additionally, we investigate the mechanism by which TTP is phosphorylated. TTP and its upstream regulators may represent novel therapeutic targets to suppress multiple pro-inflammatory mediators simultaneously with the potential to improve patient survival in HNSCC.

MATERIALS AND METHODS

University of Michigan Oral Cavity/Oropharyngeal Cancer Organ Preservation Trial. A TMA, described previously [8] from stage III/IV HNSCC treated with chemotherapy and radiation or surgery and radiation after induction chemotherapy [22], was used for these studies. The TMA was scored and interpreted as described [8, 10]. Clinical outcomes of the

patients analyzed were time to indication of surgery at primary site and time to recurrence, second primary, or death from disease. Marker values were dichotomized into low staining and high staining. Associations were evaluated with Cox proportional hazards models that related time-to-event outcomes to marker levels and other covariates. Models with each marker alone, each marker plus clinical stage, and interaction models with pairs of markers and their interactions were explored. Kaplan-Meier survival curves represent the significant findings of an interaction term between two markers in the Cox interaction model. P values represent the results of a Wald Chi-Square test of the interaction.

Cell culture: HNSCC cell lines from the University of Michigan were used for this study and were validated and cultured as described [8, 23]. HOK-16B cells (16B), a generous gift from Dr. No-Hee Park, University of California at Los Angeles, were maintained as described [10]. Primary human oral keratinocytes were cultured in oral keratinocyte medium (ScienCell, Carlsbad, CA, USA) supplement with pen/strep and supplied growth factors as described (HOK) [23].

Lentiviral Infection: For stable knockdown of TTP, HNSCC cells were infected with short hairpin RNA (shTTP and shVSVG control) in lentiviral particles containing a green fluorescent protein (GFP) (University of Michigan Vector Core) and were selected as described [10].

Transient siRNA knockdown: Individual siRNAs from the ON-target SMART pools were used to knockdown TTP, IL-6 and rap1B (Dharmacon, Lafayette, CO, USA). For MMP-9, MMP-2 and p38 knockdown, ON target *plus* siRNA SMART pools were used.

On Target *plus* siControl Smart Pool was used as a negative control. Transfections were performed as described [10].

Western Blot Analysis: Whole cell lysates were prepared with sonication on ice, electrophoresed on a polyacrylamide gel and transferred to a nitrocellulose membrane overnight as described [10]. Primary antibodies were as follows: phospho-p38, p38, rap1B, actin, and MMP-2 (Cell Signaling, Danvers, MA), MMP-9, TTP and phosphoserine (Abcam, Cambridge, MA), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (MAB374, Millipore/Upstate, Billerica, MA), IL-6 (R&D Systems, Minneapolis, MN, USA) and horseradish peroxidase conjugated donkey anti-rabbit IgG and goat anti-mouse secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Immunoreactive proteins were visualized by SuperSignal West Pico Chemiluminescent system or SuperSignal Substrate (Pierce, Thermo Fischer, Rockford, IL) and exposed to X-ray film.

Invasion assay: *In vitro* cell invasion was determined according to the manufacturer's instructions with FluoroBlok® invasion inserts or BD Invasion inserts in Matrigel® (BD Biosciences, San Jose, CA, USA). The number of cells that migrated or invaded through the FluoroBlok® inserts was determined at excitation 485 and emission at 520 on a Spectra $m2^e$ microplate reader (Molecular Devices, Sunnyvale, California) and normalized to migration. Cells were seeded on Matrigel® coated inserts in triplicate along with control non-coated inserts as described. For BD Invasion inserts, invasion and migration were determined as described [10].

Organotypic 3-dimensional equivalent: UM-SCC-1 stably transduced with shVSVG or shTTP were trypsinized and seeded on AlloDerm® (LifeCell, Branchburg, NJ) in a 48 well plate coated with human type IV collagen (Sigma Aldrich, USA) [24]. Cells were submerged in complete media for two days. The AlloDerm® organotypic cultures were raised to the air/liquid interface for four days, as described [24]. Complete DMEM media was placed below the insert to provide nutrients. The organotypic systems (oral cancer equivalents or OCE) were collected, fixed in 10% buffered formalin and stained with hematoxylin and eosin, as described [25]. Each OCE was divided into 3 parallel segments at 2-3 mm intervals prior to processing for paraffin sections. Slides with multiple 5 mm sections were stained with hematoxylin and eosin. Invasive islands and the total number of invasive cells were quantified in three sections from each OCE, each section with all three segments of tissue.

Chick Chorioallantoic Membrane (CAM) assays. Cells were seeded on an 11-day-old chick embryo CAM for two days, as described [26, 27]. Samples were frozen, sectioned and stained with hematoxylin and eosin or were immunostained for collagen IV and DAPI (Invitrogen), as described [27]. Images for GFP, collagen IV and DAPI were taken by DP Controller® and merged in DP Manager® as described [26]. Invasion colonies were quantified in multiple fields and graphed after normalization to VSVG control.

Gelatin zymography: Conditioned media (CM) was collected as described from cells seeded at 70% confluence [10]. CM was concentrated in Centrifugal Filter Units (Millipore Corporation, Billerica, MA). Gelatin enzyme activity for MMP-9 and MMP-2 was evaluated as described [8].

RNA extraction and cDNA synthesis: QIAzol® (Qiagen) was used to isolate total RNA, and cDNA was synthesized as described [10].

Quantitative-Real-Time-Polymerase Chain Reaction (Q-PCR): Quantitative Real time PCR was performed with SYBR Green Master mix on an Applied Biosystems 7600HT Real Time PCR machine as described [10] . Forward and reverse primers were designed for human IL-6 and GAPDH as described [10]. For *MMP9* (NM_004994) the primers were 5'-ttgacagcgacaagaagtgg-3 and 5'-gccattcacgtcgtccttat-3' and for *MMP2* (NM_004530) the primers were 5'-acagcaggtctcagcctcat-3' and 5'-tgaagccaagcggtctaagt-3'. Delta-Delta CT was used to determine fold change in steady state mRNA expression as described [10].

mRNA stability. 10nM of actinomycin D (Sigma Aldrich, USA) was used to halt transcription for zero, one, two or three hours. RNA was collected and processed to evaluate mRNA stability as described [10].

3'UTR luciferase transfection: UM-SCC-1 and -81B were transfected with siRNA targeting TTP (siTTP) or non-target control siRNA (NT) (described above). 48 hours later, a promoter driven firefly luciferase reporter construct containing the 3' untranslated region (UTR) of IL-6, MMP-9 or MMP-2 (SwitchGear Genomics, Menlo Park, CA) and a *Renilla* reporter construct (normalization control) were co-transfected with Lipofectomine 2000 (Invitrogen) as described by the manufacturer. Twenty-four hours later Luciferase and *Renilla* were read in the cell extracts with the firefly constructs with the Promega DualGlo® Reporter Luciferase System (Promega, Madison, WI, USA) according to the manufacturer's instructions on a LMaxx^{384} (Molecular Devices,

Sunnyvale, California). Firefly luciferase activity was normalized to *Renilla* activity and expressed as relative luminescence units (RLU).

Co-Immunoprecipitation (Co-IP): The Co-IP assay system was used to immobilize TTP following the protocol described by the manufacturer (Pierce, Thermo Fischer, Rockford, IL). Briefly, cell lysates were collected in the ice-cold lysis buffer supplemented with phosphatase inhibitors: Cocktail 2 (1:100) and Cocktail 3 (1:100) (P5726 and P0044, Sigma Aldrich, USA). Cell lysates were sonicated on ice prior to centrifugation. 10 µg of anti-TTP (SC-14030, SantaCruz Biotechnology, Santa Cruz, CA) was bound to Amino Link Plus Coupling Resin Beads® for two hours at room temperature per manufacturer's instructions. Protein was coupled with the beads. Eluted lysates were electrophoresed, immunoblotted with anti-TTP (Abcam, Cambridge, MA) and phosphoserine (Abcam, Cambridge, MA) and visualized by chemiluminescence.

Enzyme Link Immunosorbent Assay (ELISA): CM from cell lines was collected and processed as described [10]. IL-6 was measured by a non-competitive ELISA (R & D Systems).

Activation studies: For activation studies, UM-SCC-(1 and 81B) cells were serum starved for 6 hours followed by induction with IL-1β (1ng/ml) for 0, 2, 5, or 10 minutes. Whole cell lysates were collected as described [10]. Rap1B activation was determined with glutathione *S*-transferase (GST)-tagged ralGDS as described [23, 28]. The construct for ralGDS was a generous gift from Dr. Johannes L. Bos (University of Medical Centre, Ultrecht, The Netherlands).

Data analysis. Statistical analysis of *in vitro* and *in vivo* assays was performed using a Student's *t*-test. A p-value of < 0.05 was considered to be statistically significant (GraphPad®).

RESULTS

IL-6 and MMP-9 are predictive of poor outcomes in HNSCC. The interaction between IL-6 and MMP-9 was explored in terms of negative sequelae for HNSCC including short time to recurrence, second primary tumor, or surgery or death from disease (Fig. 3.1). Patients with low IL-6 and high MMP-9 or with high IL-6 and low MMP-9 had the poorest outcomes (p=0.020) followed by patients with both high IL-6 and high MMP-9 (p=0.020). Patients with low IL-6 and low MMP-9 had the best outcome with respect to tumor recurrence, second primary tumor, surgery or death from disease. These findings suggest that IL-6 and MMP-9, individually or together have a significant role in HNSCC progression.

Downregulation of TTP promotes invasion. Invasion is an essential step in tumor progression, being required for transformation of pre-cancerous lesions to HNSCC. Previously we showed that TTP inhibits multiple pro-inflammatory mediators including IL-6, but its effect on gelatinases (MMP-2, MMP-9), which promote invasion, is unknown [8, 10]. In initial *in vitro* and *in vivo* studies, we investigated whether TTP modulates invasion. As shown in Figure 3.2A, transient suppression of TTP with siRNA in UM-SCC-1, a HNSCC cell line, increased invasion almost 300% compared to non-target controls and increased migration (*p<0.01). TTP suppression was verified by immunoblot analysis (Fig. 3.2A, right panel). To recapitulate the basement membrane,

we used a three-dimensional oral cancer equivalent (OCE) system to evaluate whether TTP suppression promotes invasion. HNSCC cells were stably transduced with shVSVG or shRNA targeting TTP (shTTP) to facilitate sustained knockdown of TTP. In the organotypic OCE model system, loss of TTP significantly increased invasion compared to corresponding control cells (Fig. 3.2B). There were five times more invasive tumor islands in OCE-shTTP compared to control OCEs (OCE-shVSVG) (*p<0.01) (Fig. 3.2B, middle-left panel). There was a 16-fold increase in invasive cells in shTTP-OCEs compared to shVSVG-OCEs after normalization of invasive cells to total number of cells (Fig. 3.2B, middle-right panel) (*p<0.01). TTP knockdown was verified by immunoblot analysis in cells stably transduced with shTTP compared to shVSVG (control) (Fig. 3.2B, right panel).

The basement membrane, the first and likely the most robust structural barrier to invasion, separates the epithelium from the underlying connective tissue [29]. Given the structural complexity of the basement membrane, the effect of TTP on invasion was verified in an *in vivo* model. The chick chorioallantoic membrane (CAM) system consists of ectodermal chorionic epithelium separated by mesenchyme from the endodermal allantoic membrane [27, 30]. The chorionic epithelium is separated from the underlying connective tissue by an intact epithelial-derived basement membrane [27]. UM-SCC-1-shVSVG cells or UM-SCC-1-shTTP cells were seeded on the CAM. The tumors were harvested, sectioned and stained with DAPI and type IV collagen to highlight the nuclei and basement membrane, respectively. Invasion was evaluated on tissue sections. In Figure 3.2C (left panel), GFP-tagged tumor cells are shown on the CAM. Cells with stable TTP knockdown showed destruction of the basement membrane and more HNSCC

cells invading the underlying mesenchymal tissue compared to control VSVG cells (Fig. 3.2C, arrows on middle panel). There were 16 times more invasive islands in shTTP compared to shVSVG control tumors, which showed one or two invasive islands per field (Fig. 3.2C) (*p<0.01). Moreover, in tumors with shTTP, the basement membrane was completely disrupted (Fig. 3.S1, type collagen IV staining) while in control tumors the basement membrane appeared intact, suggesting that low TTP in HNSCC promotes aggressive and invasive tumors.

Downregulation of TTP promotes invasion via upregulation of IL-6, MMP-9 and MMP-2. MMP-2 and MMP-9 have a significant role in tumor invasion in cancer [8]. However, the role of TTP in regulating these proteins is unknown. To investigate the association between TTP expression and MMP-9 and MMP-2 secretion, MMP secretion was evaluated by gelatin zymography in conditioned medium from HNSCC cells transfected with siNT and siTTP (Fig. 3.3A). In UM-SCC-1 cells, suppression of TTP increased MMP-9 secretion by 189% and MMP-2 secretion by 142% compared to cells transfected with control siRNA. Similar findings were observed in UM-SCC-81B (Fig. 3.S2A).

Invasion has a significant role in tumor progression. Previously we showed that suppression of TTP increased IL-6 in HNSCC and high IL-6 expression is prognostic of poor patient outcome [10]. Furthermore, stable knockdown of TTP induced invasion in HNSCC. To determine whether downregulation of TTP induces invasion via overexpression of IL-6, MMP-9 and MMP-2, invasion of HNSCC cells with downregulated TTP was evaluated after suppression of these pro-inflammatory mediators. UM-SCC-1 cells stably suppressing TTP (shTTP) were transfected with

siRNA targeting IL-6, MMP-9 or MMP-2. Knockdown of IL-6, MMP-9 and MMP-2 was verified by immunoblot analysis (Fig. 3.3B, left three panels). Invasion was evaluated in a FluoroBlok® invasion assay (Fig. 3.3B, right panel). In HNSCC cells with TTP knockdown that exhibit an invasive phenotype (Fig. 3.3B, right panel), the control non-invasive phenotype was 'rescued' when IL-6, MMP-2 or MMP-9 was suppressed with siRNA. Suppression of IL-6 decreased invasion by 50%, while suppression of MMP-9 and MMP-2 decreased invasion by 55% (*p<0.01). No major changes in migration were observed in cells with knockdown of IL-6, MMP-2 or MMP-9 compared to siNT controls (data not shown). Similar results were observed in UM-SCC-81B (Fig. 3.S2C). Taken together the results suggest that low TTP in HNSCC increases secretion of cytokines and pro-inflammatory mediators to promote invasion via IL-6, MMP-9 and MMP-2 secretion.

TTP regulates the stability of transcripts for IL-6, MMP-2 and MMP-9 via the 3'-UTR. TTP is an RNA-binding protein that binds AU-rich elements (AREs) in the 3' UTR of mRNAs to promote degradation [14, 16]. AREs can be identified in multiple cytokines and pro-inflammatory factors including MMP-2 and MMP-9 [31], gelatinases that promote invasion in HNSCC [8]. However, TTP-mediated regulation of these metalloproteinases via the 3' UTR has not been investigated. In initial studies, the effect of downregulation of TTP on mRNA for IL-6, MMP-9 and MMP-2 was investigated, UM-SCC-1 cells were transfected with shTTP or shVSVG, and Q-RT-PCR was performed. As shown in Figure 3.4A, the steady state level of these mRNAs was increased when TTP was downregulated. IL-6 expression increased 3.5-fold, while MMP-9 and MMP-2 were increased 2-fold in shTTP cells compared to shVSVG control cells (Fig. 3.4A, *p<0.01). In UM-SCC-81B transient knockdown of TTP also

significantly increased IL-6, MMP-9 and MMP-2 mRNA levels compared to cells transfected with control siRNA (Fig. 3.S3A).

To determine if TTP regulates mRNA levels of IL-6, MMP-9 and MMP-2 via stabilization of transcripts, UM-SCC-1-shTTP or UM-SCC-1-shVSVG cells were treated with actinomycin D, which inhibits transcription. RNA was collected at multiple time points after actinomycin D treatment. Loss of TTP enhanced IL-6 mRNA stability (Fig. 3.4B, left panel), as shown previously in an independent experiment [10]. In contrast, in UM-SCC-1-shVSVG control cells, IL-6 transcript decreased to 50% of the baseline (Time 0) level at three hours (*p<0.01). MMP-9 mRNA was more stable in UM-SCC-1-shTTP cells at three hours compared to UM-SCC-1-shVSVG control cells which showed complete degradation by two hours (Fig. 3.4B, middle left panel) (*p<0.01). Similarly MMP-2 mRNA was also stabilized by downregulation of TTP (Fig. 3.4B, middle right panel) (*p<0.01). Downregulation of TTP was verified by immunoblot analysis (Fig. 3.4B, right panel).

To determine if TTP stabilizes the transcripts for IL-6 via the 3'-UTR, UM-SCC-1 cells with knockdown of TTP were co-transfected with a firefly luciferase reporter construct containing the 3'-UTR of IL-6 (schematic shown in Fig. 3.4C) and a *Renilla* construct for normalization. Downregulation of TTP expression increased the relative luminescence units (RLU) of IL-6 by 1.5-fold after normalization to *Renilla* compared to control cells (Fig. 3.4C, left panel) (*p<0.01). Similar studies were performed with MMP-9 and MMP-2. Downregulation of TTP increased MMP-9 and MMP-2 reporter activity by approximately 2-fold compared to control cells (Fig. 3.4C, middle-left and middle-right panels, respectively). TTP knockdown was confirmed via immunoblot in

UM-SCC-1 (Fig. 3.4C, right panel). Similar results were observed in UM-SCC-81B, an independent HNSCC cell line (Fig. 3.S3C). Taken together, these studies show that TTP promotes degradation of mRNA for IL-6, MMP-9 and MMP-2 via AREs at the 3'-UTR.

p38–mediated phosphorylation of TTP promotes secretion of pro-inflammatory mediators. p38 mitogen activated kinase ((p38)/MAPK) is an important signaling mechanism for induction of pro-inflammatory mediators. In order to determine if p38 mediates secretion of pro-inflammatory mediators in HNSCC, initial studies focused on constitutive p38 activity in HNSCC. Active, phospho-p38 was evaluated in whole cell lysates from a panel of six HNSCC cell lines (Fig. 3.5A). All six cell lines exhibited an increase in phospho-p38 expression compared to primary human oral keratinocytes (HOK) after normalization to total p38 and actin. The increase in phospho-p38 expression was greater than 200%. HOK16B, an immortalized but non-malignant cell line showed only a marginal increase in phospho-p38 expression.

In macrophages, p38 inactivates TTP via MK2-mediated phosphorylation at two serine sites [21, 32]. However the role of p38 in TTP phosphorylation in other cells, including cancer, is unknown. TTP phosphorylation was evaluated in three HNSCC cell lines that express variable levels of TTP. Because a phospho-TTP antibody was not commercially available, TTP was immunoprecipitated from these HNSCC cell lines and immunoblotted for phosphoserine. Phospho-TTP expression in UM-SCC-11A and UM-SCC-81B was compared to UM-SCC-1. After normalization to TTP eluted after immunoprecipitation, UM-SCC-1 had the lowest expression of phospho-TTP of the three HNSCC cell lines that were evaluated. UM-SCC-11A and UM-SCC-81B had approximately 10% and 40% increase in TTP phosphorylation compared to UM-SCC-1.

To determine if TTP is phosphorylated via p38, phosphorylation of TTP was evaluated after siRNA-mediated downregulation of p38 in stimulated cells. HNSCC cells were serum-starved and then induced by IL-1β, a potent agonist of the p38 MAPK pathway [33]. As expected, IL-1β induced phospho-p38 almost two-fold compared to control cells, as evaluated by immunoblot analysis (Fig. 3.5C). Moreover, siRNA targeting p38 decreased expression of p38 and phospho-p38. Stimulation of UM-SCC-1 cells with IL-1β increased phospho-TTP, normalized to total TTP, by more than two-fold compared to unstimulated cells (Fig. 3.5C, NT–IL-1β versus NT+IL-1β). Importantly, downregulation of p38 MAPK expression decreased IL-1β-mediated TTP phosphorylation by almost two-fold compared to cells transfected with non target siRNA (Fig. 3.5C, sip38+IL-1β versus NT+IL-1β). In unstimulated cells, downregulation of p38 MAPK decreased phospho-TTP compared to cells transfected with control siRNA (Fig. 3.5C, sip38 versus NT), consistent with reduced expression of constitutively active p38 MAPK. Similar results were observed in UM-SCC-81B (Fig. 3.S4A).

To determine if p38 MAPK promotes pro-inflammatory mediator secretion via TTP mediated phosphorylation, p38 MAPK was suppressed by siRNA. In HNSCC cells with knockdown of p38, IL-6 secretion was reduced by 45% in UM-SCC-1 cells (Fig. 3.5D, left panel) and by 75% in UM-SCC-81B, (Fig. 3.S4B) compared to control cells transfected with non-target siRNA. Suppression of p38 significantly decreased the secretion of IL-6 in both UM-SCC-1 and -81B by nearly 50% (Fig. 3.5D, right panel and Fig. 3.S4B) compared to NT controls, respectively (*p<0.01). Together these findings show that p38–mediated phosphorylation of TTP promotes secretion of pro-inflammatory mediators.

Rap1B activates p38 in HNSCC. We previously showed that rap1B is a critical signaling molecule in HNSCC and induces ERK/MAPK activation [8, 23, 34, 35]. To determine if rap1B mediates p38 activation in HNSCC, UM-SCC-1 cells were stimulated with IL-1β. An increase in rap1B activity preceded p38 activation, suggesting that rap1B is upstream of p38 (Fig. 3.5E). Similar results were observed in UM-SCC-81B, (Fig. 3.S4C).

To verify that rap1B activates p38 in HNSCC two siRNAs targeting rap1B expression were used. Downregulation of rap1B inhibited IL-1β-induced p38 activation by 40% in UM-SCC-1 cells (Fig. 3.5F) when compared to the corresponding stimulated cells transfected with control siRNA (siRap1B+IL-1β versus NT+IL-1β). Similar findings were observed in UM-SCC-81B (Fig. 3.S4D). Thus, rap1B activates p38 in HNSCC.

DISCUSSION

Globally, HNSCC is the sixth most common cancer with a very poor prognosis [2] This tumor secretes pro-inflammatory mediators that have been correlated with poor survival [8, 10]. However, targeted therapy against individual pro-inflammatory factors had marginal success in treating HNSCC [12]. This is likely due to the concurrent upregulation of multiple pro-inflammatory mediators with overlapping functions in tumor progression. Targeting each of these factors may improve prognosis but is impractical, emphasizing the importance of identifying a common regulatory mechanism for multiple pro-inflammatory mediators. The complementary approaches presented here conclusively show that downregulation or inactivation of TTP in HNSCC promotes tumor progression

via secretion of multiple pro-inflammatory mediators. IL-1β stimulates rap1B, which induces p38. Active p38 phosphorylates (inactivates) TTP, which leads to stabilization of mRNA transcripts. Active TTP promotes degradation of mRNA transcripts via AU-rich elements (AREs) in the 3' UTR.

Invasion is an essential phenotype of tumor progression that is required for malignant transformation of pre-cancerous lesions to HNSCC. Therefore, we focused on this phenotype. We and others have shown that loss of TTP promotes invasion in cancer [10, 36], however the mechanism by which this occurs has not been investigated. In this study, we show that downregulation or p38-mediated inactivation of TTP promotes invasion via stabilization of mRNAs of MMP-9, MMP-2 and IL-6 with subsequent enhanced secretion of these proteins.

Matrix metalloproteinases (MMPs) have a critical role in remodeling the extracellular matrix to promote invasion. These zinc-dependent proteolytic enzymes are secreted in a latent form, which has a cysteine residue in the pro-domain that masks the zinc ion in the catalytic domain [29]. Proteolytic cleavage of the pro-domain by other MMPs is required for activation. While restructuring of the surrounding matrix is important for wound healing, excessive MMP production is associated with carcinogenesis and invasion [9]. We previously showed that MMP-2 and MMP-9 are overexpressed and are prognostic of poor outcomes in HNSCC [8]. Since loss of TTP promotes invasion, we hypothesized and showed that TTP regulates the mRNA stability of MMP-9 and MMP-2. A significant but partial rescue of the effects of wild type TTP on invasion is observed in shTTP cells after siRNA-mediated knockdown of MMP-2 or MMP-9 or IL-6, consistent with an overlap in functions between multiple pro-invasive

mediators. In support of this possibility, loss of TTP enhances MMP-1, urokinase plasminogen activator (uPA) as well as IL-8 in other cancers [15, 36], which promotes tumor progression.

Small changes in stability of the mRNA of pro-inflammatory mediators can lead to large changes in secretion, and enhance proliferation, survival, invasion and tumor progression [37]. RNA-binding proteins such as HuR and TTP alter mRNA stability in opposite ways. HuR promotes mRNA stability [38], while TTP induces mRNA decay by binding and presenting mRNA to RNA degradation machinery [14, 17]. TTP is downregulated in multiple carcinomas including HNSCC, breast and colon cancer that correlate with increased secretion of IL-6, PGE_2 or VEGF [10, 15, 18, 39]. Increased secretion of pro-inflammatory mediators due to loss of RNA-BP expression can have negative outcomes for patients. For example, high levels of IL-6 and low levels of TTP are prognostic for poor disease specific survival in HNSCC patients [10].

Since p38 mediates TTP phosphorylation in macrophages and endothelial cells [40, 41], we speculated that TTP is phosphorylated and inactivated by p38 in HNSCC. In this study, every HNSCC cancer cell line evaluated exhibited constitutive phosphorylation of p38. In fact, p38 mediates TTP inactivation and suppression of p38 decreased IL-6 secretion.

Rap1, a critical signaling mediator in HNSCC, facilitates adhesion, proliferation, survival, migration and invasion in HNSCC [23, 42]. Two isoforms exist in HNSCC, rap1A and rap1B, which share >90% sequence identity [43]. Rap1B-deficient mice display decreased revascularization due to decreased VEGF secretion from deficient activation of p38 [44]. Although active, GTP-bound rap1B has a critical role in ERK/MAPK activation

in HNSCC [42], its role in modulating p38 has not previously investigated in any cancer. We established that IL-1β activates rap1B in HNSCC after two minutes of stimulation. By suppressing rap1B expression and stimulating with IL-1β, we showed that rap1B positively regulates p38 activation. This is important because the use of p38 inhibitors or the development of rap1B inhibitors as therapeutic strategies in HNSCC may improve patient survival in HNSCC. Induction of phospho-p38 by rap1B is a novel mechanism of p38 regulation in carcinomas. These epithelial-derived lesions account for the majority of cancers. p38 inhibitors are orally bio-available and some p38 inhibitors are in clinical trials to treat inflammatory diseases, however at this time, p38 inhibitors are not in use for HNSCC or oral inflammatory diseases.

Two dimensional invasion assays have been useful in investigating invasion but the lack of a basement membrane precludes duplication of the invasive process. Therefore we employ novel strategies for HNSCC to investigate how TTP promotes invasion. In the OCE 3D invasion assay that recapitulates invasion through a collagen IV barrier into human dermis, suppression of TTP increased invasion. To investigate the effect of invasion in an *in vivo* model through an intact basement membrane, we used the CAM assay [27].

Clinical implications

Treatment for HNSCC has only marginally improved in the past 40 years and current treatment protocols for HNSCC are based on tumor size, nodal involvement and metastasis. Since HNSCC secretes large amounts of pro-inflammatory mediators, inhibitors targeting pro-inflammatory mediators, are alluring treatment targets in HNSCC. However, clinical trials targeting a single pro-inflammatory mediator such as

MMP-9, MMP-2, epidermal growth factor (EGF) and PGE_2 have been unsuccessful in significantly improving patient survival [45-47]. This may be due to overlapping functions of pro-inflammatory mediators. Invasion of HNSCC is facilitated by tumor derived pro-inflammatory mediators such as IL-6, MMP-9 and MMP-2 and all of these factors are prognostic for poor survival in HNSCC [8, 10].

Our clinical correlates from a small group of HNSCC patients show that patients with tumors expressing either high IL-6 or low MMP-9 or both low IL-6 and high MMP-9, had a shorter time to tumor recurrence or second primary tumor than patients with low IL-6 and low MMP-9. This may explain why using an inhibitor against MMP-9 or IL-6 is unsuccessful because if MMP-9 is inhibited, then IL-6 can compensate for MMP-9's effects on tumor progression.

Our study highlights the role of TTP-mediated invasion in HNSCC. In a previous study, we showed that low TTP expression and high IL-6 levels correlate with poor disease specific survival in HNSCC [10]. A recent study further supported the role of low TTP in cancer progression; low TTP mRNA level is a negative prognostic indicator in breast cancer [18]. The transformation of pre-malignant keratinocytes to malignant keratinocytes is a critical step in tumor progression and loss of TTP may be a critical mediator in transformation to HNSCC. For example, overexpression of TTP decreases tumor growth and VEGF secretion in colon cancer [48] and decreases invasion in breast cancer [36]. Moreover when treating patients with deficient TTP, a synonymous polymorphism in TTP genes in a breast cancer cell line predicted failure to respond to Herceptin/Trastuzumab in HER2-positive-breast cancer patients [49].

Given that loss of TTP promotes invasion and secretion of multiple pro-inflammatory mediators simultaneously; this study may facilitate the expansion of therapeutic approaches for inflammatory-based cancers. For instance, since TTP is phosphorylated by p38 in HNSCC, upstream p38 inhibitors may also be a potential treatment strategy to decrease the pool of phosphorylated TTP in HNSCC and improve survival when used in conjunction with traditional therapeutic strategies. In a clinical trial of recurrent multiple myeloma patients treated with p38 inhibitors along with Bortezomib, some patients saw modest improvements in disease progression compared to control patients (Siegel DS, Blood, 108 (2006) abstr 3580).

Our findings collectively support the idea that loss of TTP promotes invasion in HNSCC through increased mRNA stability of IL-6, MMP-9 and MMP-2. By identifying these underlying mechanisms that promote invasion and tumor progression in HNSCC, we have uncovered a possible explanation for failure of HNSCC to respond favorably to inhibitors targeting a single pro-inflammatory mediator. Our studies provide considerable insight into the mechanisms underlying HNSCC invasion via suppression of TTP. Moreover we reveal potential treatment targets that may inhibit the secretion of multiple pro-inflammatory mediators simultaneously and improve treatment and survival.

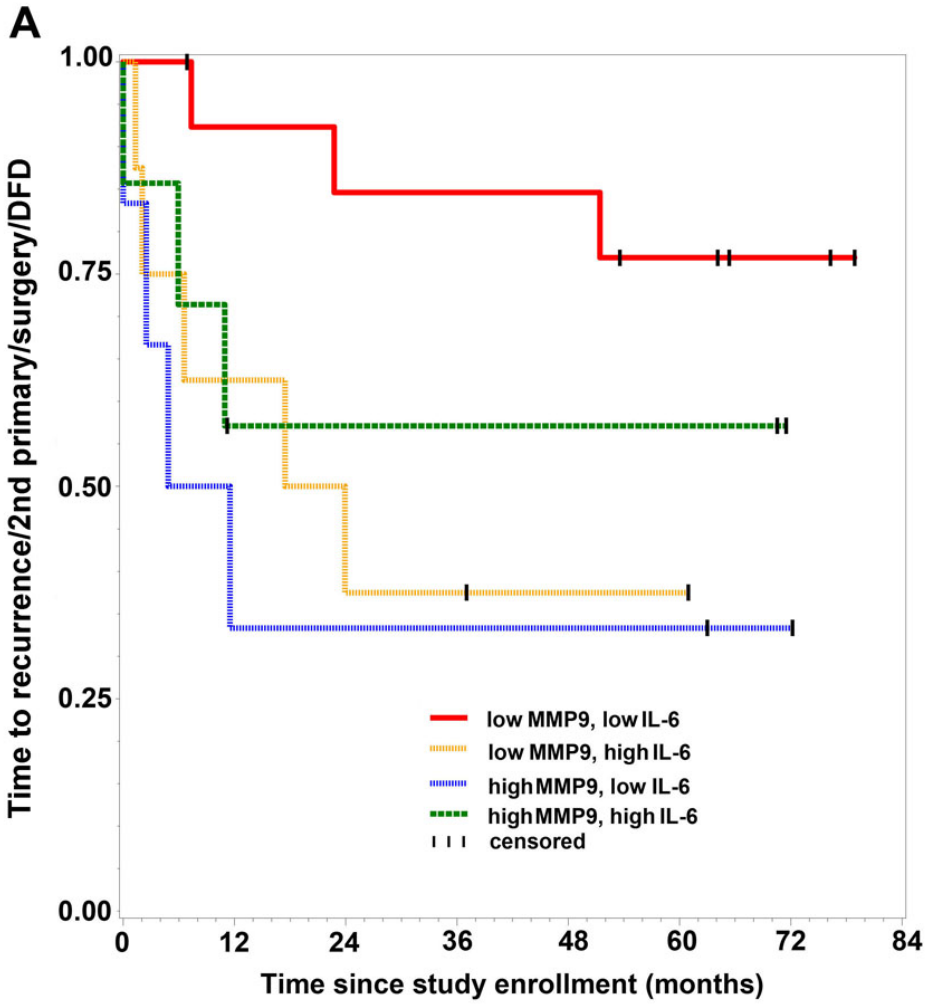

Figure 3.1: High Interleukin-6 (IL-6) and high matrix metalloproteinase -9 (MMP-9) are predictive of poor clinical sequelae in HNSCC. (A) TMA for IL-6 and MMP-9. Immunohistochemistry was performed on tissue sections of a human HNSCC tissue microarray with monoclonal antibodies IL-6 (6 μg/ml) and MMP-9 (2 μg/ml). DAB was stained and then slides were counterstained with hematoxylin. Cores were scored based on staining intensity: 1: no staining; 2: low staining; 3: moderate staining; 4: high staining. Interactions with IL-6 and MMP-9 were determined with a COX-2 interaction model. Patient groups are indicated by colored lines. Red line indicates low MMP-9 and low IL-6 staining intensity (n=14). Yellow line indicates low MMP-9 and high IL-6 staining intensity (n=8). Blue line indicates high MMP-9 and low IL-6 staining intensity (n=6). Green line indicates high MMP-9 and high IL-6 staining intensity (n=7). Subjects who did not experience the events were censored (p=.020).

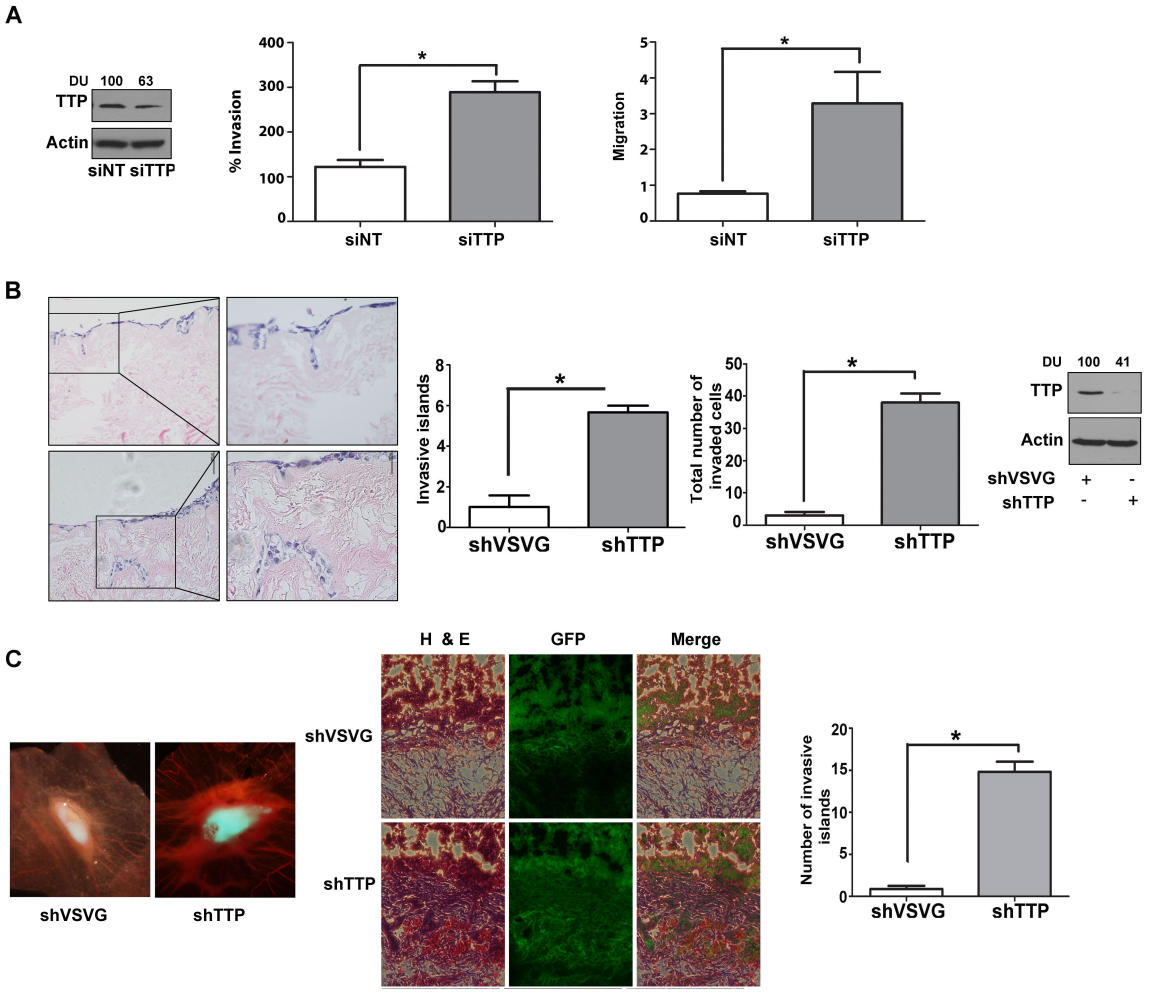

Figure 3.2: Downregulation of TTP promotes invasion. (A) Matrigel® invasion. (A, left and middle panels) Cells transfected with siNT or siTTP were plated on Matrigel®-coated or control inserts. The percent invasion was calculated after normalization to migration (not shown). Experiments were performed in triplicate in three independent experiments. (A, left panel) Immunoblot analysis. UM-SCC-1 cells were transfected with small interfering RNA for TTP (siTTP) or non-target siRNA (siNT). Whole cell lysates were electrophoresed and immunobloted with TTP and actin (loading control) antibodies. (B) Photomicrograph of 3D organotypic invasion assay. Cells stably transduced with a lentivirus containing short-hairpin RNA scramble (VSVG) or shTTP were seeded on rehydrated Alloderm® tissue. Cells were grown for three days in complete DMEM and then brought to an air/liquid interface for two more days. Alloderm® was formalin fixed and stained for hematoxylin and eosin. (H & E staining B, left panel) Dark purple represent the cells and the light pink is the Alloderm®. (B, top, left panel) 10X image of shVSVG cells on Alloderm®. (B, inset, top, right panel) 40X image of shVSVG cells on Alloderm®. (B, lower, left panel) 10X image of shTTP cells on Alloderm®. (B, inset, lower, right panel) 40X image of shTTP cells on Alloderm®. (B, middle-left graph) Quantification of total number of invasive islands in the

Alloderm® for shVSVG and shTTP. Black arrows point to invasive islands. (B, middle-right graph) Quantification of total number of invaded cells in the Alloderm® for shVSVG and shTTP normalized to total cell number. (C) CAM invasion. HNSCC cancer cells (shVSVG and shTTP; containing GFP construct) were placed on a CAM of an 11-day-old chick embryo for two days. Tissue was fixed and paraffin embedded. Cross sections were prepared and specimens were stained with hematoxylin and eosin. Images were taken under bright-field and fluorescent FITC (GFP) and images were merged. Results are representative of 3 independent experiments. (C, left panels) Gross tumor specimens from CAM assay for UM-SCC-1 shVSVG and shTTP. (C, middle panels) 20X images of H & E of cells seeded on CAM (left), GFP of cells seeded on CAM (middle) and merged images (right) for UM-SCC-1 shVSVG and shTTP. White arrows point to invasive islands. (C, right panel) Quanitfication of the number of islands that invaded past the basement membrane. (*$p<0.01$).

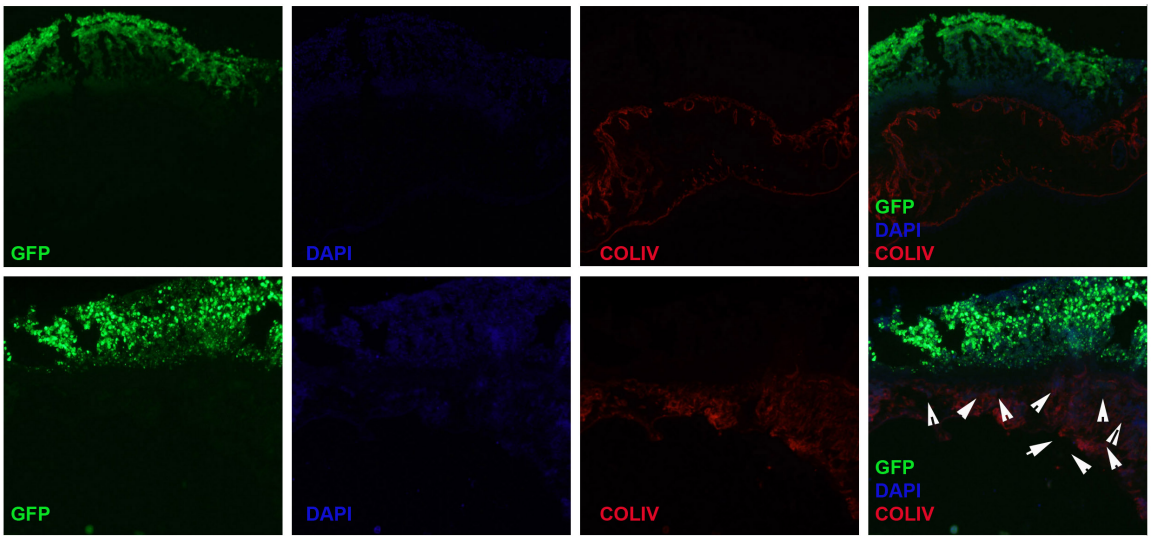

Figure 3.S1: Downregulation of TTP promotes invasion in CAM. CAM invasion. HNSCC cancer cells (shVSVG and shTTP; containing GFP construct) were placed on a CAM of an 11-day-old chick embryo for two days. Tissue was fixed and paraffin embedded. Cross sections were prepared and specimens were stained with red, type IV collagen (basement membrane); blue, DAPI. Top row represent shVSVG cells and lower row represent shTTP cells. Arrows point to the invasive islands. Results are representative of 3 independent experiments.

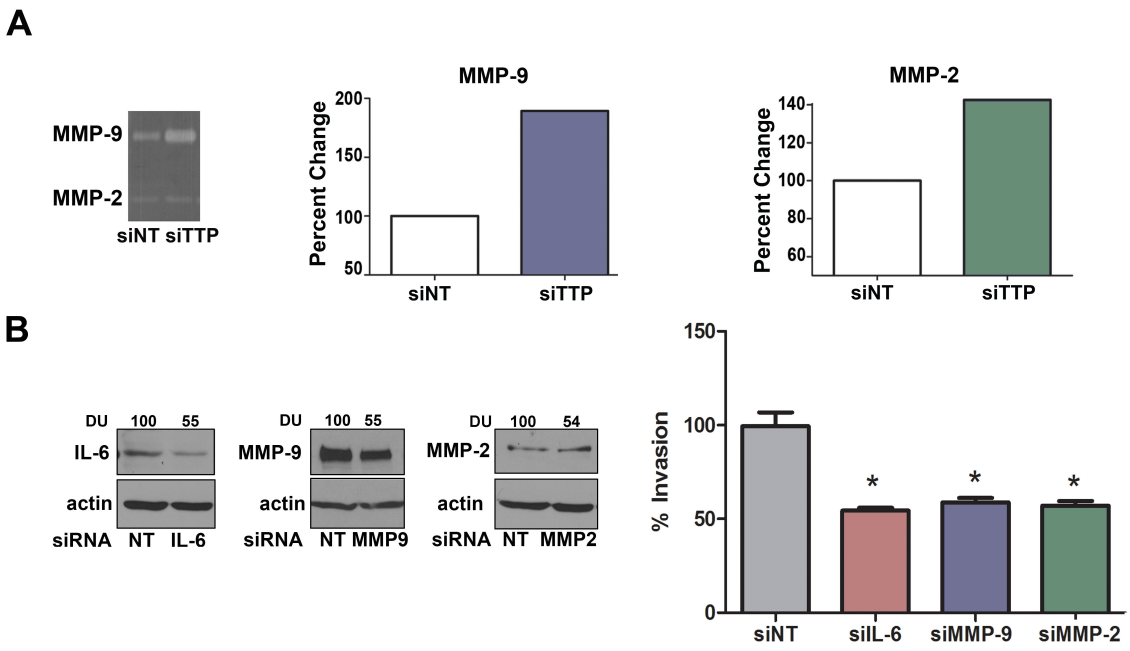

Figure 3.3: Downregulation of TTP promotes invasion via upregulation of IL-6, MMP-9 and MMP-2. (A) Gelatin zymography. HNSCC cells were transiently transfected with non-target (siNT) or siTTP. Gelatin zymography was used to quantify the activity of gelatinases (MMP-9 and MMP-2) in conditioned medium. A gelatin-containing SDS-PAGE gel was electrophoresed and stained with Coomassie blue as described. (A, middle and right panel) Percent MMP-9 and MMP-2 gelatinase activity was determined after normalizing siTTP gelatinase activity to siNT gelatinase activity and expressed as a percentage of siNT. (B, left panels) Immunoblot analysis. Whole-cell lysates were isolated from UM-SCC-1 cells stably transfected with shTTP and transiently transfected with siRNA targeting IL-6 (siIL-6), MMP-9 (siMMP-9), MMP-2 (siMMP-9) or non-target controls (siNT). Lysates were electrophorised and immunobloted with IL-6, MMP-9, or MMP-2 and actin antibodies. Densitometric units (DU) were determined after normalization to actin and expressed as a percentage of siNT. (B, right panel) Invasion in cells stably transduced with shTTP were transiently transfected with NT, IL-6, MMP-2 or MMP-9 was measured with Matrigel® coated inserts with mitomycin C. Cells were allowed to invade as described in materials in methods. Invasion was calculated as described and normalized to percent of invading siNT controls. (*p<0.01)

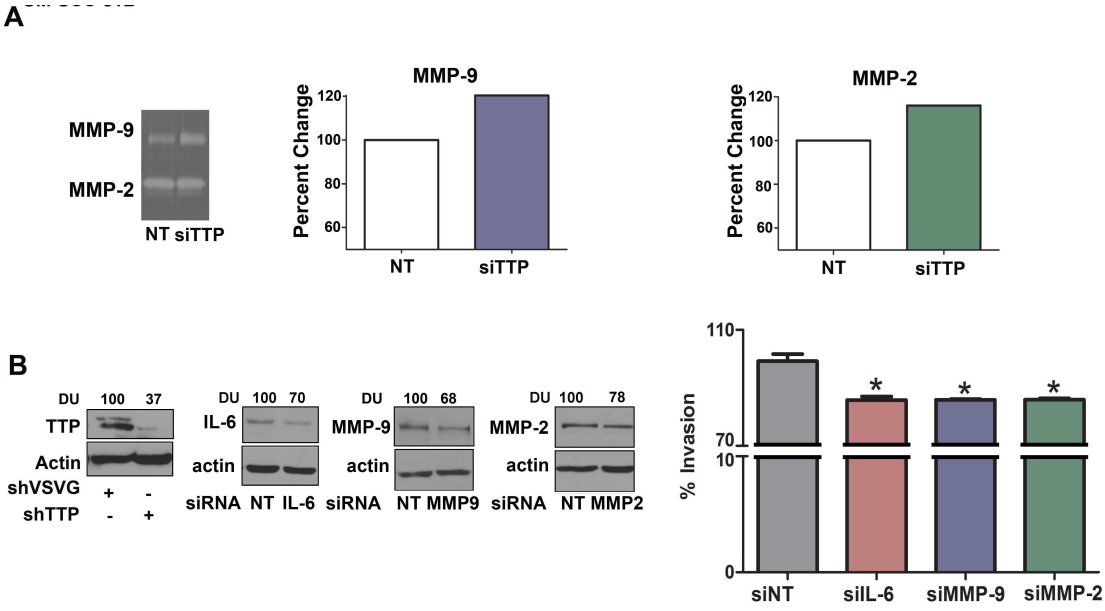

Figure 3.S2: Downregulation of TTP promotes invasion via upregulation of IL-6, MMP-9 and MMP-2. (A) Gelatin zymography. UM-SCC-81B cells were transiently transfected with siNT or siTTP. Gelatin zymography was used to quantify the activity of gelatinases (MMP-9 and MMP-2) in conditioned medium. A gelatin-containing SDS-PAGE gel was electrophoresed and stained with Coomassie blue as described. (A, middle and right panel) Percent MMP-9 and MMP-2 gelatinase activity was determined after normalizing siTTP gelatinase activity to siNT gelatinase activity and expressed as a percentage of siNT. (B, left panels) Immunoblot analysis. Whole-cell lysates were isolated from UM-SCC-1 cells stably transfected with shTTP and transiently transfected with siRNA targeting IL-6 (siIL-6), MMP-9 (siMMP-9), MMP-2 (siMMP-9) or non-target controls (siNT). Lysates were electrophorised and immunobloted with IL-6, MMP-9, MMP-2 and actin antibodies. Densitometric units (DU) were determined after normalization to actin and expressed as a percentage of siNT. (B, right panel) Invasion in cells stably transduced with shTTP were transiently transfected with NT, IL-6, MMP-2 or MMP-9 was measured with Matrigel® coated inserts. Cells were allowed to invade as described in materials in methods. Invasion was calculated as described and normalized to percent of invading siNT controls. Experiments were performed in duplicate (*p<0.01)

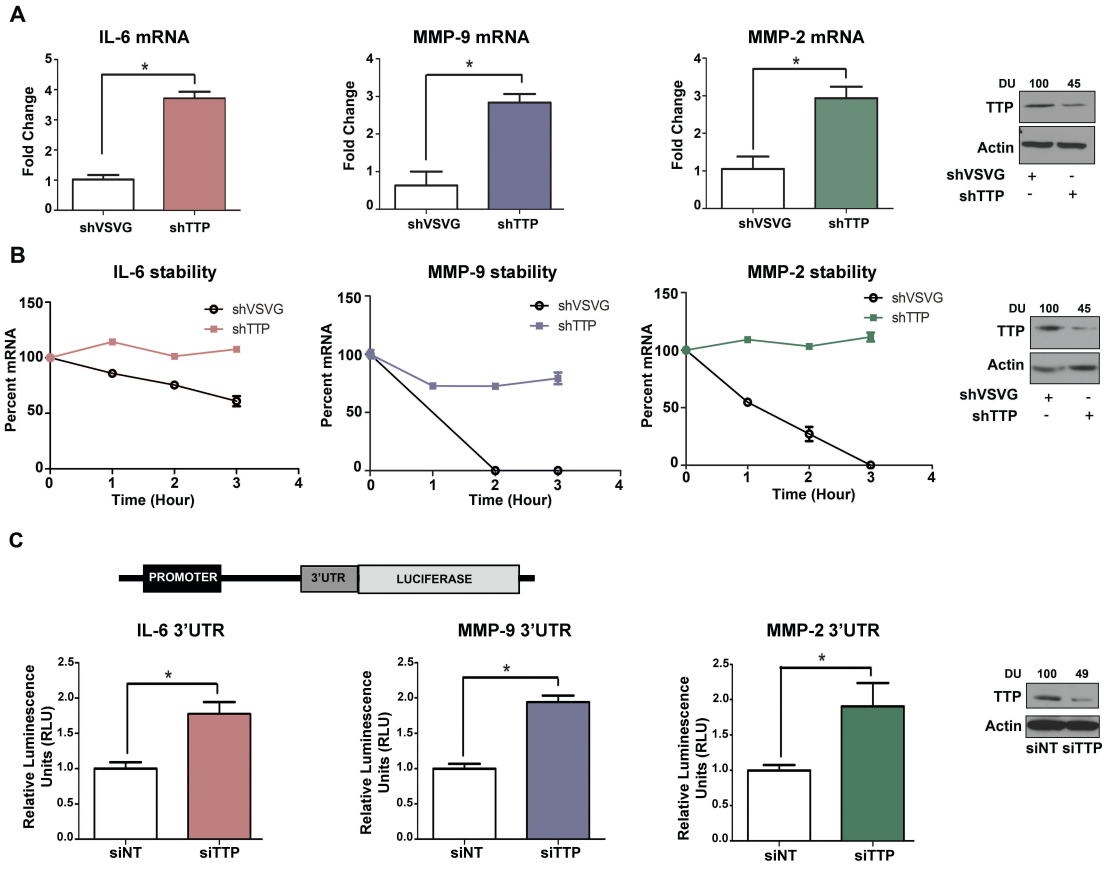

Figure 3.4: TTP regulates the stability of transcripts IL-6, MMP-9 and MMP-2 via the 3'-UTR. (A and B) Immunoblot analysis. Whole-cell lysates from the UM-SCC-1 that stably express a lentivirus containing a control VSVG vector or short hairpin RNA targeting TTP were immunoblotted with anti-TTP and anti-actin. Numbers are densitometric units with normalization to actin and expressed as a percent of corresponding control. (A) RT-Q-PCR. IL-6, MMP-9 and MMP-2 RNA levels were quantified from UM-SCC-1 shVSVG vector control or shTTP. RNA was purified, complementary DNAs (cDNAs) were prepared, and quantitative real-time–polymerase chain (Q-RT-PCR) reaction was performed using SYBR Green Master Mix. IL-6, MMP-2 and MMP-9 mRNA values were normalized to GAPDH and fold change was calculated as described in materials and methods. Analysis of gene expression was performed in triplicate in duplicate experiments. (*p<0.01) (B) mRNA decay. UM-SCC-1 shVSVG vector control or shTTP were treated with actinomycin D for 0, 1, 2 and 3 hours. RNA was collected, processed and purified as described in materials and methods. Cycle threshold values for IL-6, MMP-9 and MMP-2 were normalized to GAPDH and expressed as a percentage of time 0 as described. (C) 3'-UTR mRNA regulation of IL-6, MMP-9 and MMP-2. UM-SCC-1 cells were transfected with siRNA NT controls (siNT) or siRNA TTP (siTTP). Cells were then co-transfected with a luciferase construct containing the 3'-UTR of IL-6, MMP-9 or MMP-2 and a *Renilla* construct. Relative Luminescence Units (RLU) are expressed after luciferase normalization to *Renilla* and normalization to siNT controls. Experiments were performed in triplicate in three independent experiments (C, right panel) Immunoblot analysis. Whole cell lysates from siNT or siTTP cells were sonicated, electrophoresed and immunoblotted for TTP and

actin as described. Numbers are densitometric units with normalization to actin and expressed as a percent of corresponding control. (*p<0.01)

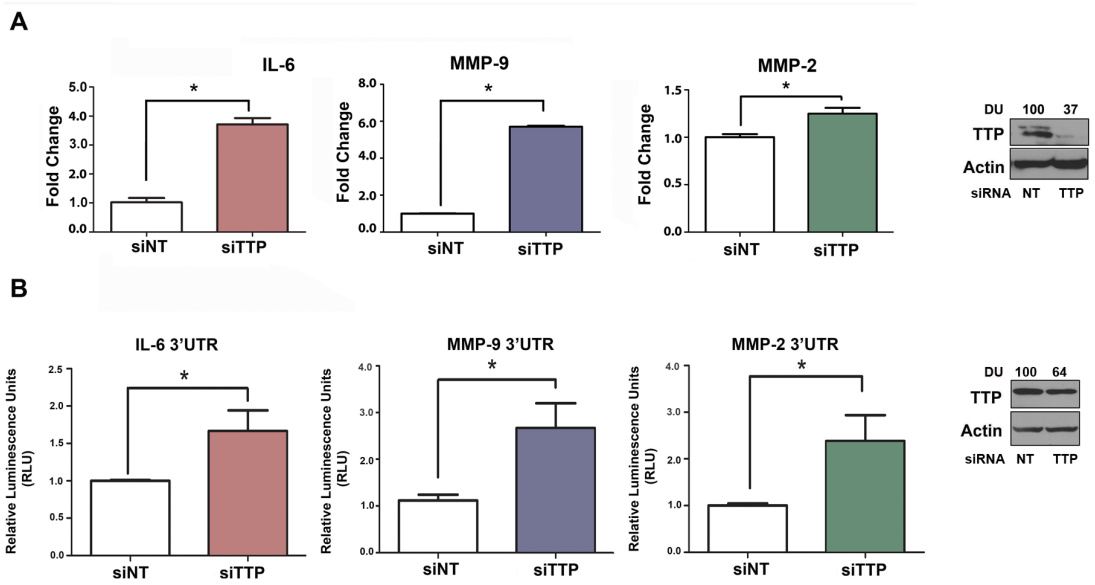

Figure 3.S3: TTP regulates the stability of transcripts IL-6, MMP-9 and MMP-2 via the 3'-UTR. (A and B) Immunoblot analysis. Whole-cell lysates from the UM-SCC-81B transiently transfected with siNT or siTTP were immunoblotted with anti-TTP and anti-actin. Numbers are densitometric units with normalization to actin and expressed as a percent of corresponding control. (A) RT-Q-PCR. IL-6, MMP-9 and MMP-2 RNA levels were quantified from UM-SCC-81B siNT vector control or siTTP. RNA was purified, complementary DNAs (cDNAs) were prepared, and quantitative real-time–polymerase chain reaction was performed using SYBR Green Master Mix. IL-6, MMP-2 and MMP-9 mRNA values were normalized to GAPDH and fold change was calculated as described in materials and methods. Analysis of gene expression was performed in triplicate in duplicate experiments. (*p<0.01) (B) 3'-UTR mRNA regulation of IL-6, MMP-9 and MMP-2. UM-SCC-81B cells were transfected with siRNA NT controls (siNT) or siRNA TTP (siTTP). Cells were then co-transfected with a luciferase construct containing the 3'UTR of IL-6, MMP-9 or MMP-2 and a *Renilla* construct. Relative Luminescence Units (RLU) are expressed after luciferase normalization to *Renilla* and normalization to siNT controls. Experiments were performed in duplicate in two independent experiments (B, right panel) immunoblot analysis. Whole cell lysates from siNT or siTTP cells were sonicated, electrophoresed and immunoblotted for TTP and Actin as described. Numbers are densitometric units with normalization to actin and expressed as a percent of corresponding control. (*p<0.05)

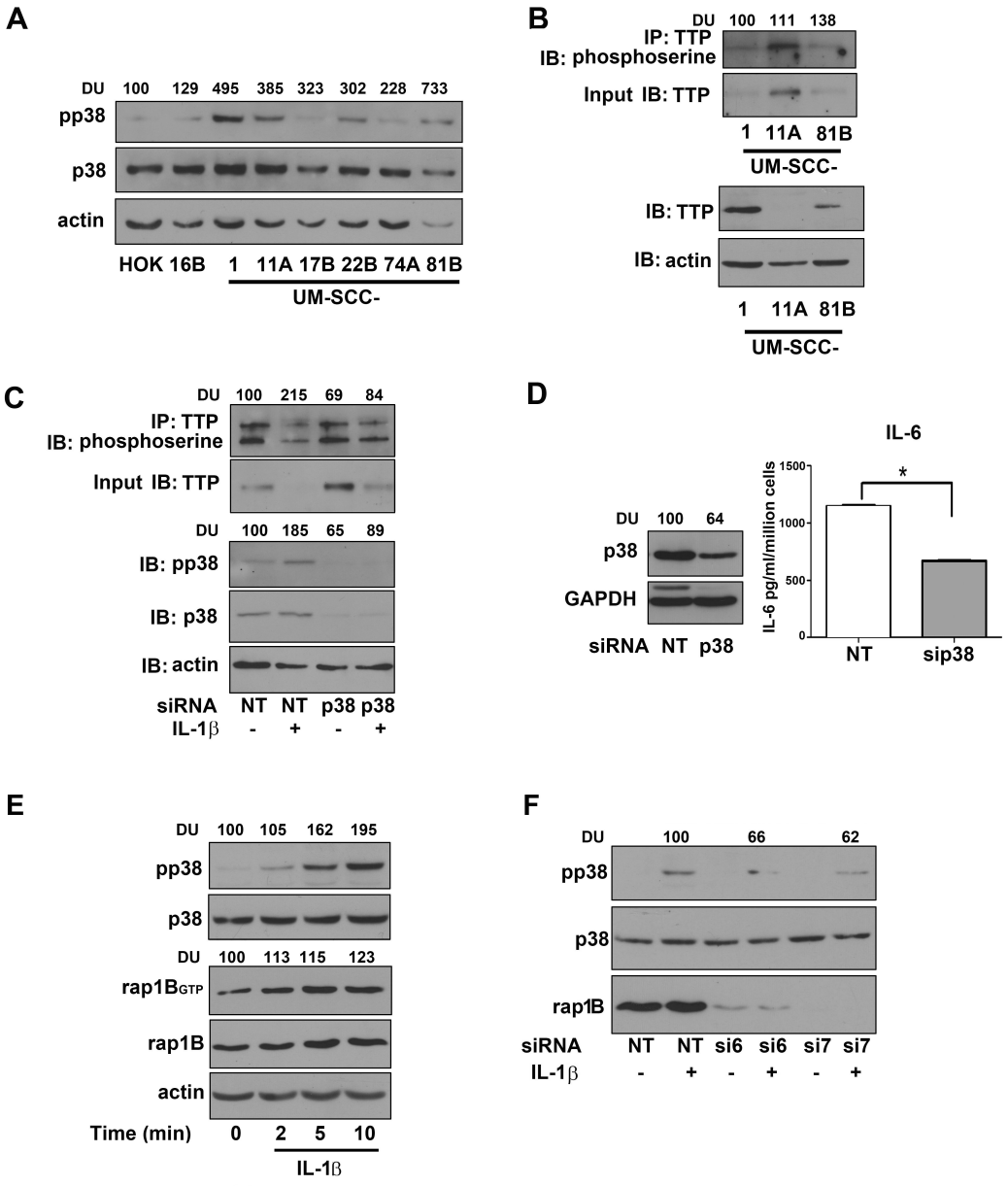

Figure 3.5: p38-mediated phosphorylation of TTP promotes pro-inflammatory mediator secretion and rap1B induces p38 activation. (A) Immunoblot (IB) analysis. Whole cell lysates from HNSCC cell lines were electrophoresed and immunoblotted for pp38, total p38 and actin. pp38 expression was normalized to total p38 and actin and pp38 expression was expressed in relative densitometric units to human oral keratinocytes. (B) Co-immunoprecipitation. Upper panel: Purified monoclonal TTP antibodies (Santa Cruz) were coupled to AminoLink® Plus Coupling Resin. Coupled TTP beads were combined with UM-SCC-1, -11A and -81B whole cell lysates overnight. Eluted substrates were electrophoresed and immunoblotted with phosphoserine or TTP. Densitometric units for phosphoTTP were determined after normalization to IB for phosphoserine and IB for TTP after Co-IP. (B, lower panel) whole cell lysates from UM-SCC-1, -11A and -81B were electrophoresed and immunoblotted with TTP and actin

antibodies in two independent experiments. (C) Co-immunoprecipitation. UM-SCC-1 were transfected with siRNA targeting p38 (sip38) or non-target controls (siNT). Cells were serum starved and treated with PBS (control) or IL-1β for 10 minutes. Whole cell lysates were coupled with TTP-bound AminoLink® Plus Coupling Resin as described above. Eluted samples were electrophoresed and immunoblotted with phosphoserine and TTP. Whole cell lysates from siNT and si38 were electrophoresed and immunoblotted with pp38, p38 and actin anti-bodies. Densitometric units for phosphoTTP were determined after normalization to IB for phosphoserine to TTP after co-IP and expressed as a percentage of unstimulated siNT. Lower whole cell lysates densitometric units for pp38 activation were normalized to total p38 and expressed as a percentage of unstimulated siNT. Three independent experiments were performed. (D) IL-6 ELISA. UM-SCC-1 cells were transfected with siRNA targeting p38 (sip38) or non-target controls (siNT). Whole cell lysates were electrophoresed and immunoblotted for anti-total p38 and anti-GAPDH. Interleukin-6 (IL-6) secretion was determined from conditioned media via ELISA in duplicate from three independent experiments. (* represents, $p<0.01$) (E) Immunoblot analysis. UM-SCC-1 cells were serum starved and treated with IL-1β for 0, 2, 5, or 10 minutes. Lysates were immunoblotted with pp38, p38, rap1B and actin (loading control). For active rap1B, whole cell lysates (50 µg of protein) were retrieved by a ralGDS pull-down assay, electrophoresed and blotted for rap1B antibody. Rap1B equivalent was verified from lysates immunoblotted with rap1B. Data represent a representative blot of three independent experiments. Densitometric units were determined by normalizing rap1B GTP to total rap1B and pp38 to total p38 and plotting them as a percentage change to time zero. (F) Immunoblot analysis. UM-SCC-1 were transfected with two siRNAs targeting rap1B (si6 and si7) or siRNA non-target controls (NT) and were stimulated IL-1β for 10 minutes or PBS (control). Whole cell lysates were immunoblotted for pp38, p38 and rap1B. Densitometric units were determined by normalizing pp38 to total p38 and expressed as a percent change of NT under stimulated conditions. Experiments were performed in triplicate.

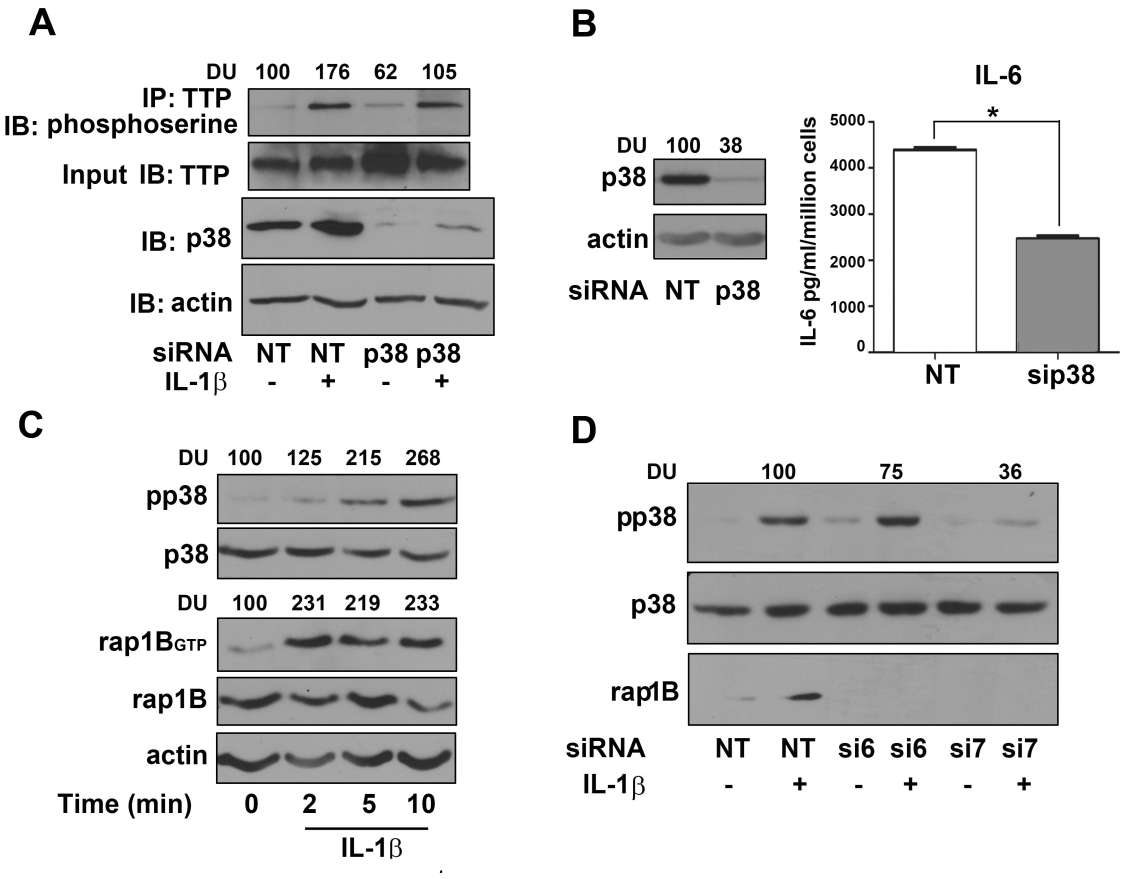

Figure 3.S4: p38-mediated phosphorylation of TTP promotes pro-inflammatory mediator secretion and rap1B induces p38 activation. (A) Co-immunoprecipitation. UM-SCC-81B were transfected with siRNA targeting p38 (sip38) or non-target controls (siNT). Cells were serum starved and treated with PBS (control) or IL-1β for 10 minutes. Whole cell lysates were coupled with TTP-bound AminoLink® Plus Coupling Resin as described above. Eluted samples were electrophoresed and immunoblotted with phosphoserine and TTP. Whole cell lysates from the same samples were electrophoresed and immunoblotted with p38 and actin antibodies. Densitometric units for phosphoTTP were determined after normalization to IB for phosphoserine to TTP after co-IP. Experiments were performed in three independent experiments. (B) IL-6 ELISA. UM-SCC-81B cells were transfected with siRNA targeting p38 (sip38) or non-target controls (siNT). Whole cell lysates were electrophoresed and immunoblotted for anti-total p38 and anti-GAPDH. Interleukin-6 (IL-6) secretion was determined from conditioned media via ELISA in duplicate from three independent experiments. (* represents, $p<0.01$) (C) Immunoblot analysis. UM-SCC-81B cells were serum starved and treated with IL-1β for 0, 2, 5, or 10 minutes. Lysates were immunoblotted with pp38, p38, rap1B and actin (loading control). For active rap1B, whole cell lysates (50 μg of protein) were retrieved by a ralGDS pull-down assay, electrophoresed and blotted for rap1B antibody. Rap1B equivalent was verified from lysates immunoblotted with rap1B. Data represent a

representative blot of three independent experiments. Densitometric units were determined by normalizing rap1B GTP to total rap1B and pp38 to total p38 and then plotted them as a percentage change to time zero. (D) Immunoblot analysis. UM-SCC-81B were transfected with two siRNAs targeting rap1B (si6 and si7) or siRNA non-target controls (NT) and were stimulated IL-1β for 10 minutes or PBS (control). Whole cell lysates were immunoblotted for pp38, p38 and rap1B. Densitometric units were determined by normalizing pp38 to total p38 and expressed as a percent change of NT under stimulated conditions. Experiments were performed in triplicate.

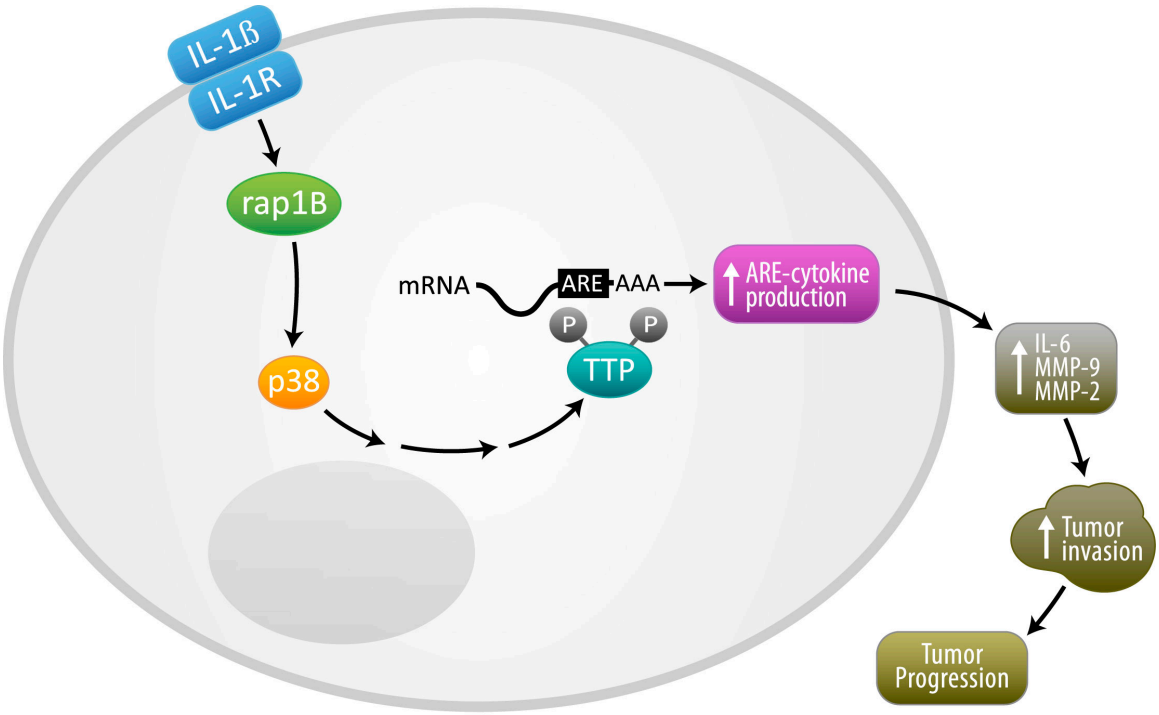

Figure 3.6: Model for interaction between rap1B, p38 and TTP in regulation of tumor progression. TTP is downregulated or is functionally inactivated by upstream proteins p38 and rap1B which promotes tumor invasion via increased secretion of IL-6, MMP-9 and MMP-2 from enhanced mRNA stability. Patients with high IL-6 or high MMP-9 are more likely to develop tumor recurrence, secondary primary, need surgery or die from disease.

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CHAPTER IV

GALANIN STIMULATES p38 MAPK TO INDUCE TTP-MEDIATED SECRETION OF PRO-ANGIOGENIC CYTOKINES AND ANGIOGENESIS

ABSTRACT

Galanin receptor 2 (GALR2) is a G-protein coupled receptor that has a significant role in progression of squamous cell carcinoma of the head and neck (HNSCC) by promoting cell survival and proliferation. Angiogenesis, invasion and metastasis are other hallmarks of tumor progression. In HNSCC, we recently showed that p38 phosphorylates tristetraprolin (TTP), an mRNA binding protein, thereby stabilizing cytokine mRNA, cytokine secretion and invasion. Despite the importance of angiogenesis in cancer progression, the role of p38-mediated TTP-phosphorylation in promoting angiogenesis via secretion of multiple pro-angiogenic cytokines has not been investigated. *Objective*: The goal of this project was to investigate whether GALR2 induces angiogenesis via p38 MAPK-phosphoTTP-mediated secretion of pro-angiogenic cytokines. *Methods*: Studies were performed in two different HNSCC cell lines stably overexpressing GALR2. Conditioned media was generated from HNSCC cell lines after knockdown of p38, TTP, or IL-6 via siRNA or shRNA. Knockdown was verified by immunoblot analysis of whole cell lysates, and pro-angiogenic cytokines were quantified by ELISA. Angiogenesis assays were performed with human microvascular endothelial cells exposed to conditioned media. *Results*: Galanin induces p38 MAPK, which promotes secretion of

IL-6, VEGF and PGE_2. Knockdown of p38 MAPK decreases the length and number of endothelial cell sprouts, whereas downregulation of TTP has the reverse effect. Furthermore, knockdown of IL6 in HNSCC cells transduced with shTTP, rescues the active TTP phenotype i.e. it reduces the length and the number of endothelial cell sprouts. *Conclusions*: Galanin stimulates p38 MAPK which promotes angiogenesis via TTP-mediated secretion of pro-angiogenic cytokines in head and neck cancer. In HNSCC, targeting upstream regulators of cytokine secretion, such as p38 or TTP, may improve response to treatment by inhibiting multiple pro-angiogenic cytokines concurrently.

INTRODUCTION

Each year nearly 650,000 individuals are diagnosed with head and neck (HNSCC), which is the sixth most common cancer globally [1]. Pro-angiogenic cytokines such as interleukin-6 (IL-6) and vascular endothelial growth factor (VEGF) promote tumor progression by supporting the development of a vascular network to supply oxygen and nutrients to the growing tumor and by promoting invasion. IL-6 is overexpressed in head and neck, myeloma and prostate cancers [2-5]. In HNSCC, IL-6 is a biomarker for poor disease specific survival [6] and patients with high VEGF have shorter time to disease recurrence than patients with low VEGF[7].

Despite the evidence that pro-angiogenic factors such as IL-6 and VEGF promote tumor progression in HNSCC, treatment has not improved significantly in over 40 years. Inhibitors of VEGF and IL-6 were developed to target the growth and vasculature of multiple solid tumors[8]. While VEGF inhibitors are used in the treatment of colorectal

cancer as an adjuvant to chemotherapy, the drug does not decrease disease recurrence [9]. Moreover, in breast cancer VEGF inhibitors have only marginally improved survival; recently the FDA revoked approval of these inhibitors for treatment of breast cancer [10]. The marginal effects of these drugs in cancer treatment may be due to redundancy in function between pro-inflammatory mediators in cancer. This highlights the importance of elucidating the mechanisms that support angiogenesis. In particular, since HNSCCs secrete multiple pro-angiogenic factors, patients would benefit from the development of therapies that target a common regulatory mechanism of multiple pro-angiogenic factors.

Pro-angiogenic factors, such as IL-6 and VEGF, are carefully regulated post-transcriptionally to enable rapid modulation of protein expression [11]. mRNA is actively degraded or stabilized by RNA binding proteins (RNA-BPs) that bind to adenine and uridine rich elements in the 3'untranslated region. Tristetraprolin (TTP) is an RNA-BP that promotes decay of the transcripts of multiple pro-angiogenic factors including IL-6, VEGF and IL-8 in cancer and inflammatory cells [4, 12-15]. In HNSCC, TTP is downregulated thereby leading to transcript stability and enhanced secretion of IL-6 and VEGF [4]. Although both IL-6 and VEGF are potent pro-angiogenic factors, the extent to which low TTP promotes angiogenesis has not been investigated in any cancer.

p38 MAPK is a critical signaling mediator that is activated during an immune response. In order to minimize tissue damage from p38 MAPK, multiple feedback mechanisms inactivate p38 MAPK activity. In HNSCC and multiple myeloma, p38 MAPK activity is constitutively active and promotes tumor growth, survival and invasion by secreting pro-inflammatory mediators[16]. However its role in angiogenesis, a hallmark of cancer [17], has not been investigated in HNSCC.

Galanin, a neuropeptide that is also secreted by HNSCC, induces the G-protein coupled receptors, galanin receptors 1, 2 and 3 [18]. Galanin receptor 2 (GALR2) has a significant role in HNSCC progression by promoting cell proliferation and survival [19]. Although galanin expression is correlated with increased microvessel density in inflammatory tissue in the dermis, a functional role for GALR2 in angiogenesis in cancer has not been investigated [20].

In this study, we show that galanin induces GALR2-mediated stimulation of p38 MAPK in HNSCC. Moreover, downregulation of p38 MAPK decreases angiogenesis due to TTP-mediated inhibition of secretion of pro-angiogenic cytokines. Given the importance of p38 MAPK-derived pro-angiogenic factors such as IL-6 and VEGF in tumor progression, inhibition of secretion of these factors by targeting a common upstream regulator may be a promising strategy to suppress angiogenesis and tumor progression.

MATERIALS AND METHODS

Cell culture. HNSCC cell lines from the University of Michigan (UM-SCC) used for this study: were UM-SCC-1, UM-SCC-22B and UM-SCC-81B. These cell lines were validated by genotyping at the DNA Sequencing Core at the University of Michigan and cultured as described [21, 22]. An immortalized human microvascular endothelial (HMEC-1) cell line was used for angiogenesis assays. HMEC-1 cells were maintained in MCDB 131 media (Gibco, Grand Island, NY, USA), supplemented with 10% FBS, 1% glutamine, 1% pen/strep and 1 ng/ml of epidermal growth factor (EGF).

Transfection. For transient transfections, *ON target SMART pool* siRNA was used to target p38 MAPK expression and individual *ON target SMART pool* siRNAs were used to target TTP or IL-6 expression. Negative controls were *ON Target plus* siControl (Dharmacon, Lafayette, CO, USA) and transfections were performed as described. [6]. UM-SCC-1 and UM-SCC-22B were stably transfected with pcDNA3.1-GALR2 (Missouri S & T cDNA Resource Center) or pcDNA (Invitrogen) as described [19]. These cells were selected with geneticin (G418).

Western Blot Analysis. Whole cell lysates were prepared in NP40 lysis buffers supplemented with phosphatase inhibitors and sonicated on ice as described [6]. After blocking membranes for 1 hour, the primary antibody was added at 4°C overnight or 1 hour at room temperature. Primary antibody concentrations were 1:1000 unless otherwise indicated. The following antibodies were used: pp38, total p38, actin (Cell Signaling, Danvers, MA), TTP (Abcam, Cambridge, MA), IL-6 (R&D Systems, Minneapolis, MN, USA) and GAPDH (MAB374, Millipore/Upstate, Billerica, MA). Secondary antibodies used were horseradish peroxidase conjugated donkey anti-rabbit IgG and goat anti-mouse (1:2,000 to 1:10,000; Jackson ImmunoResearch Laboratories). Immunoreactive proteins were visualized by SuperSignal Substrate (Pierce, Thermo Fischer, Rockford, IL) and exposed to X-ray film.

Enzyme Link Immunosorbent Assay. HNSCC were transfected with siRNA p38 MAPK, siTTP or NT controls [4]. Conditioned media (CM) from HNSCC cell lines transfected with siRNA was collected as described [6]. IL-6 and VEGF were measured by a non-competitive ELISA (R & D Systems). PGE_2 was quantified using a competitive ELISA (R & D Systems).

Matrigel In Vitro HMEC tube formation assay. Matrigel ® (BD Biosciences) thawed on ice was coated on an 8-well chamber slide (BD Biosciences) and placed in the 37˚C incubator to polymerize as described [23]. CM was concentrated, equilibrated for cell number and volume, and resuspended in MCDB 131 media containing 3% FBS. 40,000 HMEC-1 cells were seeded in triplicate in 150 µl of CM from non-target (NT), sip38, siTTP or siIL-6. VEGF (1 ng/ml) was used as a positive control. 5 µl of concentrated blank DMEM served as a negative control. Endothelial tubes (sprouts) were allowed to form for 24 hours and digital images were taken of 5 fields from each well. Endothelial tubes were counted in each field to determine sprout number. Sprout length was assigned a length from the software J Image.

Stable TTP knockdown. For stable knockdown of TTP, UM-SCC-1 and UM-SCC-81B were transduced with short hairpin RNA (shTTP and shVSVG controls) in lentiviral particles in a VSVG backbone (University of Michigan Vector Core). Stable cell lines were selected in 10µg/ml puromycin (Sigma).

GALR2 activation. Serum starved UM-SCC-1-GALR2 and UM-SCC-22B -GALR2 cells or pcDNA controls were treated with 10 nM of galanin (Gal) for zero, 2, 5 or 10 minutes as described [19].

Data analysis. Statistical analysis of *in vitro* assays was performed using a Student's *t*-test. A p-value of < 0.05 was considered to be statistically significant (GraphPad®).

RESULTS

p38 promotes secretion of VEGF and PGE_2 in HNSCC. In previous studies we showed that inactivation of TTP promotes IL-6 and VEGF secretion. To investigate whether p38 MAPK, which phosphorylates TTP, induces cytokine secretion, p38 MAPK was downregulated in HNSCC and cytokines were quantified in conditioned medium. In UM-SCC-1, siRNA-mediated knockdown of p38 MAPK reduced IL-6, VEGF and PGE_2 secretion by more than 50% compared to cells transfected with control siRNA (IL-6 previously shown in Chapter 3 Figure 3.5 and Supplemental Figure 3.4) (Figure 4.1B and 4.1C, respectively; $p<0.01$). Downregulation of p38 MAPK was verified by immunoblot analysis (Figure 4.1A and Figure 4.D). Similar results were observed in UM-SCC- 81B (Figure 4.1E through 4.F).

p38 promotes angiogenesis. Since p38 MAPK induces pro-angiogenic cytokines, its role in angiogenesis was investigated in HNSCC cell lines . In UM-SCC-1 and UM-SCC-81B, p38 MAPK expression was suppressed with siTTP but not control siRNA, as verified by immunoblot analysis (Figure 4.2A and 4.2F, respectively, left panels). Relative to control (non target) cells, p38 MAPK expression was reduced nearly ~45% after knockdown and normalization to total p38 MAPK. To assess the relationship between p38 MAPK and angiogenesis, endothelial cells (HMEC-1) were cultured on Matrigel® in conditioned media from HNSCC cells transfected with sip38 or siNT (Figure 4.2D and 4.2H, respectively, right panels). VEGF (1 ng/ml) served as a positive control while concentrated DMEM was a negative control. Sprout length and number decreased nearly 50% in HMEC-1 cells cultured in conditioned media from sip38 cells

compared to control cells in both UM-SCC-1 and UM-SCC-81B (Figure 4.2A, 4.2C 4.2F and 4.2G, respectively).

Suppression of TTP promotes angiogenesis in HNSCC. Since p38 MAPK inactivates TTP to promote cytokine secretion (Chapter 3, Figure 3.5), the role of TTP in angiogenesis was investigated. Conditioned media from UM-SCC-1 and UM-SCC-81B cells transfected with siTTP or control siRNA was collected, concentrated and seeded with HMEC-1 cells as described above. Sprout length and the number of sprouts of HMEC-1 cells increased by 2-fold when incubated with conditioned medium from siTTP cells compared to control cells (Figure 4.3A and 4.3B, respectively). In UM-SCC-81, the relative length of sprouts increased 1.5-fold while the average number of sprouts increased by more than 2-fold compared to HMEC-1 cells incubated with conditioned medium from control cells (Figure 4.3D and 4.3E, respectively). Downregulation of TTP in both cell lines was verified by immunoblot analysis (Figure 4.3C and 4.3F, respectively).

IL-6 mediates angiogenesis when TTP expression is suppressed in HNSCC. Suppression of TTP enhances angiogenesis and promotes secretion of IL-6, a pro-angiogenic cytokine. Therefore, to investigate the role of IL-6 in TTP-mediated-angiogenesis, IL-6 was suppressed in HNSCC cells with stable downregulation of TTP. UM-SCC-1 and UM-SCC-81B cells with stable suppression of TTP were transfected with siRNA targeting IL-6 or control siRNA. HMEC-1 cells on matrigel were incubated

with conditioned media isolated from UM-SCC-1-shTTP cells transfected with control siRNA or siIL-6. The length and number of sprouts decreased by ~2-fold when compared to cells incubated with conditioned media from cells transfected with control siRNA (Figure 4.4A and 4.4B, respectively). Similar results were observed in UM-SCC-81B (Figure 4.4D and 4.4E, respectively). After normalization to the actin loading control, IL-6 expression was reduced 40% in UM-SCC-1 and 30% in UM-SCC-81B cells transfected with siIL-6 compared to control siRNA (Figure 4.4C and 4.4F, respectively).

Galanin stimulates p38 MAPK in HNSCC. We recently showed that galanin-induced GALR2 promotes tumor progression in HNSCC via stimulation of ERK/MAPK and AKT pathways [19]. Given the importance of p38 MAPK in invasion and angiogenesis, hallmarks of tumor progression [17], the role of GALR2 in p38 MAPK stimulation was investigated. In order to evaluate GALR2 mediated activation of p38 MAPK, UM-SCC-1 and UM-SCC-22B cells stably transfected with GALR2 or empty vector, were incubated with 10 nM galanin. As early as 2 minutes, galanin induced p38 MAPK in UM-SCC-1-GALR2 cells after normalization to total p38 (Figure 4.5A). Galanin induced pp38 MAPK more dramatically in the GALR2 overexpressing cells compared to control cells at 2, 5 and 10 minutes. Moreover, baseline (time zero) p38 MAPK was increased in GALR2 overexpressing cells compared to pcDNA control cells by 128% after normalization to total p38 MAPK. Similar results were observed in UM-SCC-22B (data not shown).

In previous studies from our laboratory [19], we observed that GALR2 tumors were more vascular compared to control tumors (Figure 4.5B). Since galanin stimulates p38

MAPK and active p38 MAPK promotes IL-6 secretion, which promotes angiogenesis in HNSCC, we are investigating whether the GALR2-induced angiogenic phenotype is mediated by p38 MAPK.

DISCUSSION

Angiogenesis, one of the six hallmarks of cancer [17], facilitates tumor progression by supplying oxygen and nutrients. IL-6 and VEGF mediate tumor-induced angiogenesis. Tumor derived IL-6 mediates its pro-angiogenic activity via paracrine signaling to endothelial cells to promote vessel formation [24, 25]. HNSCC secretes large amounts of IL-6 which has been linked with poor patient survival [3, 4]. Similarly, VEGF is also overexpressed in HNSCC and is incompatible with patient survival[7]. VEGF, secreted by both tumor and endothelial cells, promotes the development of a blood supply [24]. A single knockout of a VEGF gene in mice is embryonic lethal suggesting that VEGF is critical for maintenance and development of the vasculature [26]. There is a need for a delicate balance of angiogenic factors to maintain proper vasculature in healthy tissue; an imbalance in favor of angiogenic factors acts like a "switch" to promote tumor growth and spread[27]. Therefore targeting angiogenesis is an enticing target to inhibit tumor growth.

Since angiogenesis is a critical event in tumor progression, drugs targeting either IL-6 or VEGF have been developed, but treatment with an individual inhibitor shows minor improvement in patient survival. In phase II clinical trials, breast cancer patients treated with concurrent monoclonal VEGF antibody (Avastin ®) and chemotherapy compared to

chemotherapy alone only marginally increased disease-free survival [28]. Surprisingly in mouse studies, short term treatment with Avastin ® lead to more aggressive tumors [29]. Since targeted therapy against either IL-6 or VEGF was only marginally successful in different cancers, these studies suggest that it would be beneficial to identify proteins that target multiple pro-angiogenic factors simultaneously. In the present study, we show that galanin induces p38 MAPK, which promotes secretion of IL-6, VEGF and PGE_2. p38 MAPK knockdown decreases the length and number of endothelial cell sprouts, whereas downregulation of TTP has the reverse effect. Furthermore, knockdown of IL6 in HNSCC cells transduced with shTTP, rescues the active TTP phenotype i.e. it reduces the length and the number of endothelial cell sprouts. Thus, galanin stimulates p38 MAPK which promotes angiogenesis via TTP-mediated secretion of pro-inflammatory mediators in HNSCC. In HNSCC, targeting upstream regulators of cytokine secretion, such as p38 or TTP, may improve response to treatment by inhibiting multiple pro-angiogenic cytokines.

p38 MAPK is a critical signaling molecule in the induction of inflammatory pathways and is constitutively active in HNSCC [30, 31]. Activation of p38 MAPK with IL-1β promotes the secretion of pro-angiogenic factors such as IL-6, PGE_2 and VEGF in HNSCC[32]. Additionally, exposure to sodium pyruvate phorbol 12-mysritate 13-acetate, a potent tumor promoter, induces pro-inflammatory secretion via p38 MAPK activation [30]. However, the role of p38 MAPK has not been investigated in cancer-related angiogenesis. This is important to investigate because p38 MAPK is constitutively active in HNSCC and angiogenesis is a critical step in tumor progression. p38 inhibitors have been developed and are used in the treatment of inflammatory diseases and may be a

potential adjuvant therapy in HNSCC treatment. Targeting p38 MAPK would likely inhibit the secretion of multiple cytokines simultaneously.

IL-6 and VEGF are inversely correlated with TTP expression in HNSCC [6], however TTP mediated angiogenesis has not been investigated in any cancer. In this study, we determined that suppression of TTP increased angiogenesis via IL-6 in HNSCC. It is likely that VEGF and other pro-angiogenic factors that are regulated by TTP, also promote angiogenesis that is induced by inhibition of TTP in HNSCC.

p38 inhibitors may be a potential molecular treatment option in HNSCC. p38 inhibitors have been used in the treatment of inflammatory diseases to limit inflammatory sequelae with some success, however their role in HNSCC is relative unexplored. A p38 MAPK inhibitor would target an upstream signaling kinase that regulates multiple downstream signaling targets. Moreover p38 inhibitors would functionally activate any remaining TTP in the cell. Studies with p38 inhibitors reduced tumor size in early and advance stages of diseases in multiple myeloma [33] and may be attributed to p38 MAPK targeted reduction of IL-6 secretion [34].

p38 MAPK is stimulated by interleukin-1β (IL-1β) in multiple cell types [35]. While macrophages, tumor cells and cells in the tumor microenvironment secrete IL-1β to stimulate p38 MAPK activation, it is unclear whether tumor derived proteins induce p38 MAPK activation. Tumor-derived galanin promotes proliferation of HNSCC cells [18]. Galanin stimulates GALR1, GALR2 and GALR3. GALR1 is a tumor suppressor that inhibits cell proliferation; it is reduced in HNSCC relative to normal tissue [18, 36] and GALR3 is variably expressed [36]. GALR2 is unchanged or overexpressed in HNSCC

compared to normal human keratinocytes [19, 36]. GALR2 is the main player in tumor progression in HNSCC; GALR2 overexpression in HNSCC promoted proliferation and survival via activation of AKT and ERK [19]. In this study, we determined that GALR2 overexpressing cells have higher baseline p38 MAPK activation compared to pcDNA controls cells. AKT and ERK pathways are active in HNSCC and multiple cancers and contribute to tumor progression [37-39]. Given that blocking AKT or ERK pathways triggers compensatory activation of alternative signaling pathways [40, 41], inhibition of GALR2 may be a potential therapeutic strategy to concurrently inhibit three signaling pathways simultaneously thereby promoting a robust anti-tumorigenic effect. Alternatively, since galanin is a tumor-derived ligand [18], targeting p38 or TTP, may be a potential therapeutic strategy to inhibit secretion of multiple tumor-derived ligands that induce AKT, ERK and p38 MAPK pathways. Future studies will investigate p38 inhibitors and HNSCC disease progression.

Taken together, elucidating the critical signaling cascades that regulate angiogenesis in HNSCC, will facilitate the development of novel treatments.

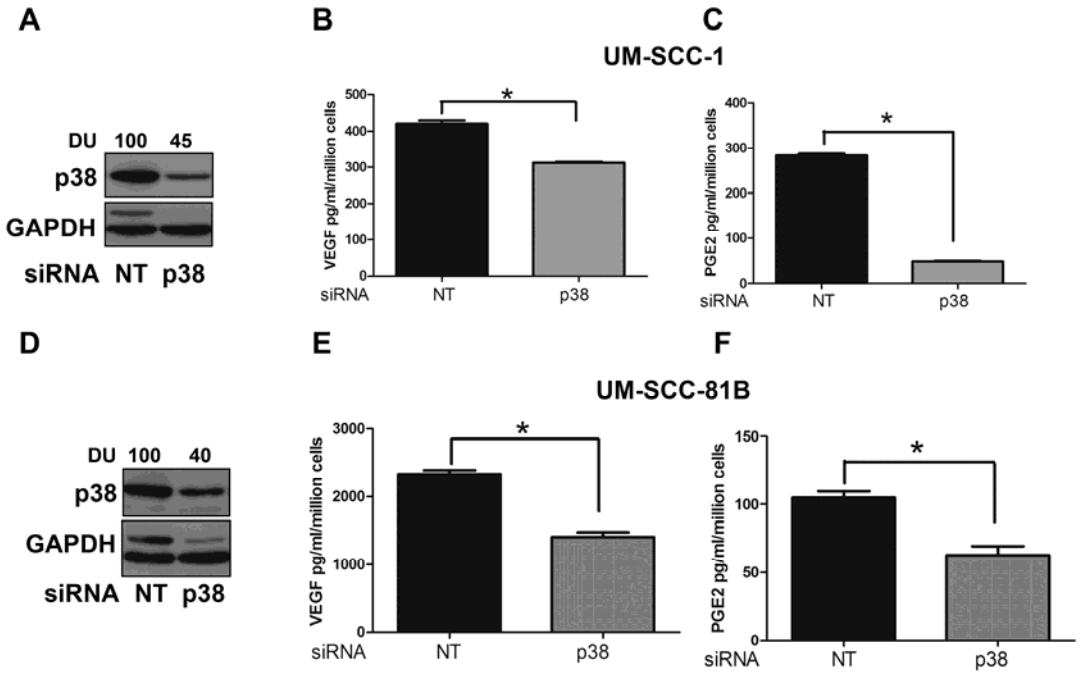

Figure 4.1: p38 promotes secretion of VEGF and PGE_2. UM-SCC-1 (A-C) and UM-SCC-81 (D-F) cells were transduced with siRNA targeting sip38 or non-target controls (NT). (A-C) The UM-SCC-1, (D-F) UM-SCC-81B cell lines were transfected with small interfering RNA (siRNA) against p38 or nontarget (nt) siRNA. (A) and (D) Whole-cell lysates were electrophoresed and immunoblotted with p38 antibody. Actin was used as loading control. p38 expression was expressed as a percentage of NT control. UM-SCC-1 (B and C) and 81B (E and F) Conditioned media was assayed in duplicate for vascular endothelial growth factor (VEGF), and prostaglandin E2 (PGE_2) by enzyme-linked immunoadsorbent assay. All experiments were performed in duplicate for UM-SCC-1 and triplicate for UM-SCC-81B. Data are representative of the mean and standard deviation of 2 replicates within an experiment (*indicates $P < .05$).

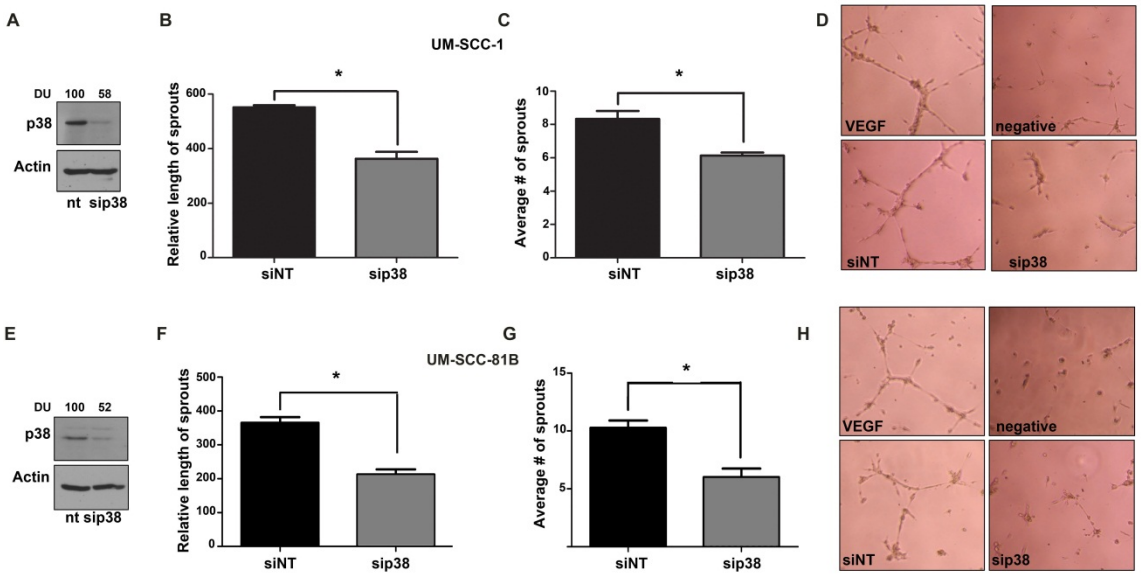

Figure 4.2: p38 promotes angiogenesis in HNSCC. (A) The UM-SCC-1, (B) UM-SCC-81B cell lines were transfected with small interfering RNA (siRNA) targeting p38 or nontarget (nt) siRNA. p38 expression was evaluated via immunoblot as described. Actin was used as a loading control. p38 expression is expressed as a percentage of NT control. Whole-cell lysates were electrophoresed and immunoblotted with p38 antibody. Actin was used as loading control. Conditioned media (CM) from siNT or sip38 cells was concentrated and total CM volume was normalized to total cell number and combined with MCDB 131 media and HMEC-1 cells that were seeded on a bed of matrigel. 1 ng/ml VEGF served as a positive control and concentrated media served as a negative control. Twenty-four hours after cell seeding, images were taken of five different fields. Sprout length and sprout number were quantified and analyzed in for significance. Data are representative of the mean and standard deviation of 2 replicates within an experiment (*indicates $P < .05$). Experiments were performed in 2 independent experiments performed in duplicated (UM-SCC-1) and 3 independent experiments performed in duplicate (UM-SCC-81B).

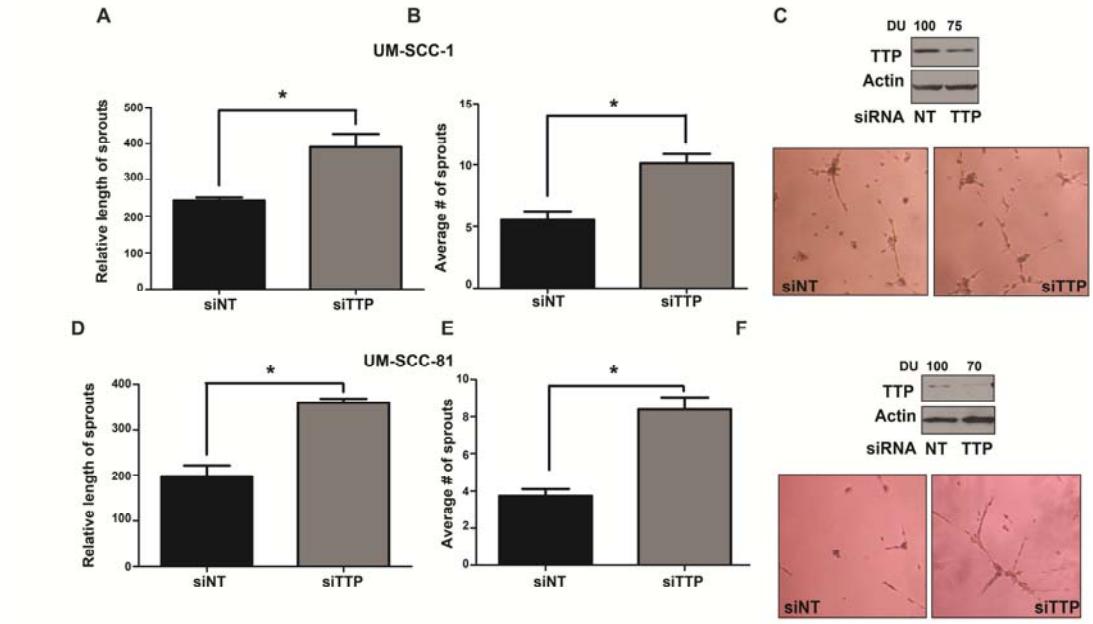

Figure 4.3: Suppression of TTP promotes angiogenesis in HNSCC. siRNA targeting TTP expression or nontarget (nt) were transfected in to (A) UM-SCC-1 and (B) UM-SCC-81B. p38 expression was evaluated via immunoblot in whole cell lysates as described. Actin was used as a loading control. p38 expression is expressed as a percentage of NT control. (CM) from siNT or sip38 cells was concentrated and total CM volume was normalized to total cell number and combined with MCDB 131 media and HMEC-1 cells that were seeded on a bed of Matrigel®. 1 ng/ml VEGF served as a positive control and concentrated media served as a negative control (not shown). Images were taken at twenty-four hours after cell seeding in five different fields per well. Sprout length and sprout number were quantified and analyzed in for significance. Data are representative of the mean and standard deviation of 2 replicates within an experiment (*indicates $P < .05$). Experiments were performed in 2 independent experiments performed in duplicated (UM-SCC-1) and 3 independent experiments performed in duplicate (UM-SCC-81B).

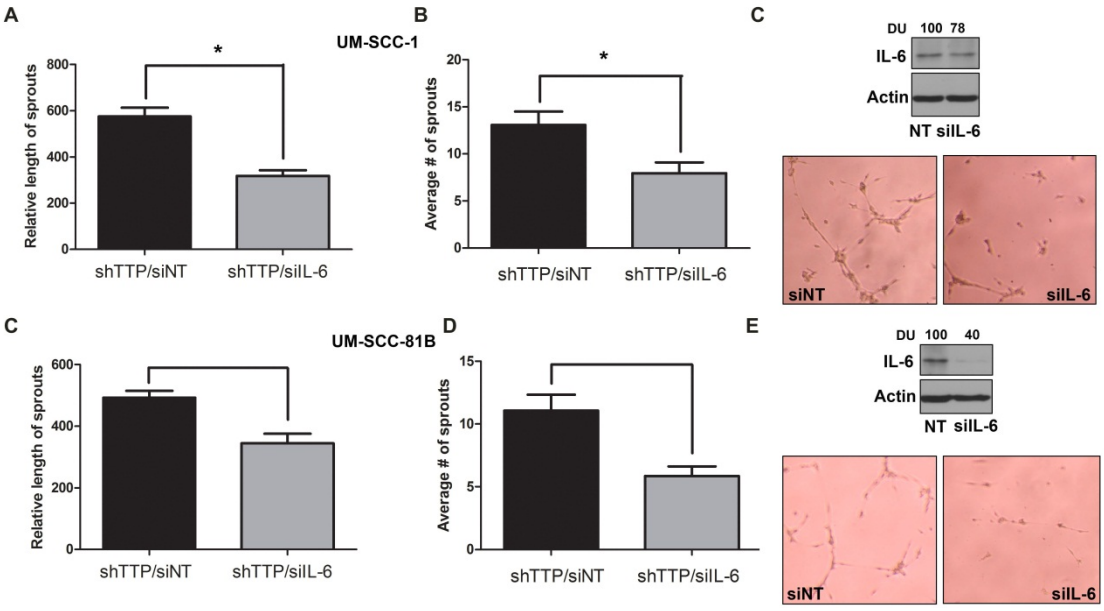

Figure 4.4: IL-6 mediates angiogenesis when TTP expression is suppressed in HNSCC. IL-6 or control nontarget (nt) siRNA were transfected in (A) UM-SCC-1 cells and (B) UM-SCC-81B cells that were stably transduced with short hairpin shRNA targeting TTP. After knockdown, concentrated CM was combined with media specific for HMEC-1 cells that were seeded on Matrigel® coated wells. 1 ng/ml VEGF served as a positive control and concentrated blank DMEM media served as a negative control (not shown). Images were taken at twenty-four hours after cell seeding in five different fields per well. Sprout length and sprout number were quantified and analyzed in for significance. Data are representative of the mean and standard deviation of 2 replicates within an experiment (*indicates $P < .05$). Experiments were performed in 2 independent experiments performed in duplicated (UM-SCC-1) and 3 independent experiments performed in duplicate (UM-SCC-81B).

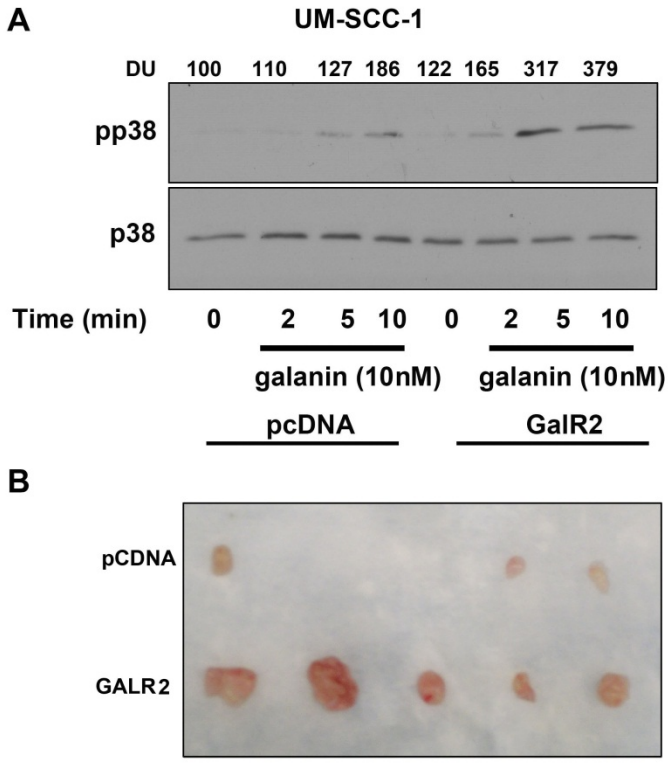

Figure 4.5: Galanin stimulates p38 MAPK in HNSCC. (A) UM-SCC-1 cells were stably transfected with pcDNA or GALR2. Cells were serum starved and treated with 10nM Galanin for 0, 2, 5, or 10 minutes. Whole cell lysates were electrophoresed, transferred to nitrocellulose and immunoblotted for pp38 and p38. Signal intensity was quantified by densitometric analysis with normalization total p38 and expressed as a ratio to time zero. Data is representative of two independent experiments. Similar results observed in UM-SCC-22B pcDNA and GALR2. (B) GALR2 promotes tumor growth and vascular appearance. UM-SCC-1 with pcDNA or pcDNA-GALR2 were injected subcutaneously in mice. After 2 weeks, the tumors were harvested and tumor volume (tumors previously collected as described [19]).

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CHAPTER V

CONCLUSION

While advances have been made in understanding the molecular basis of HNSCC progression, treatment has not significantly improved in decades. Targeting an individual pro-inflammatory mediator has not proved to be successful. This is most likely because redundant functions between pro-inflammatory mediators enable these proteins to compensate for one another. Using a cocktail of drugs to target multiple pro-inflammatory mediators is not practical due to drug toxicity. This highlights the importance of identifying a common regulatory mechanism for multiple pro-inflammatory mediators. In macrophages, tristetraprolin (TTP), an RNA binding protein, promotes the decay of multiple pro-inflammatory mediators suggesting that TTP may be a candidate protein for regulating multiple pro-inflammatory mediators in cancer [1-3]. Our objective was to attempt to identify the role of TTP in tumor progression in HNSCC.

The first aim of this dissertation was to establish the role of TTP in HNSCC. In initial studies, we explored expression of TTP and secretion of pro-inflammatory mediators in multiple HNSCC cell lines. We demonstrated that TTP expression was reduced when compared to normal human keratinocytes. Additionally, in HNSCC cell lines, TTP expression was inversely correlated with the secretion of IL-6, VEGF and PGE_2. To determine the role of TTP in modulating secretion of pro-inflammatory

mediators, we overexpressed TTP and observed that secretion of pro-inflammatory mediators was significantly reduced. This occurred regardless of the presence of IL-1β, a potent secretory agonist of pro-inflammatory mediators. Using a complementary approach, siRNA-mediated downregulation of TTP increased IL-6, VEGF and PGE_2 secretion. HNSCC cell lines stably transduced with shTTP and control shRNA were generated to evaluate the functional effect of TTP on mRNA stability. Actinomycin D was used to stop RNA transcription, which allowed evaluation of mRNA stability over multiple time points. Using this method, we found that suppression of TTP stabilized mRNA for IL-6 and PGE_2 compared to control cells. In a clinical study of patients with HNSCC, we showed that low TTP expression and high IL-6 expression is prognostic for poor disease specific survival, and moreover IL-6 is an independent biomarker for poor disease specific survival, overall survival, the development of second primary tumors, and tumor recurrence. Prior to this study RNA binding proteins had not been investigated in the context of HNSCC progression.

Once we established that TTP was inversely correlated with secretion of pro-inflammatory mediators, our next aim was to focus on the mechanism(s) of TTP mediated invasion, which had not been investigated in any cancer. To this end, we showed that low TTP facilitated invasion in a 2D matrigel invasion assay. Invasion and migration assays were functional end point studies to investigate the role of TTP knockdown in HNSCC. Loss of TTP increased invasion by over 300% and promoted cell migration in wound healing assays compared to control cells. However, the matrigel and wounding assays do not recapitulate the Type IV collagen barrier, observed in the intact basement membrane that separates a pre-cancerous lesion from the underlying

connective tissue. Therefore, a novel 3D *in vitro* assay was developed with human dermal tissue coated with Type IV collagen and seeded with cells with either TTP knockdown or control shRNA (oral cancer equivalent or OCE) . Using this method, we showed that low TTP promotes invasion when compared to control cells with high TTP. Invasion through the basement membrane is a critical step in tumor progression. Using an *in vivo* approach, we conclusively showed that TTP suppression promotes invasion through a basement membrane. Given that previous studies by the D'Silva Lab established that MMP-9 and MMP-2 have a critical role in invasion in HNSCC [4], we investigated the impact of loss of TTP on MMP-9 and MMP-2-mediated invasion. Suppression of IL-6, MMP-9 or MMP-2 in cells that have low TTP expression significantly reduced invasion compared to control cells. The interactions between IL-6 and MMP-9 were investigated in the context of negative sequelae in HNSCC: tumor recurrence, the development of second primary tumors, time to surgery, and death from disease. Both high IL-6 and high MMP-9 were independently and in combination with one another prognostic of poor clinical outcomes.

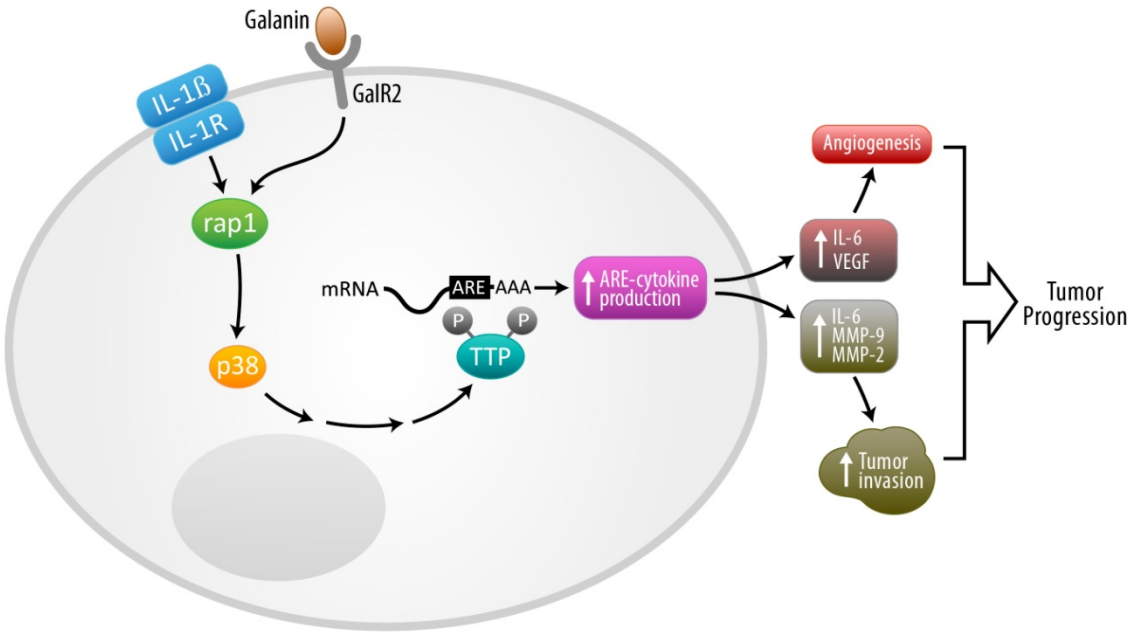
Ultimately we demonstrated that by modulating the pool of TTP we can alter the decay of multiple pro-inflammatory mediators. Therefore, TTP or upstream signaling molecules that inactivate TTP represent key therapeutic targets. In exploring this signaling cascade we established that p38 MAPK is constitutively active in a panel of HSNCC cell lines compared to normal human keratinocytes. We also determined that TTP is phosphorylated in HNSCC cells lines thereby promoting secretion of pro-inflammatory mediators, which is mediated by p38 MAPK. Finally, we established that rap1B, a small GTPase, induces p38 MAPK.

In the third aim we focused on angiogenesis, an essential phenotype of tumor progression. Inhibitors targeting an individual pro-angiogenic factor have not been dramatically successful in improving patient survival. Therefore it is critical to identify a protein that targets multiple pro-angiogenic factors simultaneously. Given that we previously established that p38 MAPK mediates TTP phosphorylation (i.e. inactivation), our initial experiments focused on whether p38 MAPK regulates secretion of pro-angiogenic mediators. We concluded that of p38 MAPK promotes secretion of multiple pro-angiogenic mediators including IL-6, VEGF, and PGE_2, and induces angiogenesis.

Although p38 MAPK inactivates TTP to promote secretion of pro-angiogenic factors and promotes angiogenesis, the role of TTP in angiogenesis had not been directly investigated. We demonstrated that suppression of TTP promotes angiogenesis via pro-angiogenic factors, such as IL-6. Our laboratory recently showed that GALR2, a G-protein coupled receptor promotes tumor progression in HNSCC via rap1-induced ERK and AKT activation [5]. Since rap1 induced p38 MAPK, which induced TTP-mediated secretion of pro-angiogenic factors and angiogenesis, we investigated the role of GALR2 in inducing p38 MAPK. We determined that stimulation of GALR2 with galanin activates p38 MAPK. Future studies will further explore the effect of GALR2 and angiogenesis.

As an overview in Figure 5.1, we propose a model to show the signaling cascade of TTP in HNSCC and how loss of TTP promotes invasion and angiogenesis by upregulation of pro-invasive and pro-angiogenic factors. In this dissertation we have demonstrated that TTP facilitates invasion via IL-6, MMP-9 and MMP-2 and promotes angiogenesis via IL-6. This is the first study to evaluate the mechanisms by which TTP

promotes invasion and angiogenesis in any cancer and to establish the upstream regulatory mechanism for TTP. In conclusion, loss or inactivation of TTP plays a key role in promoting invasion and angiogenesis, two of the six hallmarks of cancer via the modulation of multiple pro-inflammatory mediators. TTP and its upstream regulators may represent novel therapeutic targets to suppress multiple pro-inflammatory mediators simultaneously with the potential to improve patient survival in HNSCC.



Figur 5.1: Signaling pathways in HNSCC mediating TTP phosphorylation and invasion and angiogenesis. IL-1β stimulates rap1B activation and p38 MAPK activation, which mediates TTP phosphorylation. Phosphorylated TTP or suppressed TTP dissociates from the ARE region of pro-inflammatory mediator transcripts to permit enhanced mRNA stability and translation. Upregulation of pro-inflammatory mediator (i.e. cytokine) secretion promotes invasion via IL-6, MMP-9 and MMP-2 and angiogenesis via IL-6 and VEGF. Galanin mediates rap1B activation of p38 MAPK and angiogenesis via phosphorylation of TTP and IL-6 and VEGF secretion.

FUTURE DIRECTIONS

Pro-inflammatory and pro-angiogenic mediators play a significant role in tumor progression. In particular, this dissertation work showed that suppression of IL-6 decreased invasion, which is one of the hallmarks of tumor progression. IL-6 is a pleotropic cytokine that plays many important roles in normal cellular function but when overexpressed, promotes tumor growth and angiogenesis in HNSCC, breast and colon cancer [6-10]. While IL-6 is an important player in promoting invasion in cancer, the mechanism by which IL-6 promotes invasion has not been thoroughly investigated. One possible mechanism by which IL-6 could promote invasion may be by facilitating matrix metalloproteinase (MMPs) secretion and activity. MMPs are important because they can promote the degradation of the basement membrane or surrounding tissues. Recent research sheds some light on the relationship between IL-6 and MMPs. In colon cancer, IL-6 may act as a feed forward protein to promote MMP-9 and MMP-2 secretion. In particular, overexpression of IL-6 promotes invasion while an IL-6 inhibitor decreased MMP-9 and MMP-2 secretion[8]. In addition, a study in glioma cells found that constitutive IL-6/STAT3 signaling promotes invasion by potentiating an MMP-2/α5β1 integrin interaction. Furthermore, knockdown of MMP-2 decreased IL-6 levels and phospho STAT3 levels in *in vitro* and *in vivo* studies [11]. In head and neck cancer, further studies are needed to investigate the possibility that IL-6 may promote invasion via MMP secretion. More importantly, elucidating the mechanisms by which IL-6 promotes invasion may lead to the development of novel therapeutic strategies in treating HNSCC.

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