Latent Kaposi's Sarcoma Herpesvirus regulates host factors to induce Lymphangiogenesis and Angiogenesis

Kimberley Diane Gutierrez

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Reading Committee:

Michael Lagunoff, Chair

Adam P. Geballe

Timothy M. Rose

Program Authorized to Offer Degree:
Department of Microbiology

University of Washington

Abstract

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Kimberley Diane Gutierrez

Chair of the Supervisory Committee:
Professor Michael Lagunoff
Department of Microbiology

Kaposi's Sarcoma herpesvirus (KSHV), the etiologic agent of Kaposi's Sarcoma, is present in the predominant tumor cells of KS, the spindle cells. Spindle cells express markers of lymphatic endothelium and, interestingly, KSHV infection of blood endothelial cells reprograms them to a lymphatic endothelial cell phenotype. KSHV induced reprogramming requires the activation of STAT3 and PI3/AKT through the activation of cellular receptor gp130. Importantly, KSHV-induced reprogramming is specific to endothelial cells, indicating that there are additional host genes that are differentially regulated during KSHV infection of endothelial cells that contribute to lymphatic reprogramming. In this thesis we explore multiple host factors that contribute to lymphangiogenesis and angiogensis and therefore aid in the tumorogenesis of KSHV infected endothelial cells. In chapter III we explore the host transcription factor Ets-1 and found that Ets-1 is highly expressed in KS spindle cells and is upregulated during KSHV infection of endothelial cells in culture. The latent viral gene vFLIP is sufficient to induce Ets-1 expression in an NF-κB-dependent fashion. Ets-1 is required for KSHV-induced expression of VEGFR3, a lymphatic endothelial cell specific receptor important for lymphangiogenesis, and Ets-1 activates the promoter of VEGFR3. Ets-1 knockdown does not alter the expression of another lymphatic specific gene, podoplanin, but does inhibit the expression of VEGFR3 in uninfected lymphatic endothelium, indicating that Ets-1 is a novel cellular regulator of VEGFR3 expression. Knockdown of Ets-

1 affects the ability of KSHV infected cells to display angiogenic phenotypes indicating that Ets-1 plays a role in KSHV activation of endothelial cells during latent KSHV infection. Thus, Ets-1 is a novel regulator of VEGFR3 and is involved in the induction of angiogenic phenotypes by KSHV. We and others have demonstrated that KSHV infected endothelial cells display angiogenic phenotypes such as increased capillary stability. In Chapter IV we show the host cytokine TGF-beta2 is downregulated during latent KSHV infection of endothelial cells. We demonstrate that the addition of TGF-beta2 to KSHV infected cells grown in a three dimensional matrix disrupts capillary stability, indicating that KSHV downregulates TGF-beta2 in order to promote angiogenesis of latently infected endothelial cells. Furthermore, we show that the KSHV microRNAs, miR-K3 and miR-K-8 are sufficient to downregulate transcription of TGF-beta2. In summary, KSHV differentially regulates the expression of many host genes involved in promoting lymphangiogenesis and angiogenesis of latently infected endothelial cells which ultimately may contribute to KS tumorigenesis.

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CHAPTER I

Introduction

Kaposi's Sarcoma

Kaposi's Sarcoma (KS) is a highly vascularized tumor first described in 1872 by the Hungarian dermatologist, Moritz Kaposi. There are four epidemiological forms of KS; classic, African endemic, transplant associated and acute immune deficiency syndrome (AIDS)-associated. Classic KS is described as an indolent skin tumor found on the lower extremities of elderly men of Mediterranean descent. Classic KS is rarely life threatening however the other clinical forms of KS are more aggressive and present a greater challenge to public health. In parts of central Africa, KS is the most common tumor seen in hospitals, occurring in both HIV-positive and HIV-negative patients (1-5). This form of KS, African endemic, is characterized by KS lesions on the head and neck with widespread dissemination to visceral organs. African endemic KS also appears frequently in women and children (2, 3, 6) . AIDS-associated is the most aggressive form of KS and spreads rapidly throughout the body to visceral organs. In addition to cutaneous skin lesions of the upper body, AIDS-related KS presents in the oral cavity as well as lungs and gastrointestinal tract where there is often extensive edema and hemorrhaging leading to organ dysfunction and high rates of mortality and morbidity. HAART (Highly Active Antiretroviral Treatment) has led to a decline of AIDS-associated KS by delaying the onset of AIDS and controlling the depletion of the immune system (5-7). The final form of KS is associated with solid organ transplant patients during immunosuppressive therapy. Often times this form of KS regresses once the immunosuppression is halted which further supports a role for the immune system in the control of KS tumor development (5, 6).

The forms of KS are classified based on presentation and epidemiological variances however, all forms of KS are histologically indistinguishable. KS tumors are highly vascularized, exhibiting extensive

neoangiogenesis, the formation of new blood vessels, which is thought to be critical to the development of the tumor (8). The vasculature of KS tumors is malformed, with leaky vascular slits resulting in the extravasation of red blood cells giving the KS tumor its characteristic reddish purple, bruise-like appearance. In addition to erythrocytes, the KS tumor also contains infiltrating inflammatory cells. However, the main cell type found within the KS tumor is the spindle cell, a cell of endothelial origin (9, 10). Specifically, KS spindle cells display markers of lymphatic endothelium including Vascular Endothelial Growth Factor receptor 3 (VEGFR3), podoplanin and Prox1 (11-13). VEGFR3 is the receptor for VEGF-C, a cytokine critical for the induction of lymphangiogenesis, the formation of new lymphatic vessels. The gene expression profile of KS spindle cells most closely matches that of isolated lymphatic endothelial cells (LECs), further indicating that KS is a lymphatic endothelial cell disease (14, 15). KS tumors also have elevated levels of proangiogenic cytokines and growth factors such as TNF, INFγ, VEGFs, IL-6, and IL-8. Many of the cytokines are secreted by spindle cells and are essential for the spindle cell growth in culture suggesting that deregulation of cytokines and growth factors is essential for KS tumor development and maintenance.

Discovery of KSHV as the infectious agent of Kaposi's Sarcoma

In 1972, electron microscopy revealed the presence of Herpesvirus-like capsids in KS tissue. However, there was no strong evidence to indicate a Herpesvirus was the cause of KS (16). The AIDS-related KS epidemic provided large-scale epidemiological studies that provided strong evidence that the development of KS may be due to an infectious agent. Early in the AIDS epidemic of the 1980s, KS was widely recognized as a complication of the disease and became the first AIDS defining illness. The suspected cause of KS quickly became HIV. However, the development of KS could not be explained by HIV infection alone. Firstly, the majority of spindle cells from KS biopsies of AIDS patients did not harbor the HIV genome. Secondly, despite being severely immunodeficient due to HIV infection, the risk of

developing KS varied among the AIDS population with more than 20% of homosexual men with untreated AIDS developed KS while less that 2% of hemophiliacs with AIDS and essentially no children with AIDS presented KS (5). The risk associated with KS development was strongly correlated with how the individual was infected with the HIV virus. Homosexual men primarily acquired HIV via a sexual route while hemophiliacs and children acquired the virus from a blood transfusion or through maternal transmission. This suggested that although KS can be transmitted via blood products there may be an alternative sexual route of transmission involved in the development of KS (5, 17).

In 1994, Chang and Moore utilized representational difference analysis to identify unique DNA sequences between KS tissue and normal uninfected tissue (18). It was reasoned that if an infectious agent was responsible for KS then comparing KS tissue to normal tissue would reveal differences at the DNA level. Short fragments of DNA from diseased and normal tissue are amplified by PCR and then hybridized to DNA from normal tissue and diseased tissue. This method enriches for unique DNA sequences obtained from diseased tissue by differentiating the sequences that hybridize to both disease and normal tissue. Chang, Moore and colleagues obtained two distinct sequences that did not hybridize to normal tissue. It was revealed that these sequences shared distant homology to Epstein-Barr Virus (EBV), a human gamma Herpesvirus and Herpesvirus saimiri. These were the first sequences of a newly discovered virus named Kaposi's Sarcoma Herpesvirus (KSHV) or human herpesvirus-8 (HHV-8), which was classified as a gamma Herpesvirus (18). The KSHV genome was cloned and sequenced which lead to the development of clinical PCR tests to detect viral DNA and serological tests for the presence of recombinant antigens (5, 19). Following the identification of KSHV, the presence of the virus was detected in endothelial cells lining the vascular slits of the KS lesion as well as within the predominant cell type of the lesion, the spindle cell. The virus was found in all four epidemiologic forms of KS and KSHV viral DNA is always found in KS tumors including HIV-negative forms of KS (20-24).

Two B cell lymphoproliferative diseases, primary effusion lymphoma (PEL) and Multicentric Castlemen's disease (MCD) are also associated with KSHV (6, 25). PEL, or body cavity based lymphoma, is a rare non-Hodgkin's B-cell lymphoma that appears in the pleural, pericardial and abdominal cavities. PELs lack *c-myc* rearrangement which distinguishes them from Burkitt's lymphomas (26, 27). Multicentric Castlemen's disease is a rare multiple lymph node angiolymphoproliferative hyperplasia. KSHV association with both PEL and MCD is commonly seen in AIDS patients further supporting the involvement of immunosuppresion as a factor for KSHV tumorigenesis (6, 26). Interestingly, most PELs are co-infected with KSHV and EBV, suggesting that the two viruses cooperate to promote cellular transformation. However, while all PELs are KSHV-positive, 30% of PELs are EBV-negative therefore PEL is classified as a KSHV malignancy. Unlike the spindle cells from KS tumors, PEL cell lines are able to maintain KSHV infection when cultured *ex vivo* (6, 28, 29). Therefore KSHV virions were first propagated from a PEL cell line and KSHV-positive/EBV-negative PEL cell lines are currently the source of most viral stocks (6, 30).

KSHV latent and lytic gene expression

KSHV has a double stranded DNA genome approximately 165 kilobase pairs in length encoding over 90 ORFs. Most genes encoded by KSHV are conserved with other herpesviruses and include structural genes and viral genes necessary for DNA replication and viral gene expression. However, there are more than 15 unique genes within the KSHV genome that include a K prefix in their name. These genes encode homologs of cellular genes that affect host cell growth, alter cell signaling pathways, or promote immune evasion (6). As with all herpesvirsues, KSHV has both a lytic and a latent phase. Latency is the default mode of KSHV infection as determined by viral transcript analysis and Immunofluorescence (IFA) detection of viral proteins. Latent DNA is maintained as a circular episome in

the nucleus. In cultured endothelial cells and in KS tumors, the virus establishes latency in over 95% of infected cells, while only 1-5% of the cells support lytic replication of the virus (31).

During latency only a handful of genes are expressed, including latency-associated nuclear antigen (LANA-1), viral cyclin (vCyc), viral FLICE inhibitory protein (vFLIP), the Kaposin family members A, B, and C, as well as numerous viral microRNAs (miRNAs) expressed from 12 loci (32-36). LANA-1, vCyc and vFLIP are all expressed from the same locus transcriptionally regulated by the KSHV latent promoter that produces a differentially spliced polycistronic mRNA. The latent promoter of KSHV is bidirectional and controls the constitutive expression of the latent genes and during lytic reactivation controls expression of K14 and vGPCR. In addition, studies have suggested that other viral genes may be expressed at low levels during latency as well (37). All of the latent proteins have growth stimulatory or pro-survival functions that can contribute to the pathogenesis of KSHV. Numerous functions for LANA-1 have been described in KSHV pathogenesis. LANA-1 tethers the viral episome to cellular chromatin to ensure the proper segregation of viral genomes during cell division and promotes latent viral genome replication. Additionally, LANA binds and inhibits the functions of the tumor suppressors p53 and pRb, and can act as a transcriptional repressor or activator (38). Another latent viral gene, vCyc, is a homolog of cyclin D and promotes G1 to S phase cell cycle progression and is resistant to the cyclin dependent kinase inhibitors, $p27^{Kip1}$, $p21^{Cip1}$ and $p16^{Ink4}$ (39). vFLIP inhibits induction of FADD-mediated apoptosis and activates NF-kappaB dependent transcription of anti-apoptotic proteins and cytokines (36). Kaposin A and kaposin B alter the mitogen activated protein kinase (MAPK) pathways to control the expression of cellular genes, including cytokines (40, 41). Finally, the 12 microRNAs (miRNAs) of KSHV have effects on viral replication, immune evasion, apoptosis and angiogenesis. Thrombospondin, a regulator of endothelial cell adhesion, migration, and angiogenesis is targeted by three KSHV microRNAs (42).

Lytic reactivation of KSHV leads to an ordered cascade of viral gene expression resulting in virion production and cell lysis. Lytic replication can be experimentally induced by phorbol esters such as 12-O-

tetradecanoylphorbol-13-acetate (TPA) or sodium butyrate, which activate AP-1 transcription factors and inhibit histone deacetylases respectively (6). The lytic-switch protein replication and transcriptional activator (Rta/ORF50) initiates the lytic program and transactivates the promoters of lytic genes. Upon primary infection of endothelial cells, there is an initial burst of lytic gene expression, which is quickly replaced by a predominant latent gene expression profile (43). LANA and the viral microRNA miR-K12-7-5p can bind to the Rta promoter and repress activity, which promotes the establishment and maintenance of latency (44, 45).

Blood versus Lymphatic Endothelial Cells

It is generally accepted that KS-derived spindle cells originate from endothelial cells, although they also express markers of many other cell types including smooth muscle cells, macrophages, fibroblasts and dendritic cells (9, 46). In particular, spindle cells display markers of the lymphatic endothelium including vascular endothelial growth factor receptor 3 (VEGFR-3) and podoplanin (12, 47, 48). The gene expression profile of KS tumors also most closely matches that of lymphatic endothelial cells (14, 15). Our lab and others previously found that KSHV infection of blood endothelial cells leads directly to cellular reprogramming to a more lymphatic endothelial cell phenotype (14, 15, 49).

Blood endothelial cells (BECs) and lymphatic endothelial cells (LECs) differentially express over 300 genes and make up the two major vascular systems (50). The lymphatic vasculature, comprised primarily of LECs, is responsible for the uptake of lymph, activated immune cells and fluids leaked into peripheral tissues, and returning this to the blood circulation. The blood vascular system of veins and arteries, comprised of BECs, is completely separate from the lymphatic system and is responsible for delivering oxygen and nutrients throughout the body (51). There are differences in both the architecture of blood and lymphatic vessels, as well as the cell surface markers found on the respective cell types, BECs and LECs (Figure 1-1A). Blood vessels are surrounded by pericytes and smooth muscle cells and

have a readily apparent basal lamina. Lymphatic vessels lack smooth muscle cells, and have overlapping intracellular junctions and a less defined basal lamina, allowing for more efficient uptake of immune cells and fluid from peripheral tissues.

During embryogenesis, the blood vessel system, lined by blood endothelial cells, forms first, followed by the lymphatic vessel system, which is lined by lymphatic endothelial cells, forms. The blood vasculature is established from progenitor cells in a process termed vasculogenesis during embryonic development. Endothelial precursor cell chemotaxis, cell proliferation and differentiation, and vascular structure assembly is directed predominantly by the growth factor VEGF-A. VEGF-A acts by binding to the receptors VEGFR-1 and VEGFR-2 found on BECs. In the adult, VEGF-A also controls the maintenance, remodeling and development of new blood vessels, a process termed angiogenesis (51, 52).

The lymphatic vasculature is derived from the venous system after the start of blood vascular development (Figure 1-1B). In mice, lymphatic endothelium is first detected at embryonic day 9.5 in a subset of cells in the cardinal vein that express the homeobox transcription factor Prox1 (53-55). After induction of Prox1 expression, early LECs express the lymphatic markers VEGFR-3, LYVE-1, and podoplanin. VEGF-C produced from mesenchymal cells, directs these early LECs to bud from the anterior cardinal vein and migrate to form primitive lymph sacs and sprout to form lymphatic vasculature (56).

The transcription factor Prox1 is the master regulator of lymphatic differentiation (53-55). Prox1 knockout mice lack a lymphatic vasculature, indicating the default pathway for differentiation is blood endothelium (53, 57). Additionally, ectopic expression of Prox1 in differentiated BECs upregulates the expression of lymphatic markers, such as VEGFR-3, and downregulates the expression of blood endothelial markers (54, 55). Thus, Prox1 functions as both a transcriptional repressor and activator. Prox1 expression in other cell types does not upregulate lymphatic genes, suggesting the need for additional endothelial-cell-specific factors (54). The initial signals that induce Prox1 expression remain unknown.

While Prox1 mediates the initial steps in lymphatic differentiation, the other lymphatic markers are involved at later stages in the development of the lymphatic vasculature. VEGFR-3 is widely expressed in blood vascular endothelial cells in the early stages of development, but expression is restricted to lymphatic vessels later in the adult. Like VEGFR-1 and VEGFR-2, VEGFR-3 is a class III receptor tyrosine kinase characterized by seven extracellular immunoglobulin-like domains and an intracellular tyrosine kinase domain (58, 59). Activation of VEGFR-3 by the ligands VEGF-C and VEGF-D, initiates a series of intracellular signaling pathways to promote cell growth, survival, and migration of LECs. VEGFR-3 is required for initial budding and formation of the lymphatic vasculature and subsequent lymphatic vessel growth in response to VEGF-C (60). In addition to VEGFR-3, the surface glycoprotein Podoplanin may contribute to lymphatic endothelial cell adhesion and migration in later stages of development (61). The function of the lymphatic vessel hyaluronan receptor LYVE-1 in lymphatic development has yet to be determined.

In the adult, new blood and lymphatic vessel growth is stimulated by damage to the vasculature, inflammation and immune responses and tumorigenesis (62). VEGF-A promotes angiogenesis through the receptors VEGFR-1 and VEGFR-2 found on blood endothelium, while VEGF-C and VEGF-D promote lymphangiogenesis through binding the receptors VEGFR-3 and VEGFR-2 found on lymphatic endothelium (52). In addition to VEGF/VEGFR signaling, several other signaling systems have been shown to mediate angiogenesis and lymphangiogenesis, including angiopoietin signaling, ephrin signaling, and the Notch pathway (51). Tumor cells stimulate a number of these pro-angiogenic and pro-lymphangiogenic signaling pathways to recruit new blood and lymphatic vessel formation, which in turn contributes to tumor growth and metastasis (56, 63).

KSHV infection alters endothelial cell gene expression and cell signaling networks

Currently there is no widely accepted reproducible animal model available in order to study KSHV tumor development and pathogenesis. Therefore, cell culture systems have been developed as model systems in order to study KSHV alterations to host cell signaling pathways and host gene expression. High-level KSHV infection can be established in human primary dermal microvascular endothelial cells (d-HMVEC) as well as in Tert-Immortalized Microvascular Endothelial (TIME) cells (31). Primary d-HMVEC cultures consist of a mixture of LECs and BECs, from which pure LEC and BEC populations can be isolated by magnetic activated cell sorting (MACS) with antibodies to the extracellular domain of VEGFR-3 or podoplanin (64). The gene profile of TIME cells has determined they most closely resemble BEC's in origin. Because of the Hayflick limit, primary d-HMVECs can only be passaged 10-15 times in culture before senescence whereas TIME cells can grow well over 100 passages in culture. Infection in these cells recapitulates what is observed in KS lesions where over 90% of the cells establish a latent infection with only 1-5% expressing lytic genes.

KSHV infection of different populations of endothelial cells alter host genes that can promote cell survival, proliferation, migration, extracellular matrix remodeling, and angiogenesis, which can potentially contribute to KS tumor formation (14, 15, 49, 65-68). It has been hypothesized that KSHV infects LECs in vivo to establish KS tumors because KS spindle cells express markers of lymphatic endothelium. (12, 47, 48) Furthermore, KS does not occur in organs that lack lymphatic vessels, such as the brain or eye (69, 70). However, gene expression profiling has determined that KSHV can drive the differentiation of BECs to LECs (14, 15, 49). KSHV infection induces many lymphatic specific genes including Prox1, VEGFR-3, LYVE-1 and podoplanin in both primary BECs and TIME cells (14, 15, 49). Prox1 knockdown by siRNA partially inhibited KSHV-induced lymphatic reprogramming, suggesting the involvement of both Prox1-dependent and -independent mechanisms of lymphatic marker induction (14). Moreover, all endothelial cells latently infected with KSHV express lymphatic markers, suggesting

that latent viral gene expression contributes to lymphatic differentiation (49). Therefore, it is possible that KSHV infects blood or circulating endothelial cells and drives differentiation to lymphatic endothelium as they become spindle cells, which may be of major importance to KS tumor formation.

Ectopic expression of Prox1 in BECs induces the expression of lymphatic specific genes and downregulates the expression of blood endothelial genes such as VEGFR-1 and VEGF-C. However, KSHV infection of TIME cells upregulates the expression of VEGFR-1 and VEGF-C, genes that are normally expressed at lower levels in LECs versus BECs (49). Therefore, the cellular reprogramming observed during KSHV infection of endothelial cells is a unique and complex process that may be important in KS tumor formation. The induction of VEGFR-1 is a result of KSHV-mediated induction of hypoxia-induced factors (HIFs) at the mRNA level (71). Moreover, KSHV-mediated HIF upregulation depends on Src-family kinase activity (71). Both HIF1α and HIF2α are highly expressed in latently infected TIME cells and within KS tumor biopsies (71). Similar to LEC marker induction, these results suggest that KSHV latent genes are partially responsible for activating HIF transcriptional programs in KSHV-infected endothelial cells and within KS tumors.

KSHV's alteration of endothelial gene expression may be the result of viral proteins directly binding host DNA or modulating cellular transcription factors. The latent protein LANA-1 has been shown to act as a transcriptional activator and repressor, and also acts to epigenetically silence promoters through the recruitment of DNA methyltransferases (72). The 12 viral miRNAs can also directly regulate host cell gene expression by binding host mRNAs and suppressing their translation. Finally, host cell gene expression can also be regulated by modification of cell signaling cascades by viral proteins, eventually leading to activation of cellular transcription factors.

KSHV infection deregulates a number of cell signaling pathways including the MAPKs, phosphatidyl-inositol 3-kinase (PI3K)/Akt, NF-kappaB, Notch, Wnt/β-catenin, and JAK/STAT3 pathways. These pathways are altered upon expression of individual viral genes, in the context of KSHV infection,

and within KS tumors and PEL cells and have been shown to contribute to host cell proliferation, survival, and immune response modulation (73). How KSHV alters these cell signaling pathways and how these pathways in turn affect host cell gene expression can help unveil the mechanisms of KSHV tumorigenesis. It is important to understand how individual viral genes contribute to alterations in cell signaling pathways and gene expression. However, these changes must be verified in the context of KSHV infection where there is crosstalk between signaling pathways stimulated by the individual viral genes.

KSHV infected endothelial cells support angiogenic phenotypes

KS is an angioproliferative disease and KS tumors are highly vascularized, therefore it has been proposed that KSHV may induce angiogenesis. Angiogenesis is a normal process in wound healing but is hijacked and manipulated under various disease conditions such as cancer. Angiogenesis is a tightly regulated process that results in the formation of new blood vessels and is a hallmark of tumor formation and metastasis. The angiogeneic process is summarized in Figure 1-2. Under non-diseased states, endothelial cells that make up the vasculature are maintained in a non-proliferating state, or quiescence (74). During tumor formation, pro-angiogenic cytokines secreted by tumor cells can activate receptors on nearby endothelial cells to induce growth and proliferation. These activated endothelial cells begin to release proteases such as matrix metalloprotease that degrade the basement membrane to allow endothelial cells to migrate from the pre-existing vessels. The endothelial cells then proliferate into the surrounding environment and form solid sprouts or capillaries that connect to neighboring vessels. As these capillaries extend toward the source of the angiogenic stimulus, endothelial cells migrate and attach to neighboring cells or vessels using adhesion molecules or integrins (74, 75) . Integrins not only aid in attachment and adherence but are important for cell-cell communication and signaling (76). These capillaries form loops to become new vasculature and undergo remodeling,

differentiation and stabilization. During remodeling, the capillaries that do not connect to another vessel receive signals to undergo apoptosis. Eventually theses new vessels undergo maturation and become fully functional vasculature (74, 75). Many of the characteristics of angiogenesis and activated endothelial cells are induced upon KSHV infection of endothelial cells including proliferation, migration, and the expression of metalloproteases (8, 77). Many pro-angiogenic cytokines and genes involved in driving angiogenesis are induced in KSVH-infected endothelial cells suggesting that the combination may drive angiogenesis and KS tumorigenesis (8, 49, 78).

Among the pro-angiogenic cytokines induced during KSHV infection of endothelial cells are Vascular Endothelial Growth Factors (VEGFs), Angiogpoietin-2, COX-2, and Angiogenin (49, 78-80). The VEGFs are a family of cytokines that play a major role in the regulation of angiogenesis. VEGF-A and its receptors are required for embryonic vascular development and are important for vascular permeability, proliferation and survival of newly formed vasculature (81). VEGF-A is detected in KS spindle cells and its secretion is known to be increased by inflammatory cytokines that are present in the KS tumor (82). Infection of endothelial cells with KSHV directly leads to increased expression of VEGF-A (83-85). KSHV conditioned media from PEL cells has been shown to regulate angiogenic phenotypes in endothelial cells, therefore, KSHV induction of VEGF-A is likely to be critical for both the induction of angiogenesis as well as the activation of infected spindle cells (86-88). The mechanisms by which KSHV induces VEGF-A expression and secretion remain unresolved. However, several KSHV genes have been implicated including LANA-1, vGPCR, K1, K8.1 and vIL-6 and glycoprotein B (88-92). In addition to VEGF-A, VEGF-C is also induced during KSHV infection (83). Signaling of VEGF-C through its predominant receptor, VEGFR3, is involved in the proliferation and migration of lymphatic endothelial cells. VEGF-C is required for Prox1 expressing LEC precursors to migrate and develop the lymphatic vasculature during embryogenesis (56, 93). The main receptors for VEGFs are the VEGF receptors 1, 2 and 3 (81). As previously noted, VEGFR1, the receptor for VEGF-A, is upregulated during KSHV infection (49). The altered expression of VEGFR2,

another receptor for VEGF-A, has not been reported during KSHV infection. VEGFR1 has been described as both a positive and a negative regulator of angiogenesis (94). More research is needed to delineate the importance of induced VEGFR1 expression during KSHV infection. KSHV also induces expression of the receptor for VEGF-C, VEGFR3, during infection of endothelial cells (49, 78). The expression of VEGFR3 is specific to lymphatic endothelial cells and critical for lymphangiogenesis. Interestingly, endothelial tip cells at the leading edge of newly formed vasculature are the only adult cell type known to express both VEGFR1 and VEGFR3, whereas KSHV induces the simultaneous expression of both receptors (95).

Endothelial cells that line the vasculature form coordinated junctions to maintain barrier function. Breakdown of these junctions is necessary for the initiation of angiogenesis, immune cell extravasation and tumor cell metastasis (74). Several studies have found KSHV endothelial cells to have altered or perturbed adherens junctions which may result from the degradation of VE-cadherin and/or the disruption of the VE-cadherin/beta-catenin signaling pathway (8). KSHV can directly initiate the breakdown of cell-cell adherence but the exact mechanism is unknown. However, several viral genes could be involved such as vGPCR, due to its ability to induce cell permeability by down-regulating VE-cadherin (96). K5, a viral ubiquitin ligase protein also targets VE-cadherin and other cellular proteins important for cell to cell communication including platelet/endothelial cell molecule-1 (PECAM-1, CD31) (97).

Endothelial cells latently infected with KSHV also exhibit enhanced migration and invasion as evidenced via transwell assays (98, 99). During angiogenesis, endothelial cells migrate from the pre-exisiting vasculature toward the site of angiogenic stimuli. The extracellular matrix that surrounds the existing vasculature must be broken down in order to allow for invasion and migration of new cells and allow for the formation of new vessel development (74). The matrix metalloprotease (MMP) proteins aid in the breakdown of the extracellular matrix during angiogenesis and KSHV induces the expression of

MMP-1, -2, and -9 (77). Expression of MMPs induced by KSHV allows for increased invasion of both infected and uninfected endothelial cells (77, 91).

Endothelial cells grown in three-dimensional cultures migrate and organize into capillary-like structures. This activity is dependent at least in part on growth factors and cytokines present in the matrix or growth media. KSHV-infected cells are able to undergo capillary morphogenesis in low growth factor conditions to a greater extent than uninfected cells (85, 98). This could be due to increased cytokine secretion from KSHV–infected cells. Cultured endothelial cells in the presence of conditioned media from BCBL-1 cells have an increased ability to for capillary-like networks. The effect of the conditioned media was greater on KSHV infected cells than on mock infected cells suggesting that infected cells are more receptive to angiogenic growth factors (85). Capillary like structures from KSHV infected endothelial cells are more persistent than mock infected cells indicating the promotion of cell survival and continual angiogenesis by KSHV. DiMaio et al. showed that latent KSHV infection induces expression of the pro-angiogenic integrin, integrin β3 (98). The induction of integrin β3 leads to increased cell surface expression of the αvβ3 integrin heterodimer. Latently infected endothelial cells preferentially adhere to the integrin ligands fibronectin and vitronectin and are more migratory than mock infected cells. These induced phenotypes require RGD-binding integrins, specifically integrin β3. Furthermore, infected cells are more sensitive to inhibitors of integrin β3 and its downstream signaling molecules, Src kinases. This suggests that during latent KSHV infection there is a shift in endothelial cell signaling that results in a more angiogenic phenotype dependent on αvβ3 expression. KSHV alterations of endothelial cell signaling pathways can alter how the cell responds to intra as well as extra cellular signals.

The mechanisms by which KSHV induces angiogenic phenotypes remain largely unknown. However, many alterations in endothelial cell signaling pathways induced by KSHV promote cell growth, migration and survival. These changes lead to alterations in angiogenic properties that are likely to play

a role in KS tumor formation. Furthermore, the induction of lymphatic specific gene expression during latent KSHV infection suggests that signaling pathways that induce lymphatic reprogramming may also be involved in promoting angiogenic phenotypes (8). Therefore, delineating the cellular pathways that are required for lymphatic reprogramming may give insights to the mechanism of virally induced angiogenic phenotypes and ultimately KS tumor formation.

gp130 signaling is required for lymphatic differentiation by KSHV

KSHV infection of blood endothelial cells induces lymphatic specific gene expression and KS spindle cells by gene expression, most closely resemble LECs. Our lab demonstrated that activation of the phosphatidylinositol 3-OH-kinase (PI3K)/Akt cell signaling pathway, through the IL-6 cytokine family transmembrane receptor gp130, leads to the expression of the lymphatic specific markers VEGFR3, LYVE-1, podoplanin and Prox1 and that KSHV induced lymphatic reprogramming requires continued latent viral gene expression (100). Activation of gp130 by KSHV also induced persistent activation of STAT3. The gp130 receptor-mediated activation of both the JAK2/STAT3 and PI3K/Akt cell signaling pathways during KSHV infection is necessary for induction of the lymphatic specific transcription factor Prox1 and subsequent lymphatic reprogramming of endothelial cells. However, the cell signaling pathway by which gp130 activation leads to the induction of Prox1 remains incomplete. In addition, the latent gene, KaposinB, has been shown to stabilize Prox1 mRNA but does not actively induce expression of the gene (101). The activation of gp130 receptor signaling during KSHV infection is a novel mechanism for blood to lymphatic endothelial cell differentiation and therefore may be relevant to KSHV pathogenesis.

It has been demonstrated that the viral homolog to human IL-6 (vIL-6) is sufficient to induce lymphatic reprogramming of blood endothelial cells. However vIL-6 is not required for blood to lymphatic endothelial cell differentiation in the context of KSHV infection (102). Therefore other viral

factors are involved in driving KSHV induced reprogramming of blood endothelial cells. Importantly, the induction of lymphatic endothelial cell specific markers is observed in KSHV infected blood endothelial cells but not in infected cells of different origins, for example HEK 293 cells (VA Morris and M Lagunoff unpublished observations). Therefore, we sought to identify additional host genes that are differentially regulated during KSHV infection of blood endothelial cells that could contribute to lymphatic reprogramming.

Our lab performed a microarray analysis of Mock or KSHV infected TIME cells transfected with negative control or gp130-specific siRNA. Of the genes differentially expressed upon KSHV infection, between 28.6% and 60% were inversely expressed in the KSHV-infected gp130 knockdown cells (103). The microarray data confirmed that KSHV-mediated induction of gp130 and VEGFR-3 expression was blocked by gp130 siRNA and that gp130 receptor knockdown blocked all identified genes involved in LEC differentiation during KSHV infection. Furthermore, gp130 receptor knockdown does not affect latent infection or the establishment of latency by KSHV. Knockdown of the gp130 receptor also did not affect the expression of the latent viral protein LANA or genes known to be repressed by LANA activity. The signal transducer NF-κB, known to be activated during KSHV infection and modulated by the latent gene vFLIP, was also unaffected by gp130 knockdown.

In addition to the LEC-specific genes, gp130 receptor knockdown during KSHV infection altered other genes involved in angiogenesis, cell signaling, cell adhesion, cell migration, and tumorigenesis. This indicates that KSHV activation of the gp130 receptor leads to changes in global gene expression patterns in KSHV-infected endothelial cells and further work is needed to understand how gp130 signaling contributes to angiogenesis as well as lymphatic differentiation.

The microarray also identified genes differentially expressed upon KSHV infection that are not affected by gp130 receptor knockdown. We have identified some of these genes as being commonly upregulated during solid tumor formation and angiogenesis. Analysis of the microarray suggests that

gp130 receptor signaling is important for lymphatic differentiation and likely plays a role in the induction of pro-angiogenic factors. However, the array also indicates that signaling via gp130 is not the sole mechanism by which KSHV alters host gene expression. Therefore work is needed to uncover the role of host genes differentially regulated by gp130 and not by gp130 and how these genes contribute to KSHV pathogenesis and KS formation. This thesis focuses on key host genes altered during KSHV infection and explores the viral mechanism of regulation and how these genes promote angiogenesis, lymphangiogenesis and KSHV pathogenesis. Ets-1 is a transcription factor highly expressed in many solid tumors and was found to be induced by KSHV in a gp130 independent fashion (104). TGF-beta2 is an anti-angiogenic cytokine found to be downregulated by KSHV infection and controlled by gp130 receptor expression. The regulation of Ets-1 and TGF-beta2 and the role these host factors play in promoting angiogenesis and lymphangiogenesis during latent KSHV infection of endothelial cells will be discussed.

Ets-1 transcription factor

The transcription factor Ets-1 is a proto-oncoprotein highly expressed in a variety of solid tumors and has been implicated in tumor invasion and progression (105-109). Ets-1 is found in a variety of cell and tissue types including lymphocytes, fibroblasts, mesenchymal cells, and endothelial cells of the developing vasculature (110-114). Ets-1 is also expressed in B and T cells during all stages of their development. Ets-1 induces angiogenic and invasive phenotypes in endothelial cells, including wound healing, cell proliferation, and cell survival during embryonic angiogenesis (115-118). Ets-1 belongs to the ETS family of transcription factors due to the presence of the ETS DNA binding domain. This domain recognizes a GGAA/T core DNA sequence. Ets-1 is the first of the cellular homologues of the viral oncogene *v-ets* in the avian transforming retrovirus E26. There are two forms of the Ets-1 protein generated from one RNA, full length p54-Ets-1 and p42-Ets-1 lacking exonVII which contains an auto-

inhibitory sequence as well as calcium dependent phosphorylation sites. Ets-1 is predominantly found in the nucleus but can be found in the cytoplasm of quiescent endothelial cells and in a number of cancers, including gastric and ovarian. The nuclear localization sequence of Ets-1 is located in the C-terminus. The N-terminus of Ets-1 contains a Ras-responsive site at threonin-38 which when phosphorylated increases the transcriptional activity of Ets-1. Phosphorylation of threonin-38 induces expression of matrix metalloprotease 3 and urokinase-type plasminogin activator (uPA) which breakdown and degrade the extracellular matrix. Because of this function, the MMPs and uPA are implicated in cellular invasion and tumor progression.

Several kinases have been shown to modulate the activity of Ets-1 both positively and negatively. Calmodulin-dependent kinase II (CaMKII) can block Ets-1 DNA binding activity by phosphorylating the exonVII domain and blocking Ets-1 transcription. However, protein kinase Cα (PKCα) was shown to phosphorylate exonVII and increase transcriptional activity of Ets-1. Ets-1 cooperatively interacts physically and functionally with a number of proteins and transcription factors including AP-1, NF-κB, HIF2α and STAT5 (119-123). Ets-1 and AP-1 activate the Ras/ERK cell signaling pathway and upregulated among other genes, matrix metalloprotease 1 (MMP1). Ets-1 has also been shown to bind and directly activate the promoter of VEGFR1, an important growth factor receptor of blood endothelial cells. Other known gene targets of Ets-1 include Early growth response (Egr1), Tie 1 and 2, p53, VE-cadherin and Osteopontin (124-130). Many of these genes are angiogenesis related and promote survival, migration and invasion of endothelial cells. Therefore, it is not surprising that aberrant Ets-1 activity has been reported to promote angiogenesis and tumor development.

Ets-1 is upregulated by another human gamma herpesvirus, Epstein Barr virus (EBV), mediated by the viral latent membrane protein-1 resulting in the upregulation of c-Met and proliferation of virally infected epithelial cells (131). In KSHV, the latent gene LANA-1 was shown to interact with Daxx, a negative regulator of Ets-1, leading to the increased expression of VEGFR1 and VEGFR2 (132). Ets-1 was

previously shown to be induced by KSHV infection of endothelial cells and was necessary for the induction of the pro-angiogenic growth factor, Angiopoietin 2 (Ang2) (133). Ets-1 was also shown to be required for Ras mediated lytic reactivation of KSHV, indicating that Ets-1 may play a role in both latent and lytic phases of KSHV infection (134, 135). Microarray analysis from our lab indicated that Ets-1 is upregulated during latent KSHV infection of TIME cells (103). Chapter III of this thesis explores the role of Ets-1 during latent KSHV infection of endothelial cells.

Transforming Growth Factor-beta2 (TGF-beta2)

TGF-beta2 is a member of the transforming growth factor beta (TGFB) family of cytokines which are multifunctional peptides that regulate proliferation, differentiation, adhesion, migration, and other functions in many different cell types (136). TGF-beta proteins signal through transmembrane type I and type II receptors (TGFBR1 and TGFBR2) which activate the downstream Smad proteins. Smads can bind directly to DNA and modulate genes involved in the activation of endothelial cells as well as enhance migration, invasion and proliferation (137). Disruption of the TGF-beta/SMAD pathway has been implicated in a variety of human cancers.

The TGF-beta family of proteins are synthesized and secreted as large pro-peptide molecules consisting of three regions; an amino terminal signaling sequence, a pro-domain and a mature protein domain located in the carboxy terminus (138). Once synthesized, the TGF-β homodimer interacts with a Latency Associated Peptide (LAP) forming a complex called the Small Latent Complex (SLC). This SLC is then bound by another protein called Latent TGF-beta-Binding Protein (LTBP), forming the Large Latent Complex (LLC) which gets secreted to the ECM. After secretion, the LLC remains in the extracellular matrix as an inactivated complex containing both the LTBP and the LAP which must be removed in order to release active TGF-beta. Activation of TGF-beta can be mediated by pH, reactive oxygen species (ROS), thrombospondin, urokinase plasminogen activator (uPA), MMPs and integrins. Active TGF-beta

can now signal through the TGFB receptors which are serine/threonine kinases. TGF-beta initially binds to TGFBR2 which has a constitutively active kinase. TGFBR1 then binds to the TGF-beta molecule and becomes phosphorylated by TGFBR2 (136).

There are three isoforms of TGF-beta (TGF-beta1, 2 and 3) which display 60-80% homology at the amino acid level (138). TGF-beta1 is the most well studied and best characterized of the three isoforms. TGF-beta1 regulates a number of genes including integrins, matrix proteases, fibronectin, collagen, and thrombospondin. TGF-beta1 can exhibit both inhibitory and stimulatory effects on angiogenesis. An in vitro angiogenesis model suggests TGFBR2 mediates the anti-proliferative effects of TGF-beta1 while TGFBR1 mediates the pro-angiogenic matrix response. TGF-beta1 is also a potent chemoattractant for monocytes, macrophages, lymphocytes, neutrophils and fibroblasts and can also stimulate the release of pro-angiogenic cytokines IL-1, IL-6, TNFα and bFGF (138). TGF-beta1 maintains homeostasis and integrity of the ECM by stimulating collagen deposition and promoting the formation of fibrous tissue while also inhibiting proteases that degrade the ECM. TGF-beta1 is also implicated in embryogenesis in a number of cell types including endothelial cells and hematopoietic precursor cells (136, 138).

TGF-betas are involved in the process of wound healing and the regulation and remodeling of angiogenic capillaries. Aberrant regulation of TGF-betas and TGF-beta signaling has been described in a number of solid tumors and cancer progression. Two dimensional *in vitro* assays show that TGF-beta1 inhibits growth and migration of endothelial cells and also inhibits growth of cells transformed with the middle T oncogene of Polyoma virus (136). The anti-proliferative effects of TGF-beta1 can be overcome by a down-regulation of TGFBR2 (138). In vitro three dimensional culture assays show that TGF-beta1 inhibits invasive phenotypes of endothelial cells. When seeded on Matrigel, a proteinaceous basement membrane matrix, endothelial cells organize into networks and form connections or capillary junctions. Over time, due to the depletion of growth factors, the capillaries will regress whereas transformed cells,

cancer cells, will maintain these capillary networks. TGF-beta1 has been shown to accelerate the regression of capillary networks, however, it has also been reported that when endothelial cells are cultured in collagen gels, TGF-beta1 can accelerate the organization of the cells into capillary networks (136). This suggests that TGF-beta1 may have different functions on vessel formation at different stages of the angiogenic process. It is suggested that once a new vessel has formed TGF-beta1 promotes capillary regression and vessel maturation through the organization of a functional basement membrane (136).

A number of viruses including KSHV have been reported to regulate the TGF-beta pathway (139-143). During lytic infection, the KSHV viral genes vIRF1 and k-bZIP block Smad mediated transcriptional activity (144, 145). The TGF-beta pathway is also disrupted by KSHV latent gene products. The latent gene LANA-1 can epigenetically silence the TGFBR2 thereby inhibiting TGF-beta signaling in PEL cells (142). The KSHV microRNAs from pre-miR-K10 have also been shown to inhibit TGF-beta signaling by blocking Smad signaling and targeting the TGFBR2 (143).

Our lab identified a down-regulation of TGF-beta2 during latent KSHV infection of endothelial cells. TGF-beta2 is a homolog of TGF-beta1 and exhibits 41% homology at the cDNA level (146). There are two isoforms of TGF-beta2 created by alternative splicing of the mRNA (147). TGF-beta2 signals via the same receptors as TGF-beta1 and activate Smad signaling cascades (137). Chapter IV of this thesis discusses the mechanism of KSHV down-regulation of TGF-beta2 and the role this cytokine plays in KSHV induced angiogenesis.

Hypotheses

KS spindle cells, the predominant cells found within KS lesions most closely resemble lymphatic endothelial cells by gene expression profiling (49, 78, 148). KSHV infection of blood endothelial cells induces lymphatic reprogramming and the expression of lymphatic specific markers Prox1, VEGFR3,

podoplanin and LYVE-1 (49). Signaling via the IL-6 family receptor gp130 is necessary for the induction of lymphatic reprogramming during latent KSHV infection of endothelial cell (100). Furthermore, lymphatic reprogramming by KSHV latent infection is endothelial cell specific suggesting there are host factors or host phenotypes that are specific to endothelial cells that contribute to this process. Microarray analysis of KSHV infected endothelial cells indicates that gp130 receptor signaling directs global gene expression patterns that regulate cellular processes beyond lymphatic differentiation such as angiogenesis (103). However, many cellular pathways altered during latent KSHV infection are unaffected by gp130 knockdown.

I hypothesize that during latent KSHV infection, host genes regulated independent of gp130 signaling contribute to lymphatic reprogramming and promote lymphangiogenesis. Furthermore, in addition to lymphatic reprogramming, gp130 signaling regulates host factors that promote angiogenesis during latent KSHV infection. Therefore, latent KSHV infection alters host pathways to induce lymphatic reprogramming, lymphangiogenesis and angiogenesis that together promote KSHV pathogenesis and ultimately lead to KS spindle cell and tumor formation.

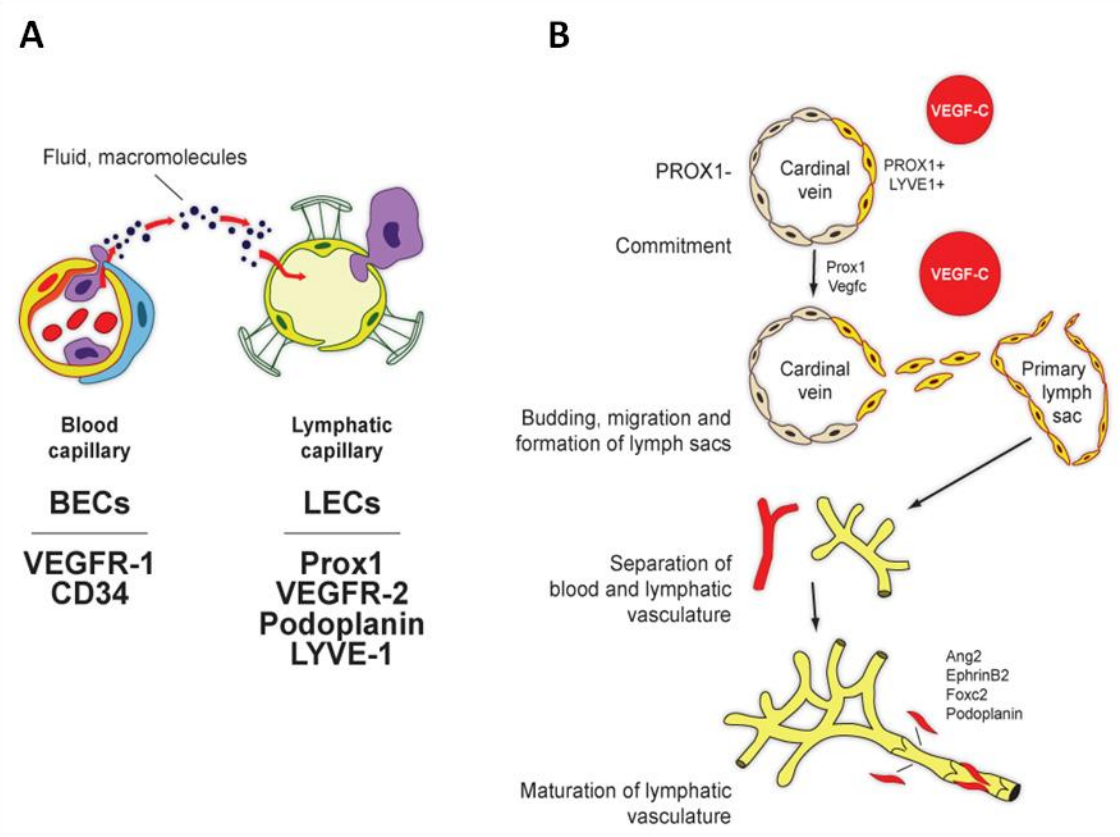


Fig 1-1. Lymphatic endothelial cell and vessel development A) Cellular architecture of blood and lymphatic capillaries and cell surface markers of BECs and LECs that make up the respective vascular systems. Immune cells and fluid leaked from blood vessels into the peripheral tissues enter the lymphatic vessels through loose valve like openings. Lymphatic vessels are linked to the extracellular matrix by anchoring filaments, which also prevent vessel collapse. B) Lymphatic vessels develop from the pre-existing blood vasculature. Prox1 and LYVE1 expressing cells respond to VEGF-C and bud from the cardinal vein to form primary lymph sacs. Other factors, Ang2, Podoplanin, Foxc2 and EphrinB2 direct the maturation of the lymphatic vasculature. (adapted from (149)).

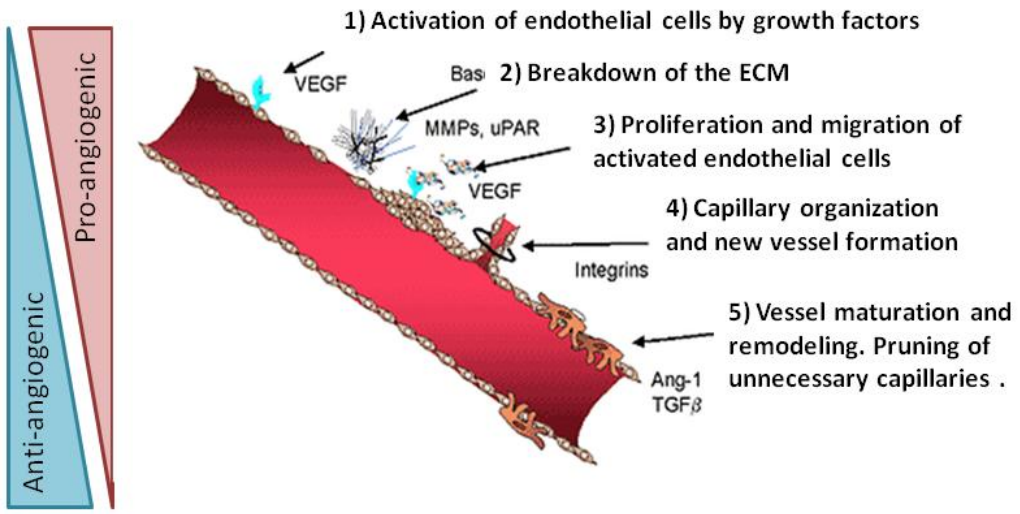


Figure 1-2 The Angiogenic process. (1) Quiescent endothelial cells of mature vasculature receive signaling in the form of VEGFs that activate cellular proliferation and pro-survival pathways. (2) MMPs and proteases are expressed and begin to break down the extra cellular matrix so that activated endothelial cells can (3) proliferate and migrate towards the source of pro-angiogenic stimulus and begin to organize into capillary-like cellular protrusions. (4) The capillary-like protrusions continue to elongate and form a lumen. Cell surface proteins, such as integrins, anchor the preliminary vessels to the ECM. (5) Maturation of the preliminary architecture is necessary for stabilization of the vasculature as well as removal of superfluous vessels. Cytokines like TGF-betas and Ang-1 aid in the maturation of the new vasculature by signaling the regression of unnecessary new capillaries, activating protease inhibitors to stop matrix degradation and stimulating collagen deposition to aid in ECM development. Additionally, supporting cells, such as pericytes and smooth muscle cells, are recruited to line the new mature vasculature (adapted from (150)).

CHAPTER II

Materials and Methods

Cell lines

TIME cells, LANA expressing TIME cells, pGreen and shEts-1 TIME cell lines and primary lymphatic endothelial cells (LEC) were maintained as monolayer cultures in EGM-2 medium (Lonza). HEK293 cells were maintained as monolayer cultures in DMEM (Cellgro, Mediatech, Inc.) supplemented with 10% fetal bovine serum, penicillin, streptomycin and glutamine. Stable LANA expressing TIME cells were previously described (102). GFP expressing TIME cell lines pGreen and shEts-1 were created using pGreenPuro shRNA cloning and expression lentivector (System Biosciences). The following oligonucleotide sequence was used to create the short hairpin RNA for Ets-1: forward, 5'-GAT CCG GAT GTG AAA CCA TAT CAA CTT CCT GTC AGA TTG ATA TGG TTT CAC ATG CTT TTT G-3'; reverse, 5'-AAT TCA AAA AGG ATG TGA AAC CAT ATC AAT CTG ACA GGA AGT TGA TAT GGT TTC ACA TCC G-3'. Lentivirus was created and used to infect TIME cells as previously described (102). Stable cell lines expressing GFP were selected for puromycin resistance in EGM-2 supplemented with 10 μg/ml of puromycin. KSHV inoculum, from BCBL-1 cells was used to infect all cell lines as previously described (31). Stable 3'UTR luciferase cell lines {pMirTarget control, TGF-beta2 and mutant TGF-beta2, (OriGene Technologies)} were created by transfecting 2 x 10^6 HEK293 cells with 20ug of plasmid using TransIT-293 reagent (Mirus) according to manufacturer's protocol. Stable cell lines expressing RFP were selected for neomycin resistance in complete DMEM supplemented with 50ug/ml of neomycin.

KSHV virus and infections

KSHV infections were performed in serum-free EBM-2 medium for 4 hours, after which the medium was replaced with complete EGM-2 medium containing serum and supplements. Infection rates

were assessed by immunofluorescence using antibodies against LANA and the lytic protein ORF59. In all infections performed with wild-type KSHV >90% of the cells were LANA-positive and <1% were ORF59-positive.

Plasmids

Lentiviral expression plasmids pSIN-MCS containing KSHV genes LANA, vFLIP or vCyclin (151) pCGSW (152) encoding GFP were described elsewhere. Lentiviral expression plasmids (GFP, vFLIP-HA, and mutant A57L-vFLIP-HA) were a kind gift from Thomas Schultz and were described elsewhere (153). Ets-1 construct (pcDNA-p51ETs-1) was a kind gift from Satoshi Yamagoe (National Institute of Infectious Diseases, Tokyo, Japan) and was propagated in DH5α competent E.coli grown in Luria-Bertani broth (LB broth) supplemented with 100ug/ml ampicillin. pMirTarget Control (#PS100062), pMirTarget-TGF-beta2 (#SC220584) 3'UTR luciferase constructs were purchased from OriGene Technologies (Rockville, MD). Plasmids were propagated in DH5α competent E.coli grown in LB broth supplemented with 100ug/ml Kanamycin and used to create stable cells line in HEK293 cells. KSHV microRNA lentiviral constructs were a kind gift from Ofer Mandelboim (154). Mutant-TGF-beta2 3'UTR was designed and constructed by custom gene synthesis {order # B13321-1/C37434 (Genewiz Inc.)}. Mutant-TGF-beta2 3'UTR was cloned into pMirTarget vector using restriction sites, 5', EcoRI and 3', MluI.

Antibodies and Reagents

VEGFR1 and podoplanin antibodies (Abcam) and VEGFR3, gp130 and Ets-1 antibodies (Santa Cruz Biotechnologies) and Beta actin and HA antibodies (Sigma) were used in immunoblot analysis as described below. Rabbit polyclonal Ets-1 (Abcam ab26096), Rat monoclonal (LANA-1) to KSHV ORF73 (Advanced Biotechnologies Inc) and purified rabbit IgG (Jackson ImmunoResearch) were used in immunohistochemistry analysis as described below. The LANA antibody used in the cell culture assays

was a kind gift of Don Ganem and the vCyclin antibody used was a rabbit polyclonal raised to the full length vCyclin protein (Morris and Lagunoff, unpublished). Kinase inhibitors AG490, LY294002, BAY 11-7082 (Calbiochem), and PD98059 (Cell Signal) were reconstituted in dimethyl sulfoxide (DMSO; Sigma) and used at the indicated concentrations. IKKγ NEMO Binding Domain (NBD) Inhibitor Peptide and control peptide (Imgenex) were re-suspended in DMSO and used at the indicated concentrations. The rhTGF-beta2 (Peprotech) and used in the three dimension culture of endothelial cells described in Chapter IV. miRCURY LNA microRNA inhibitors (Exiqon), negative control (#199020),KSHV-mir-K12-3 (#427946) and KSHV-miR-K12-8 (#427954) were resuspended in water and used at the indicated concentrations.

Immunoblot analysis

Cells were harvested and resuspended in RIPA lysis buffer (50 mM Tris-HCl, pH7.6, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate, 1 mM sodium orthovanadate, 1mM sodium fluoride, 40 mM β-glycerophosphate, and Complete Mini protease inhibitor tablet [Roche]), and cell debris was removed after a 30 minute incubation by centrifugation. Protein concentrations were determined by the bicinchoninic acid assay (Pierce), and 10 ug protein was fractionated on a 4-20% sodium dodecyl sulfate-polyacrylamide gradient gel, transferred to Immobilon P polyvinylidene difluoride membranes (Millipore), blotted with the appropriate antibody (dilutions were 1:1000 for anti-VEGFR3, anti-VEGFR1, anti-Ets-1, anti-podoplanin, anti-gp130, and anti-HA; 1:10,000 for anti-β-actin), and subsequently probed with horseradish peroxidase-conjugated goat anti-mouse or –rabbit immunoglobulin G (Jackson ImmunoResearch Laboratories). Immunoreactive proteins were visualized by chemiluminescence using Amersham ECL Plus (GE Healthcare).

RNA isolation and quantitative RT-PCR

Total RNA was isolated from TIME cells and LECs using the NucleoSpin RNA II (Mackerey-Nagel). One hundred nanograms of total RNA was used in a SuperScript III, Platinum SYBR green, one-step, quantitative reverse transcription PCR (RT-PCR; Invitrogen) according to manufacturer's protocols with the primer for either GAPDH (glyceraldehydes-3-phosphate dehydrogenase) (forward, 5'-AAG GTG AAG GTC GGA GTC AAC G-3'; reverse, 5'-TGG AAG ATG GTG ATG GGA TTT C-3') or VEGFR3 (vascular endothelial growth factor receptor 3) (forward, 5'-GAC AGC TAC AAG TAC GAG CAT CTG-3'; reverse 5'-CTG TCT TGC AGT CGA GCA GAA-3') or Ets-1 (forward, 5'-TCC TGC AGA AAG AGG ATG TG-3'; reverse 5'-GCT CTG AGA ACT CCG ATG GT-3') or LANA (forward, 5'- TTG CCA CCC ACG CAG TCT-3'; reverse 5'- GGA CGC ATA GGT GTT GAA GAG TCT-3') or vFLIP (forward, 5'- AGC TGT GTG CGA GGG ATA TT-3'; reverse, 5'-GGC GAT AGT GTT GGG AGT GT-3') or vCyclin (forward, 5'- ACG AGG TCA ACA CCC TGA TT-3'; reverse 5'-GCC TGT AGA ACG GAA ACA T-3') or TGF-beta2 (forward, 5'- CGC TAC ATC GAC AGC AAA GT-3'; reverse, 5'- TCC CAG GTT CCT GTC TTT ATG-3'). Relative abundances of mRNA were normalized by the delta threshold cycle method to the abundance of GAPDH, with mock-infected for control treated cells set to 1. Error bars reflect standard errors of the means of three independent experiments.

Immunohistochemistry

KS tumors from archived patient derived tissue samples were obtained with appropriate institutional review board oversight and deparaffinized in xylenes and allowed to rehydrate in TBS. Antigen retrieval consisted of incubation of slides in Tris-EDTA buffer (pH 9) at 95^{o}C in a water bath for 20 min. Primary antibodies were added overnight at room temperature (Ets-1 [1:250], Orf73 [1:400], or purified rabbit IgG [1;250]). Ets-1 antibody binding was revealed using a peroxidase-based staining kit (EnVision, Dako). Sections were counterstained with haematoxylin. Stained tissue sections were digitized

using a virtual microscope (NanoZoomer, Olympus) and images were analyzed using NDPviewer (Hamamatsu) and prepared for publication using Adobe Photoshop (CS).

Transfection of siRNA and LNAs

siRNA specific to gp130,Ets-1 and negative-control oligonucleotides were designed and synthesized by Ambion (Austin, TX). The following oligonucleotide sequences were used: gp130 (Ambion identification [ID] no. 106709; sense, 5'-GGC AUA CCU UAA ACA AGC UdTdT-3'), Ets-1 (ID no. 146635; sense 5'-GCA UAG AGA GCU ACG AUA GdTdT-3') and negative-control siRNA (sense, 5'-AGU ACU GCU UAC GAU ACG GdTdT-3'). TIME cells or LECs were transfected with 3ug of siRNA using Amaxa's Nucleofector kit (Cologne, Germany) according to the manufacturer's protocol. At 24 hrs post transfection, cells were mock or KSHV infected and subsequently harvested for analysis after an additional 48 hours. LECs transfected with siRNA were harvested 48 hours post transfection for analysis. Five micrograms of LNA specific to KSHV miR-K3, KSHV miR-K8 or a negative control were transfected into TIME cells using Amaxa's Nucelofactor kit (Cologne, Germany) according to the manufacturer's protocol. At 24 hours post transfection cells were transduced with Lentivirus to miR-K3, miR-K8 or control vector pSIN by spinoculation for 2 hours followed by 4 hour incubation at 37^{o}C in 5% CO^{2}. Cells were harvested 48 hours post transduction and harvested for analysis.

Promoter Luciferase Assays

TIME or HEK293 cells were seeded in a 6 well tissue culture dish and transfected with VEGFR1, VEGFR3 or an empty promoter firefly luciferase construct (Switchgear genomics) along with an empty vector control or an Ets-1 construct. Renilla luciferase was used to normalize transfection efficiency between samples. TIME cells were transfected using TransIT-Jurkat reagent (Mirus) and HEK293s were transfected using TransIT-293 reagent (Mirus). Twenty-four hours post transfection cells were lysed and

analyzed by Dual-Luciferase Reporter Assay (Promega) according to the manufacturer's protocol. Luciferase expression was measured using a Glomax 20/20 Luminometer (Promega) and reported as fold increase in luciferase expression of Ets-1 compared to empty vector. Error bars reflect standard errors of the means of five independent experiments.

3'UTR Luciferase Assays

Stable cells lines expressing either control 3'UTR, TGF-beta2 3'UTR or mutant TGF-beta2 3'UTR were seeded in 6-well tissue culture dishes at 5 $x10^5$ cells per well. Wells were transduced with indicated KSHV microRNA lentivirus. Lentiviral transduction was performed by 2 hour spinoculation followed by 4 hour incubation at 37^{o}C in 5% CO^2. Mock infected wells were used to measure the basal level of luciferase expression. Cells were harvested 48 hours post transduction, lysed and analyzed by Dual-Luciferase Reporter Assay (Promega) according to the manufacturer's protocol. Protein concentrations were determined by the bicinchoninic acid assay (Pierce), and used to normalize against relative luciferase units measured. Luciferase expression was measured using a Glomax 20/20 Luminometer (Promega) and reported as fold increase in relative luciferase units per ug of protein in microRNA treated samples compared to mock infected. Error bars reflect standard errors of the means of three independent experiments.

Three-dimensional culture of endothelial cells

Matrigel (10 mg/ml; BD Biosciences, Bedford, MA) was applied at 0.5 ml/35 mm in a tissue culture dish and incubated at 37°C for at least 30 min to harden. Mock- or KSHV-infected cells were removed using trypsin-EDTA and resuspended at 1.5×10^5 cells per ml in growth medium. One ml of cells were gently added to the Matrigel-coated plates, incubated at 37°C, monitored at 4, 6 and 24 hrs, and photographed in digital format using a Nikon microscope. Capillaries were defined as cellular processes

connecting two bodies of cells. Capillary morphogenesis assayed with rhTGF-beta2 was performed as described with 30ng/ml of rhTGF-beta2 added to the cells at the time of plating on matrigel.

Osteopontin ELISA

TIME cells were mock or KSHV infected and treated with the indicated pharmacological inhibitors at the indicated concentrations at 16 and 40 hours post infection. 48 hours post infection cell supernatents were harvested and centrifuged to remove cellular debris. Osteopontin levels in the supernatents were measured by ELISA (RnDSystems, # DOST00) according to the manufacturer's instructions. Samples were compared to a standard curve of Osteopontin and data is reported as mg/ml of Osteopontin.

CHAPTER III

Ets-1 is required for the activation of VEGFR3 during latent Kaposi's Sarcoma Herpesvirus infection of endothelial cells

Originally published as an article in the Journal of Virology:
Kimberley D. Gutierrez, Valerie A. Morris, David Wu, Serge Barcy and Michael Lagunoff

SUMMARY

Kaposi's Sarcoma herpesvirus (KSHV), the etiologic agent of Kaposi's Sarcoma, is present in the predominant tumor cells of KS, the spindle cells. Spindle cells express markers of lymphatic endothelium and, interestingly, KSHV infection of blood endothelial cells reprograms them to a lymphatic endothelial cell phenotype. KSHV induced reprogramming requires the activation of STAT3 and PI3/AKT through the activation of cellular receptor gp130. Importantly, KSHV-induced reprogramming is specific to endothelial cells, indicating that there are additional host genes that are differentially regulated during KSHV infection of endothelial cells that contribute to lymphatic reprogramming. Here we show that Ets-1 is highly expressed in KS spindle cells and that KSHV infection of blood endothelial cells strongly upregulates Ets-1 expression at the RNA and protein levels. The latent KSHV gene vFLIP is sufficient to upregulate Ets-1 expression in an NF-κB-dependent fashion, and inhibitors of NF-κB activity also block KSHV induction of Ets-1. Knockdown of Ets-1 by siRNA blocks the KSHV induction of the lymphatic specific growth factor receptor, VEGFR3, and Ets-1 expression is sufficient to induce the VEGFR3 promoter. Upregulation of Ets-1 is independent of gp130 signaling, indicating that there are at least two distinct mechanisms required for KSHV regulation of VEGFR3. The loss of Ets-1 results in defective capillary stabilization in KSHV infected blood endothelial cells. Additionally, the knockdown of Ets-1 in uninfected primary lymphatic endothelial cells results in a decrease in VEGFR3 expression but does not affect expression of another lymphatic specific gene,

podoplanin, indicating that Ets-1 is not a global regulator of lymphatic differentiation but is specific to VEGFR3 expression. Our data demonstrate that Ets-1 is a novel regulator of VEGFR3 and likely plays an important role in KSHV activation of infected endothelial cells through the activation of multiple endothelial cell activation pathways.

RESULTS

Ets-1 is highly expressed in the KS tumor

Previous studies demonstrated that KS tumor spindle cells express high levels of Ang2, a gene shown to be upregulated by KSHV infection of macrovascular endothelial cells in an Ets-1 dependent fashion (133). However, it has not been shown if Ets-1 itself was expressed in KS spindle cells. We obtained unstained tissue sections derived from formalin fixed, paraffin-embedded tissue samples from 4 KS patients with confirmed histopathologic diagnosis of KS and immunostained them with antibodies to LANA and Ets-1 (Fig 3-1). Two of the tumors were from the oral cavity and two were nodular KS skin lesions. Shown in figure 1 is one oral cavity nodule tumor (fig. 3-1 A-E) and one skin nodule (fig. 3-1 F-J). In tumors from all four patients there was high level staining with the Ets-1 antibody. Importantly, throughout the four biopsy samples it is clear that there is strong Ets-1 staining in the regions where there are high levels of LANA staining (predominantly spindle cells), while in regions of the biopsy where there is little or no LANA nuclear staining, only a very low percentage of the cells stain with the Ets-1 antibody. In the spindle cells, Ets-1 stains throughout the cell with high levels of nuclear staining, as indicated by the very dark staining nuclei, with some cytoplasmic staining as indicated by the light cytoplasmic staining. In cells from areas of the biopsies that did not stain for LANA, there is little or no staining seen for Ets-1. Whole cell staining of Ets-1 has previously been demonstrated in gastric, breast and colorectal carcinomas (107, 155-157). Ets-1 located in the nucleus in

bound to DNA while Ets-1 is overproduced in the cytoplasm (157). In summary, Ets-1 stains highly in KS tumor cells in regions where there are high levels of spindle cells that express LANA indicating that Ets-1 is highly expressed in KS spindle cells.

Ets-1 is upregulated during KSHV infection

It was previously reported that Ets-1 is upregulated during KSHV infection (133). Analysis of our microarray studies comparing mock and KSHV infected endothelial cells also indicated that Ets-1 was significantly upregulated in our KSHV infected samples (data not shown). We confirmed this result in TIME cells and primary dermal microvascular endothelial cells (DMVECs) using quantitative real-time RT-PCR to measure transcript levels of Ets-1. Cells were mock or KSHV infected for 48 hours, which allows for the establishment of latency in the KSHV infected cells, total mRNA was extracted and used to measure transcript levels using quantitative real time RT-PCR. KSHV infected TIME cells have elevated Ets-1 transcript levels, approximately 3.5-fold higher than mock infected cells, and Ets-1 mRNA levels were similarly elevated in KSHV-infected primary dermal microvascular endothelial cells (Fig. 3-2A, and data not shown). VEGFR3 mRNA is upregulated 6-fold in KSHV infected TIME cells as compared to mock infected cells (Fig 3-2A) as we have described previously (49, 100). We next examined protein expression levels of both Ets-1 and VEGFR3 using Western blot analysis. Ets-1 and VEGFR3 protein levels are significantly higher in TIME cells latently infected with KSHV (Fig 3-2B) as compared to mock-infected cells. Importantly, in the KSHV infected samples, at least 95% of the cells express the latent gene LANA while less than 1% express ORF59, a marker of lytic infection in all experiments used, indicating the effects seen are predominantly due to latent infection.

KSHV vFLIP is sufficient to induce Ets-1

Because Ets-1 upregulation occurs predominantly during latent infection, we asked if a single latent viral gene could lead to Ets-1 expression. We transiently expressed the KSHV latent genes LANA, vFLIP and vCyclin in TIME cells using lentiviral expression vectors, and we also utilized a TIME cell line that was infected with the LANA expressing lentivirus and was selected for long term high level expression of LANA. TIME cells were transduced with lentiviruses expressing KSHV genes, and 48 hours post infection RNA was harvested from cells and subjected to real time RT-PCR. RT-PCR with primers to LANA, vFLIP and vCyclin were able to detect transcripts for each of the individual lentiviral constructs tested (data not shown). Western blot analysis of LANA and vCyclin indicated that vCyclin was expressed to higher levels than in KSHV infected cells but the transient expression of LANA was lower than in cells from KSHV infected cells (data not shown). Therefore, we used the LANA expressing TIME cell line in subsequent experiments where we have demonstrated high level expression. We were unable to compare protein expression of vFLIP in lentivrial transduced cells to KSHV infected cells due to the lack of an appropriate antibody. However, we demonstrated vFLIP expression by both real time RT-PCR and by demonstrating activation of NF-κB in vFLIP transduced cells (data not shown). Cells expressing vCyc and low levels of LANA from the lentivirus showed no significant increase in Ets-1 expression while there was a significant increase in Ets-1 expression in cells transduced with the lentivirus expressing vFLIP. The LANA expressing cells that were selected for high expression levels of LANA also yielded increases in Ets-1 protein levels (Fig 3-3A). Our results with LANA are in agreement with a previous study that reported that LANA interacts with an inhibitor of Ets-1, Daxx during KSHV infection (132). Currently, we cannot rule out the possibility that other viral genes or miRNAs may also be able to induce expression of Ets-1 during latency. However, this data indicates that the latent gene vFLIP is sufficient to induce Ets-1 and when selected for high level expression LANA is also sufficient to induce Ets-1.

KSHV vFLIP protects cells from apoptosis through the activation of the NF-κB pathway. To determine if vFLIP upregulates Ets-1 through the NF-κB pathway, we expressed vFLIP as before except at 24 hours post-lentivirus infection we treated the cells with the NF-κB inhibitor Bay11-7082. As demonstrated in Figure 3-3B, the NF-κB inhibitor reproducibly abrogated the ability of vFLIP to induce Ets-1 in multiple experiments. We also used a vFLIP construct, A57L-vFLIP-HA, that contains a point mutation yielding decreased NF-κB activation (153). When transduced into TIME cells the mutant was expressed to over 80% of wild type in two experiments while Ets-1 expression levels were approximately 50% that of the wild type transductants indicating that the mutant was unable to induce Ets-1 expression to wild type vFLIP levels (Fig 3-3C).

To determine if KSHV activation of Ets-1 is also dependent on NF-κB, at 24 hours post infection with KSHV, we treated TIME cells with Bay11-7082 and harvested cells at 48 hours post infection. Inhibition of NF-κB blocked the induction of Ets-1 by KSHV indicating that as with vFLIP, KSHV requires NF-κB for the induction of Ets-1 (Fig 3-3B). In order to confirm this result, we used a NEMO binding peptide inhibitior (NBD peptide) to specifically block NFkB activation during KSHV infection. TIME cells were mock or KSHV infected and 24 hour post infection the cells were treated with 50uM of control peptide or NBD peptide. Cells were harvested 48 hours post infection and levels of Ets-1 protein expression were analyzed by western blot. As seen in figure 3-3D, the NBD peptide blocked the expression of Ets-1 in both mock and KSHV infected cells. The control peptide had no effect on Ets-1 expression and there was no noticable toxicity with either peptide. Therefore, vFLIP is likely the predominant viral gene inducing Ets-1 during KSHV latent infection. Additionally, another cellular gene known to be induced by KSHV, podoplanin, was not significantly altered by Bay11-7082 treatment (Fig 3-3B) indicating that in the presence of this inhibitor KSHV was still able to activate cellular proteins.

Ets-1 regulates expression of the lymphatic specific gene VEGFR3

To determine the role of Ets-1 during infection and explore host genes controlled by Ets-1 during KSHV infection of blood endothelial cells, we knocked down Ets-1 expression with siRNAs directed specifically to Ets-1 sequences. TIME cells were transfected with Ets-1 siRNA, gp130 siRNA, or a control non-specific siRNA. The cells were subsequently mock or KSHV infected 24 hours post transfection, and cell extracts were harvested 48 hours post infection for Western blot analysis. The siRNA directed to Ets-1 efficiently knocked down Ets-1 expression in KSHV infected cells to mock-infected cell levels (Fig. 3-4). Ets-1 knockdown results in a loss of VEGFR1, a growth factor receptor previously shown to be regulated by Ets-1. Interestingly, expression of the lymphatic specific gene, VEGFR3 was also knocked down by Ets-1 siRNA in KSHV infected cells. We have previously published that activation of gp130 by KSHV is required for lymphatic reprogramming and for the expression of lymphatic specific genes Prox1, podoplanin, LYVE-1 and VEGFR3 (100). Knock down of gp130 by siRNA results in a decrease in VEGFR1, VEGR3 and podoplanin but does not significantly alter the expression levels of Ets-1 (Fig. 3-4). This suggests that upregulation of Ets-1 by KSHV and Ets-1 control of VEGFR3 is independent of gp130 signaling. Interestingly, knockdown of Ets-1 appears to slightly increase expression of gp130. While the mechanism of gp130 upregulation when Ets-1 is knocked down is not clear, however, this provides further evidence that Ets-1 induction of VEGFR3 is a separate pathway from gp130 activation.

While Ets-1 knockdown decreases expression of VEGFR3, it does not significantly alter the expression of another lymphatic specific gene podoplanin, indicating that Ets-1 is not a global regulator of lymphatic gene expression but is specific to the lymphatic endothelial gene VEGFR3 (Fig 3-4). Taken together this data suggests that activation of the transcription factor Prox-1 through gp130 signaling is required for lymphatic differentiation of blood endothelial cells and expression of VEGFR3, while Ets-1 is necessary for the optimal expression of VEGFR3 but not lymphatic reprogramming of blood endothelial cells (see discussion).

Ets-1 activates the VEGFR3 promoter

Ets-1 is a transcription factor known to induce the expression of growth factors and has been described to directly activate the promoter of VEGFR1 (116, 119, 126, 130). Therefore, we next examined the ability of Ets-1 to activate the promoter of VEGFR3. Firefly luciferase reporter constructs driven by either VEGFR1 or VEGFR3 promoters were transfected into HEK293 and TIME cells. A construct containing the full length Ets-1 protein was tested for the ability to increase luciferase production as compared to empty vector transfection in both cell types. A plasmid construct with the Renilla-luciferase gene under control of thymidine kinase promoter was co-transfected and used to normalize for transfection efficiency. Ets-1 is able to strongly activate the VEGFR1 promoter driving luciferase production in both HEK293 and TIME cells, confirming that Ets-1 activates the VEGFR1 promoter as shown in figure 3-5A where the average induction from five separate biological replicate experiments is shown (Fig. 3-5A). Ets-1 expression is also able to active the VEGFR3 promoter and increase luciferase production 3-fold in HEK293 cells and 4.5-fold in TIME cells (Fig. 3-5B). Ets-1 regulation of the expression and promoters of VEGFR1 and VEGFR2 has been described previously. This is the first report that Ets-1 regulates the promoter of the lymphatic endothelial cell specific growth receptor, VEGFR3.

Ets-1 controls VEGFR3 expression in uninfected primary lymphatic endothelial cells

We next wanted to determine if physiological levels of Ets-1 controls VEGFR3 in primary lymphatic endothelial cells where there are high levels of VEGFR3 expression. We transduced primary lymphatic endothelial cells with a plasmid expressing control or Ets-1 specific siRNA and performed Western blot analysis on cell lysates. Ets-1 siRNA efficiently knocked down expression of Ets-1 in LECs demonstrating the efficacy of the siRNA used. Knockdown of Ets-1 led to a decrease in VEGFR3 protein expression by approximately 50% (Fig. 3-6A). Using real time RT-PCR, VEGFR3 mRNA levels were decreased 2-fold and Ets-1 mRNA was decreased 6-fold in LECs treated with Ets-1 siRNA (Fig 3-6B). This

data suggests that Ets-1 is a global regulator of VEGFR3 expression and may play a role in the sustained expression of VEGFR3 in lymphatic vasculature.

Loss of Ets-1 results in defective capillary morphogenesis of KSHV infected endothelial cells

It was previously published that KSHV leads to stabilization of endothelial tubule formation at 24 hours after plating in a three dimensional matix (85, 98). Ets-1 has been described as a pro-angiogenic factor that activates genes that contribute to various stages of the angiogenic process such as matrix metalloproteases, Ang 1 and 2, and Tie 1 and 2 (111, 112). This suggests that Ets-1 may play a functional role in the ability of KSHV infected endothelial cells to induce angiogenic phenotypes. For these studies we created TIME cell lines with a shRNA specific to Ets-1 and a control vector only cell line expressing GFP. The protein level of Ets-1 is significantly knocked down in both mock and KSHV infected cells with the Ets-1 shRNA as compared to the control cell line and as expected from above, VEGFR3 expression is also decreased (Fig 3-7E). Infection levels of both control (pGreen) and Ets-1 shRNA (shEts-1) cell lines were not significantly different as measured by IFA to determine the percentage of cells expressing LANA (data not shown). To investigate the angiogenic role of Ets-1, we mock and KSHV infected the shEts-1 cell line and tested to ability of these cells to organize and form capillary-like structures in a three-dimension culture on Matrigel. Mock infected TIME cells begin to spread in the Matrigel by 4 hours post-plating and form capillary structures by 6 hours. The shEts-1 knockdown cells demonstrate a delay in cell spreading but form similar numbers of capillary like structures by 6 hours post-plating. However, in both cell lines the majority of capillary-like connections regress by 24 hours and no longer form a structured network (Fig. 3-7A and 3-7B and quantified in Fig. 3-7E). KSHV-infected TIME cells are able to attach and form capillary-like structures in Matrigel similar to the uninfected cells. However, unlike the uninfected cells, at 24 hours post plating in Matrigel, intact capillary-like networks were still present suggesting KSHV promotes a prolonged angiogenic phenotype (Fig. 3-7C and quantified in fig. 3-

7E) and as seen previously (85, 158). Interestingly, KSHV infected shEts-1 knockdown TIME cells did not exhibit capillary-like structures 24 hours post plating (Fig 3-7D). This data suggests that Ets-1 contributes to the ability of KSHV infected cells to display enhanced angiogenic phenotypes.

DISCUSSION

Ets-1 is highly expressed in many solid tumors and is associated with tumor progression and invasion (105, 106, 109). Ets-1 is also present at high levels in the developing vasculature and is associated with tumor vasculature. KS spindle cells are endothelial in origin and therefore have many activated endothelial cell phenotypes common to angiogenesis and to tumor formation. A previous study examining gene expression of KS tumors by microarray found that there were high levels of Ets-1 mRNA in tumor biopsies (99). However, the KS tumor consists of multiple cell types and therefore, we examined protein expression by immunohistochemistry. We found that there are high levels of Ets-1 expression in LANA positive KS spindle cells as well as in some the cells lining the vascular slits in the tumor, indicating that Ets-1 expression is associated with both the increased vascularization of KS tumors and with the tumor cells themselves. Therefore, Ets-1 may play an important role in KS tumor initiation and progression.

Previous studies have shown that Ets-1 expression is increased by KSHV infection of cultured endothelial cells (99, 133). Additionally, it was shown that KSHV infection of lymphatic endothelial cells decreased the expression of two cellular miRNAs that could downregulate Ets-1 (99). Our cell culture models of latent KSHV infection confirmed previous studies showing that KSHV infection of endothelial cells significantly increased Ets-1 expression at both the mRNA and protein levels (99, 133). While this does not demonstrate a direct correlation between KSHV gene expression and high levels of Ets-1 in the KS spindle cells, it is suggestive that KSHV latent gene expression plays a role in the high levels of Ets-1 expression in the tumor. In endothelial cells, the latent gene vFLIP was sufficient to induce expression

of Ets-1 in an NF-κB-dependent fashion. An endothelial cell line expressing high levels of LANA also showed some induction of Ets-1, however, transient expression of LANA was not sufficient to induce Ets-1 (data not shown). Importantly, inhibition of NF-κB by the pharmacological inhibitor Bay11-7082 or with NEMO binding inhibitory peptides blocked KSHV-induced Ets-1 expression. Because NF-κB activation is necessary for KSHV induction of Ets-1 and vFLIP activation of NF-κB is sufficient for induction of Ets-1, vFLIP is likely the dominant KSHV activator of Ets-1 expression during latent infection and the role of LANA induction may be auxiliary or needed for maximal expression.

Ets-1 regulates genes involved in many different cellular processes such as migration, proliferation and apoptosis (111, 117, 118). We found that KSHV induced Ets-1 expression was necessary for the full induction of VEGFR3. VEGFR3 is the receptor for VEGFC, a cytokine important for the growth and differentiation of lymphatic endothelium. VEGFR3 expression is switched on during blood to lymphatic endothelial cell differentiation. We, and others, previously showed that KSHV infection of blood endothelial cells induces reprogramming to lymphatic endothelium and the expression of VEGFR3 (14, 15, 49). During development this process is known to require Prox-1 which activates VEGFR3 among many other lymphatic specific genes. While Ets-1 is necessary for the full induction of VEGFR3 by KSHV, knock-down of Ets-1 did not completely abrogate VEGFR3 expression, likely due to KSHV induced expression of Prox-1. Interestingly, podoplanin, another lymphatic endothelial specific gene, is highly induced by KSHV even when Ets-1 is blocked, indicating that Ets-1 is specific to VEGFR3 and is not involved in overall lymphatic reprogramming. A previous study in macrovascular endothelial cells showed that Prox-1 acts synergistically with Ets-2 to induce lymphangiogenesis and that Ets-2 and to a much lesser extent Ets-1 can bind to prox-1 and potentiate its activation of VEGFR3 (85). We found that Ets-1 activates the promoter of VEGFR3 in the absence of Prox-1 expression in endothelial cells as well as in non-endothelial cells however, relatively little is known about the regulation of the VEGFR3 promoter. While it is not currently known if Ets-1 directly

binds to the VEGFR3 promoter, we identified two consensus Ets-1 binding sites upstream of the VEGFR3 TATA box. Further work to determine if these sequences are involved in the Ets-1 activation of the VEGFR3 promoter is necessary to determine if there is direct activation. However, it is not clear if Ets-1 expression alone is enough to activate VEGFR3 from its native promoter. It is possible that VEGFR3 may need to be turned on by other factors and that Ets-1 boosts expression of VEGFR3 through activation of an open promoter. KSHV also induces Prox-1 in blood endothelial cells and therefore, Prox-1 and Ets-1 could synergize to induce the VEGFR3 promoter in KSHV infected cells to achieve optimal VEGFR3 expression. This was reported to be the case in HUVEC cells (85). However, in TIME cells or HEK 293 cells, Prox-1 did not synergistically increase the VEGFR3 promoter activation by Ets-1 (data not shown). Further work to understand the interactions of Ets-1 and Prox-1 in microvascular endothelial cells and how the two factors activate VEGFR3 is warranted. Importantly, knockdown of Ets-1 in primary lymphatic endothelial cells also led to a significant decrease in VEGFR3 expression, indicating that Ets-1 is necessary for high level VEGFR3 expression in naturally differentiated lymphatic endothelium. While it has been reported that Ets-1 is required for embryonic endothelial cell survival Ets-1 is apparently not required for survival of differentiated endothelial cells as we were able to create a stable shRNA Ets-1 knockdown cell line (115). As VEGFR3 is critical for growth of lymphatic endothelium, Ets-1 expression is, by correlation, likely to be important for growth of lymphatic endothelium as well.

We, and others, have published that KSHV infection promotes cell survival and proliferation and supports angiogenic phenotypes such as capillary formation (85, 98, 158). Ets-1 clearly plays a role in KSHV induced angiogenesis. We found that Ets-1 activates VEGFR3 and VEGFR1 in KSHV infected endothelial cells, and previously others reported that Ets-1 induction leads to upregulation of Ang2 (133). All of these genes play roles in the induction of angiogenesis and/or lymphangiogenesis. We found that Ets-1 was shown to have a direct role in the ability of KSHV to stabilize and prolong endothelial capillary-like tube formation in Matrigel. Thus, Ets-1 plays a direct role in the induction of

angiogenic phenotypes by KSHV. Whether this is through VEGFR3, VEGFR1 or Ang2 regulation is currently under investigation. Regardless, it is apparent that Ets-1 is highly expressed in KS spindle cells and KSHV induction of Ets-1 by vFLIP is likely to play a direct role in formation of this endothelial cell-based tumor.

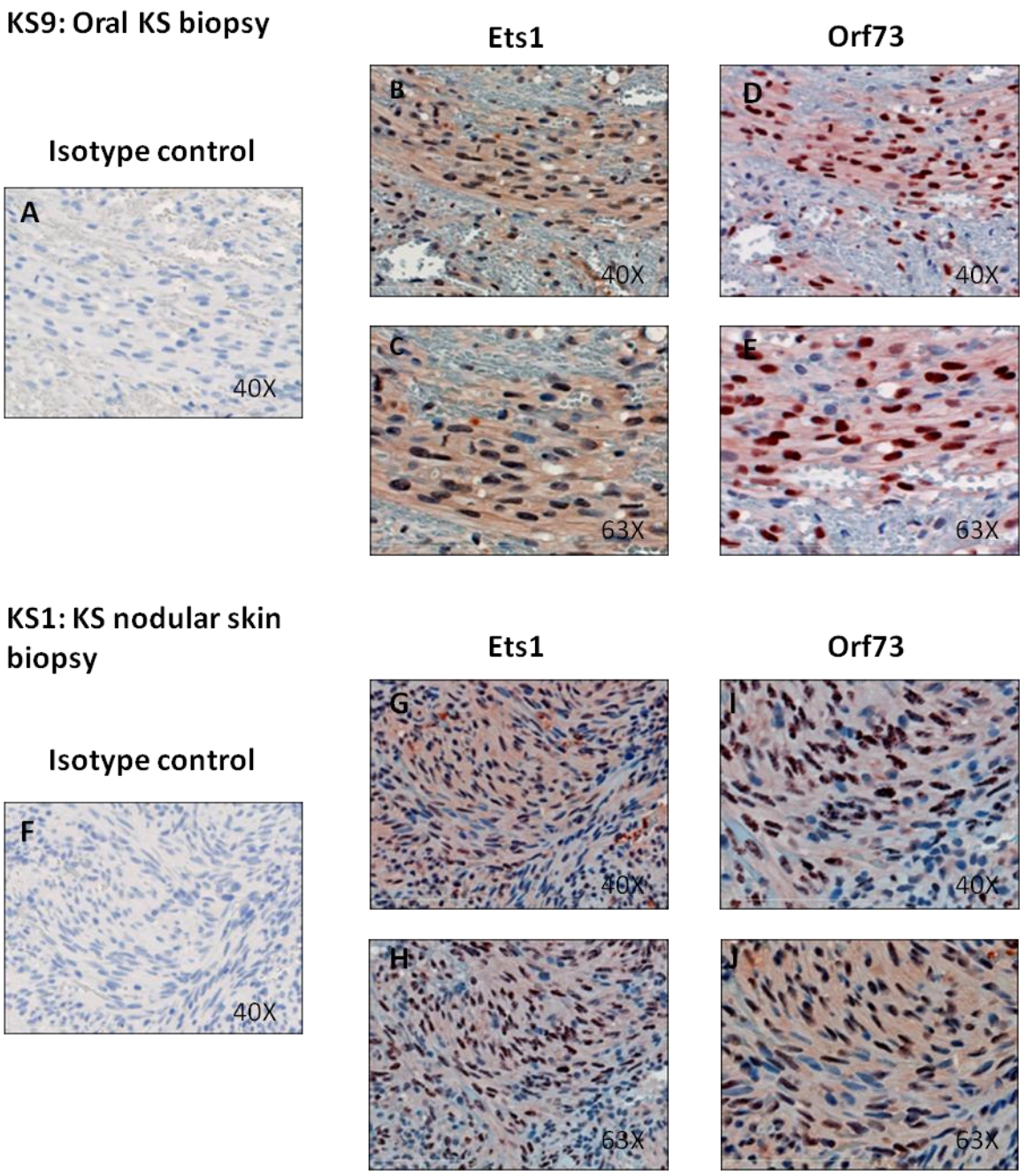


Figure 3-1: **Ets-1 is highly expressed in LANA positive regions of KS tumors**. Serial KS tumor sections were stained with antibodies to Ets-1 isotype control rabbit IgG or KSHV Orf 73 (LANA) or Ets-1 and counterstained with haematoxylin. (A-E) 40x and 63x magnification of serial sections of an nodule oral KS biopsy from the right maxillary gingiva are shown with the same regions aligned and is representative of staining seen in 2 different oral KS nodules. (F-J) 40x and 63x magnification of the same aligned regions of a nodular KS skin biopsy are shown and is representative of staining seen in 2 different KS skin biopsies.

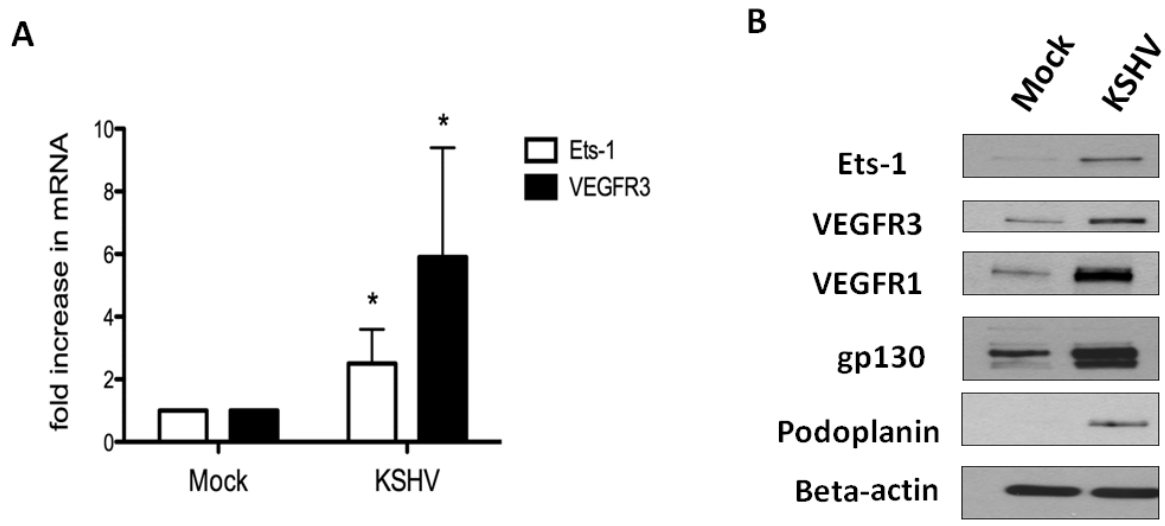
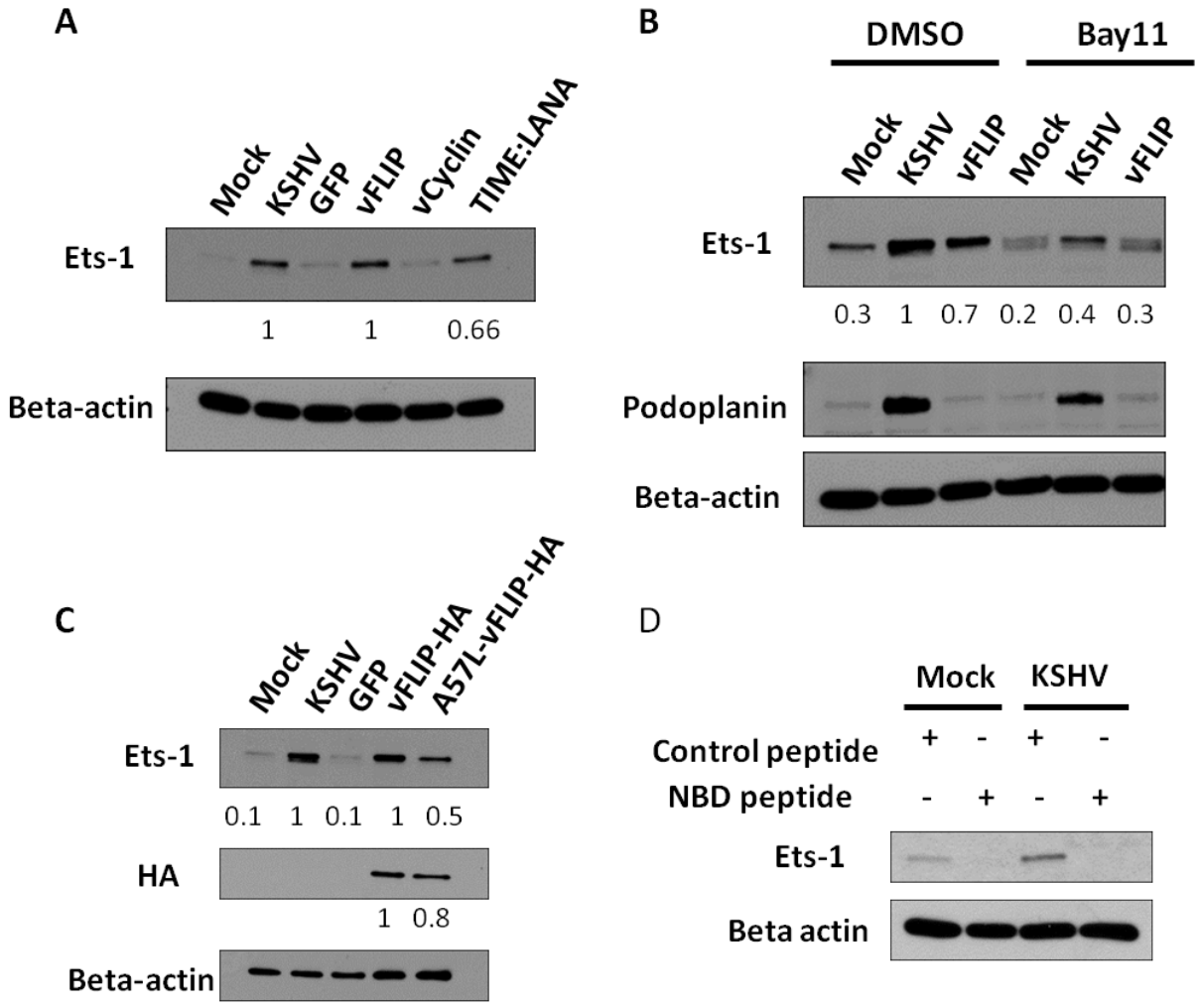


Figure 3-2: **KSHV upregulates Ets-1 in endothelial cells.** Tert Immortalized Microvascular Endothelial (TIME) cells were mock or KSHV infected for 48 hours in order to establish latency. (A) Cells were harvested and mRNA was isolated and analyzed by quantitative real time RT-PCR. Samples were normalized to GAPDH and reported as fold change over mock-infected cells. Error bars reflect standard errors of the means of three independent experiments. *, $P < 0.05$. (B) 48 hours post-infection mock and KSHV infected TIME cells were harvested and cell extracts were analyzed by Western blot with antibodies against Ets-1, VEGFR1, VEGFR3, gp130, podoplanin and Beta actin as a loading control.


A
Mock
KSHV
GFP
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1
1
0.66
Beta-actin
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Mock
KSHV
vFLIP
Mock
KSHV
vFLIP
Ets-1
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1
0.7
0.2
0.4
0.3
Podoplanin
Beta-actin
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Mock
KSHV
GFP
vFLIP-HA
A57L-vFLIP-HA
Ets-1
0.1
1
0.1
1
0.5
HA
1
0.8
Beta-actin
D
Mock
KSHV
Control peptide
+
-
+
-
NBD peptide
-
+
-
+
Ets-1
Beta actin

Figure 3-3: **The latent gene vFLIP induces Ets-1 expression through NF-κB activity.** (A) TIME cells were either mock or KSHV infected, or infected with lentivirus containing GFP or the latent genes vFLIP and vCyclin for 48 hours and cell lysates were harvested. TIME cells stably expressing LANA were also harvested and all cell lysates were analyzed by Western blot with antibodies directed to Ets-1 or beta-actin as a load control. Both KSHV infection and expression of the individual latent genes vFLIP and LANA induce Ets-1 expression. (B) TIME cells were mock or KSHV infected or infected with lentivirus expressing the latent gene vFLIP. 24 hours post infection, cells were treated with either a vehicle control (DMSO), an inhibitor of NF-**κB** (Bay11-7082, 5 μM). At 48 hours post-infection cell extracts were analyzed by Western blot with the indicated antibodies. KSHV infected TIME cells treated with Bay11-7082 displayed a 2.5 fold decrease in Ets-1 expression compared to KSHV infected cells treated with DMSO. vFLIP transduced TIME cells displayed a 2.3 fold decrease in Ets-1 expression when treated with Bay11-7082 as compared to DMSO treatment. Ets-1 protein data was quantitated using ImageJ and is shown below the Ets-1 panel. (C) TIME cells were mock or KSHV infected or transduced with lentivirus expressing either GFP, vFLIP-HA or the NF-κB activation-deficient mutant A57L-vFLIP-HA. After 48 hours, cells were harvested and cell lysates were analyzed by Western blot with antibodies directed to Ets-1 and beta actin as a load control. vFLIP and A57L-vFLIP expression was detected with an antibody to the HA tag. KSHV and vFLIP-HA induce expression Ets-1 while A57L-vFLIP-HA displays an approximately 50% reduced ability to induce Ets-1 expression. A57L-vFLIP was expressed to over 80% of wild type levels. Data was quantitated using ImageJ and is displayed below the Ets-1 panel. (D) TIME cells were mock or KSHV infected and treated with either a control peptide or IKKγ NEMO Binding Domain (NBD) Inhibitory peptide 24 hours post infection at a final concentration of 50uM. 24 hours post treatment, cells were harvested and cell extracts were analyzed by Western blot with antibodies to Ets-1 and beta actin as a load control.

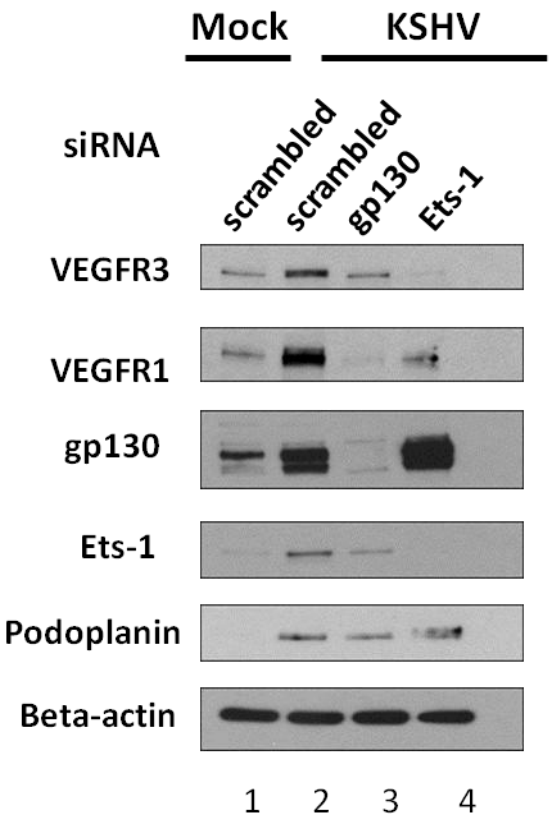


Figure 3-4: **Ets-1 induction by KSHV is necessary for VEGFR3 expression.** TIME cells were transfected with control, gp130 or Ets-1 siRNA by Amaxa and 24 hours post-transfection were mock or KSHV infected. Cells were harvested 48 hours post-infection and cell extracts were analyzed by Western blot with the indicated antibodies. While gp130 siRNA knocks down KSHV-induced gp130, VEGFR1, VEGFR3 and podoplanin expression, Ets-1 siRNA decreases KSHV-induced expression of Ets-1, VEGFR1 and VEGFR3 but not podoplanin.

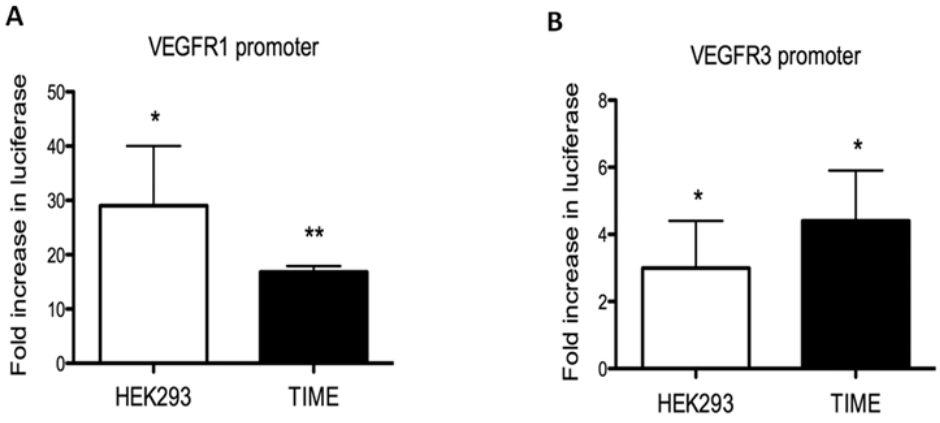


Figure 3-5: **Ets-1 activates the VEGFR1 and VEGFR3 promoters**. Luciferase reporter contructs with the VEGFR1 (left panel) or VEGFR3 (right panel) promoter driving firefly luciferase expression were transfected into TIME and HEK293 cells with either an empty vector or Ets-1 expression plasmid. A Renilla-luciferase reporter construct was co-transfected and used as a control for transfection efficiency. The graph indicates the fold increase in relative luciferase expression (Firefly/Renilla) of the VEGFR1 or VEGFR3 promoter constructs in the presence of Ets-1 expression vector versus the empty vector control. Error bars reflect standard errors of the means of five independent experiments. *,$P < 0.05$; **, $P < 0.005$.

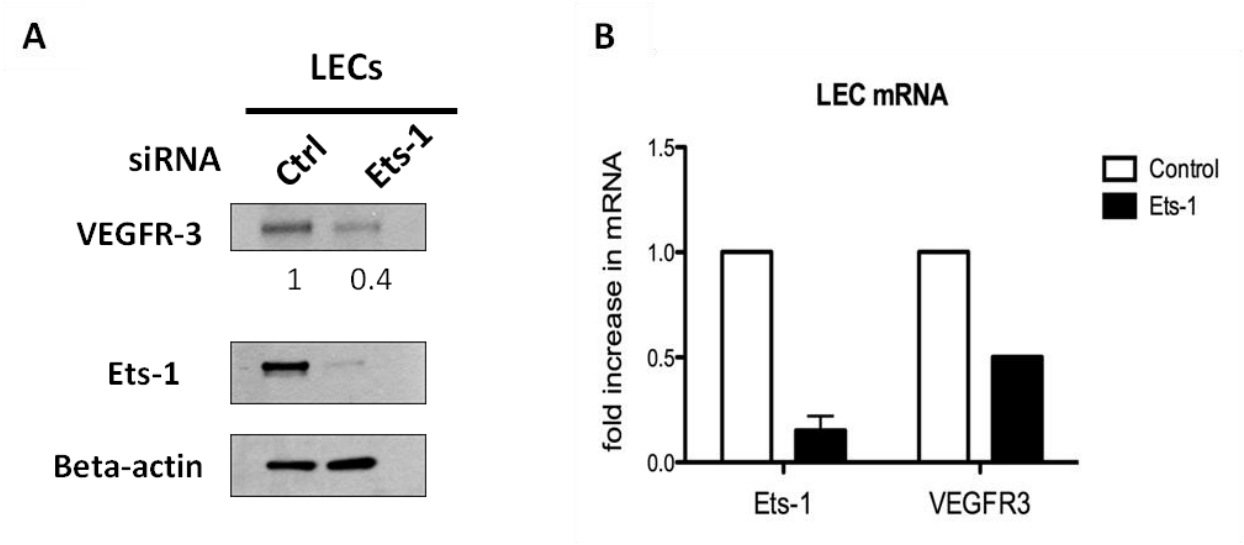
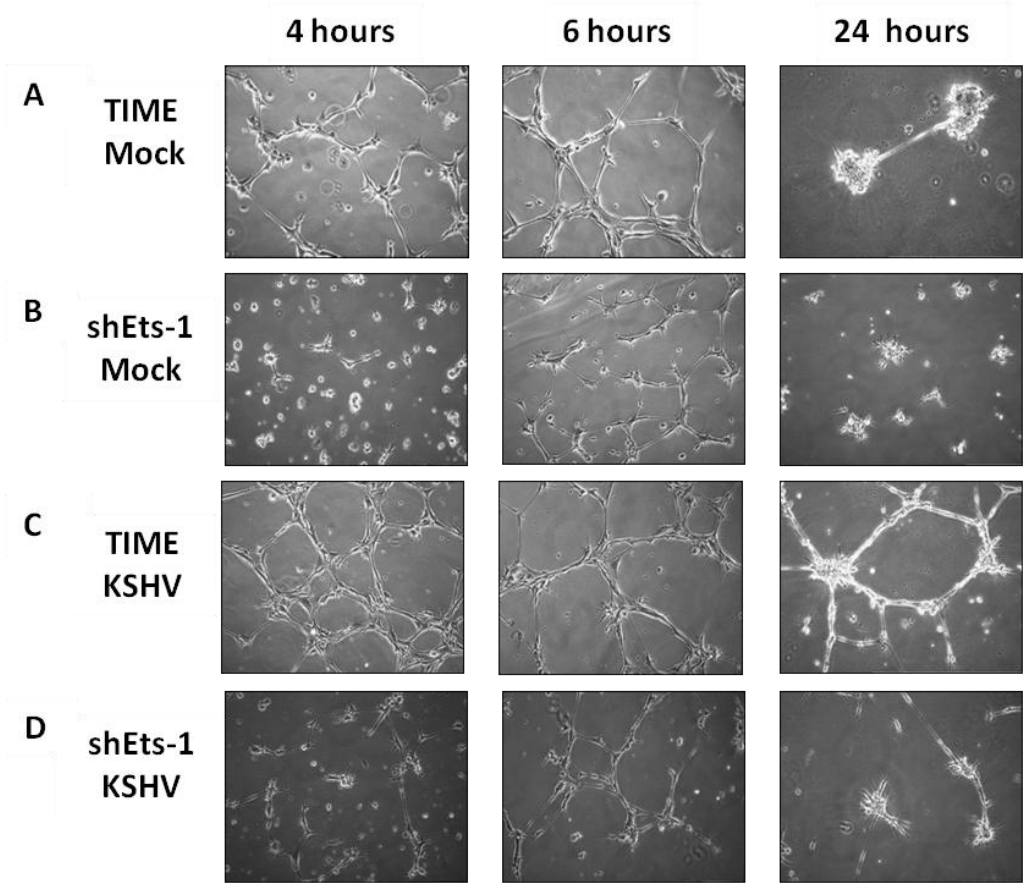


Figure 3-6: **High level VEGFR3 expression in lymphatic endothelial cells requires Ets-1**. Primary lymphatic endothelial cells were transfected by Amaxa with either a control siRNA or Ets-1 siRNA. (A) 48 hours post-transfection cell lysates were analyzed by Western blot with the indicated antibodies. VEGFR3 protein data was quantitated using ImageJ comparing cells transfected with Ets-1 siRNA vs. control siRNA and is shown below the VEGFR3 panel. (B) Quantitative real time RT-PCR was used to analyze Ets-1 and VEGFR3 mRNA expression levels of control siRNA or Ets-1 siRNA transfected LECs 48 hours post transfection. Data is reported as fold change in mRNA of Ets-1 siRNA vs. control siRNA. Error bars reflect standard errors of the means of three independent experiments.


4 hours
6 hours
24 hours
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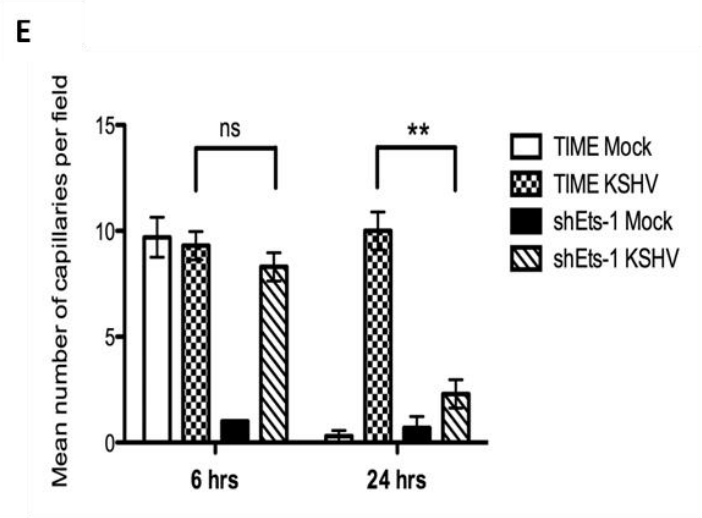
TIME
Mock
B
shEts-1
Mock
C
TIME
KSHV
D
shEts-1
KSHV


E
Mean number of capillaries per field
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10
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**
6 hrs
24 hrs
TIME Mock
TIME KSHV
shEts-1 Mock
shEts-1 KSHV

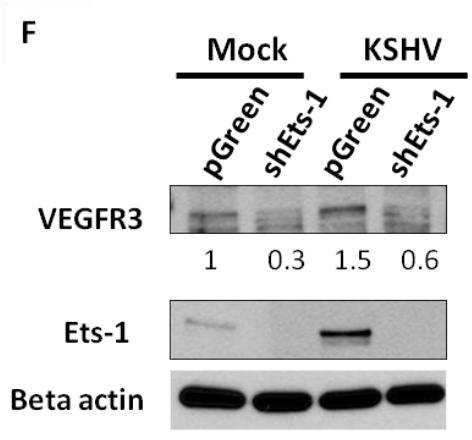
F
Mock
KSHV
pGreen
shEts-1
pGreen
shEts-1
VEGFR3
1
0.3
1.5
0.6
Ets-1
Beta actin

Figure 3-7: **Ets-1 contributes to the angiogenic phenotype of KSHV infected endothelial cells**. (A-D) TIME cells and shEts-1 transduced TIME cells were mock or KSHV infected. 48 hpi cells were plated on Matrigel 3-dimensional matrix and incubated at 37^0C. Representative Capillary morphogenesis pictures taken at 4, 6 and 24 hours post plating are shown. (E) Quantitation of capillaries from three independent experiments similar to the one seen in A-D. Three fields from each experiment (9 total) were analyzed and the number of capillaries per field were counted. Error bars indicate the standard errors of the means of three independent experiments. ns, not significant; **, P <0.005 (evaluated by Students *t*-test). (F) Western blot of a TIME cell line expressing a shRNA to Ets-1 (shEts-1) and a control cell line (pGreen) mock and KSHV infected for 48 hours. Control pGreen cell line displayed results similar to TIME cells where infection with KSHV increased expression of Ets-1 and VEGFR3. Mock and KSHV infected shEts-1 cell line showed efficient knock-down of both Ets-1 and VEGFR3. VEGFR3 protein data was quantitated using ImageJ and is shown below the VEGFR3 panel.

CHAPTER IV

Downregulation of TGF-beta2 during latent Kaposi's Sarcoma Herpesvirus infection promotes capillary morphogenesis of endothelial cells

SUMMARY

KSHV is the etiological agent of KS, a highly vascularized endothelial cell tumor. KS tumors exhibit malformed, leaky vasculature and enhanced inflammation and edema. These characteristics, together with histopathology, support a likely role for angiogenesis in KS tumor formation. Angiogenesis is a tightly regulated process involved in embryogenesis and wound healing. However, angiogenesis is also a fundamental step in tumor formation and many cancer cells support angiogenic phenotypes. Transformed or tumor cells, when plated on a three-dimensional matrix such as matrigel, will form capillary-like networks and these networks are more stable and remain intact for a longer period of time than non-transformed cells indicating these cells have an increased angiogenic potential. Similar to cancer cells, endothelial cells infected with KSHV display an increased ability to form capillary-like networks when plated on Matrigel *in vitro* (85, 98). Furthermore, they are able to maintain stable networks longer than uninfected cells. The formation of capillary-like networks, or capillary morphogenesis by KSHV infected cells, requires the RGD-binding integrin, integrin β3 and downstream signaling via Src kinases (98). The host transcription factor Ets-1 also plays a direct role in the ability of KSHV latently infected endothelial cells to stabilize and maintain capillary-like networks (104). This work indicates that KSHV latent infection enhances the angiogenic potential of endothelial cells. However, the mechanism by which KSHV promotes vascular stabilization remains unclear. We found TGF-beta2, a host cytokine involved in aspects of angiogenesis, to be downregulated during latent KSHV infection. TGF-beta cytokines are involved in wound healing and repair, during which they aid in re-establishing cellular homeostasis and endothelial cell quiescence. Expression of TGF-betas and TGF-beta signaling are commonly altered in many tumors. Therefore, we sought to determine the viral mechanism of TGF-

beta2 downregulation and what impact this cytokine has on angiogenic phenotypes of KSHV infected endothelial cells. Here we find that the addition of exogenous TGF-beta2 decreases the angiogenic potential of infected cells by decreasing the stability of capillary-like tube formation of KSHV infected cells. The addition of exogenous TGF-beta2 to KSHV infected cells plated on Matrigel leads to regression of capillary-like tubules formed by KSHV infected endothelial cells at 24 hours, to similar levels as uninfected cells.

MicroRNAs are endogenous 21-24 nucleotide non-coding RNA molecules that negatively regulate gene expression in plants and animals. MicroRNAs function via the RNA-induced silencing complex (RISC) to target messenger RNA in post-transcriptional gene silencing by base pairing which leads to mRNA cleavage or translational repression depending on the extent of sequence complementarity (159). KSHV encodes 18 mature miRNAs from 12 loci and several of the KSHV miRNAs have been shown to downregulate host mRNAs (154, 160-164). We show that the KSHV microRNAs, miR-K3 and miR-K8 are sufficient to induce TGF-beta2 downregulation. Sponges to miR-K3 and miR-K8 together are capable of restoring TGF-beta2 transcripts. This work indicates that the viral microRNAs, miR-K3 and miR-K8, are involved in the downregulation of TGF-beta2 in order to promote angiogenic phenotypes of KSHV latently infected endothelial cells.

RESULTS

TGF-beta2 is downregulated during latent KSHV infection

Microarray analysis of KSHV latently infected endothelial cells indicated that the cytokine TGF-beta2 is down-regulated at the transcript level. Based on the location of our real time RT-PCR primers we are confident that both variants of TGF-beta2 are downregulated. We validated this finding in TIME and primary HDVECS by quantitative real-time RT-PCR. Cells were mock or KSHV infected for 48 hours,

which allows for the establishment of latency in the KSHV infected cells, total mRNA was extracted and used to measure transcript levels using quantitative real time RT-PCR. There is a 90% decrease in TGF-beta2 mRNA expression in KSHV infected cells compared to mock infected cells (Fig. 4-1). Similar levels of TGF-beta2 mRNA were found in primary HDVECs infected with KSHV (data not shown). We next examined protein expression of TGF-beta2 in mock and KSHV infected TIME cells by Enzyme-linked Immunosorbent Assay (ELISA). TGF-beta2 is a cytokine secreted by endothelial cells and activated by a number of host factors in the extracellular space. The activated protein then binds to the TGF-beta type 2 receptor (TGFBR2) to activate downstream signaling pathways (137). Supernatents from mock or KSHV infected TIME cells were harvested 48 hours post infection and levels of TGF-beta2 were measured by ELISA. KSHV infected TIME cells secrete half the amount of TGF-beta2 as mock infected cells (data not shown). Taken together this data indicates that KSHV downregulates TGF-beta2 mRNA and which leads to a decrease in the amount of protein secreted by the latently infected endothelial cell. It should be noted that TGF-beta1, a more commonly studied homolog of TGF-beta2, was not found to be altered by KSHV infection at either the mRNA or the protein level (data not shown). IFA was used to determine infection rates and all KSHV infected samples were at least 95% latently infected, expressing the latent gene LANA, while less than 1% expressed ORF59, a marker of lytic infection. This suggests that TGF-beta2 downregulation is likely due to latent infection and latent KSHV gene expression.

TGF-beta2 disrupts capillary stability of KSHV infected cells

It has been previously published that KSHV induces the stabilization of capillary-like tubule networks at 24 hours after plating on a three dimensional matrix (85, 98). TGF-betas are involved in wound healing and matrix repair and remodeling of angiogenic capillaries. During late stages of angiogenesis and vessel remodeling in non-disease states, TGF-beta1 promotes capillary regression and

apoptosis of capillaries that are not in physical contact with the newly formed vessel (136, 138). Regression of non-joining capillaries is part of vessel remodeling and maturation of the newly formed vessel (74). Therefore, TGF-beta1 functions to inhibit cell proliferation, migration and survival and it is hypothesized that TGF-beta2 may function in a similar anti-angiogenic fashion.

To investigate the angiogenic role of TGF-beta2, we measured the ability of these mock and KSHV infected cells to organize and form capillary-like structures in a three-dimensional culture on Matrigel in the presence of recombinant human TGF-beta2 (rhTGF-beta2). Primary HDVECs were mock or KSHV infected and plated on Matrigel 48 hours post infection in order to allow for the establishment of latency. At the time of plating, rhTGF-beta2 or a vehicle control was added to the cells and media. All cells begin to spread in the Matrigel by 4 hours post-plating and form capillary structures between 6-7 hours post plating (Fig 4-2A). This indicates that the addition of exogenous TGF-beta2 does not interfere with attachment or cell spreading on the ECM. By 24 hours post plating in Matrigel, the capillaries of mock cells treated with either the vehicle or rhTGF-beta2 have destabilized and regressed resulting in a decrease in the number of capillary junctions (Fig 4-2A and quantified in fig 4-2B). This phenotype for mock infected cells has been previously reported and indicates that the addition of exogenous TGF-beta2 does not enhance capillary regression of normal uninfected endothelial cells. KSHV intact capillary-like networks were still present in the vehicle control sample suggesting KSHV promotes a prolonged angiogenic phenotype (Fig. 4-2A and quantified in fig. 4-2B) and as seen previously (85, 98, 104). However, capillaries formed by KSHV infected cells treated with rhTGF-beta2 were not maintained by 24 hours post plating and regressed to similar levels as mock infected cells (Fig 4-2A and quantified in fig 4-2B). A similar phenotype was observed with TIME cells (data not shown). This data suggests that TGF-beta2 exhibits anti-angiogenic effects on KSHV infected endothelial cells and destabilizes capillary-like networks.

KSHV microRNAs, miR-K3 and miR-K8, downregulate TGF-beta2

Latent KSHV infection downregulates the expression of TGF-beta2 therefore we sought to uncover the viral mechanism of downregulation. During latency, KSHV expresses 6 viral genes and expressed up to 18 mature microRNAs (163). MicroRNAs are small non-coding RNAs between 21-24 nucleotides that function in transcriptional and post-transcriptional regulation of gene expression. MicroRNAs function via base-pairing with complementary sequences within mRNA molecules which results in gene silencing either by mRNA degradation or by preventing mRNA from being translated. A given miRNA may have multiple mRNA targets, and a given target might similarly be targeted by multiple miRNAs (165-167).

Because miRNAs function in gene silencing we asked if any of the KSHV miRNAs could downregulate TGF-beta2 transcripts. We obtained 15 of the 18 KSHV miRNAs cloned as individual lentiviral constructs expressing GFP and a lentiviral construct that contains 10 of the 12 KSHV miRNA loci from the KSHV genome referred to as KSHV-cluster. TIME cells were mock or KSHV infected or transduced with the individual miRNAs, the KSHV-cluster or vector control, pSIN. Transduction efficiency was measured by GFP expression where at least 90% of the cells expressed GFP. Cells were harvested 48 hours post infection or transduction and total RNA was harvested and used to measure both variants of TGF-beta2 transcript levels using quantitative real-time RT-PCR. Several KSHV miRNAs, including miR-K5 and miR-K11, have been reported to downregulate thrombospondin transcript levels therefore thrombospondin was used as a positive control (168). MiR-K5 and miR-K11 are able to downregulate thrombospondin transcript levels verifying the miRNAs are functional in our system (data not shown). Compared to mock infected cells, TGF-beta2 transcript levels were down by 90% in KSHV infected cells (Fig. 4-3). The KSHV-cluster did not have a significant effect on TGF-beta2 transcripts. However, this construct lacks any selectable marker in order to assay for transduction efficiency, therefore we have not yet determined if this construct is efficiently expressed in cells in culture.

Interestingly, the individual KSHV miRNAs did display varying effects on TGF-beta transcript levels. Of the KSHV microRNAs tested, miR-K3 and miR-K8 had the greatest effect on TGF-beta2, demonstrating an 80-90% decrease in transcript levels compared to mock infected cells (fig. 4-3). We therefore decided to pursue miR-K3 and miR-K8 and determine the mechanism of TGF-beta2 downreguation by these two KSHV microRNAs.

The 3'UTR of TGF-beta2 is not targeted by miR-K3 or miR-K8

MicroRNAs commonly bind to the 3'untranslated region (UTR) of a gene or the coding DNA sequence (CDS) to direct gene silencing (165, 169). A seed sequence of 6-8 nucleotides located at the 5' end of the miRNA sequence (nucleotides 2-9) is important for determining target specificity. A perfect complementary seed match is not required for miRNA binding and several combinations of seed matches have been previously described. Anywhere between 5 and 7 nucleotide matches can elicit binding of a miRNA to a target sequence and the presence of an adenine 5' adjacent to the seed sequence of a target gene has been shown to enhance binding of the miRNA to the target sequence (167).

We determined the seed sequences of miR-K3 and miR-K8 (Fig. 4-4A) and looked for seed matches within the 3'UTR of TGF-beta2 and identified 5 potential seed matches to miR-K3 and 1 perfect seed match to miR-K8 (Fig. 4-4B). In order to assay for translational repression of TGF-beta2, we incorporated the use of a 3'UTR luciferase reporter system. If the 3'UTR of TGF-beta2 is the target of miRNA translational regulation, transduction of miR-K3 and miR-K8 will result in a decrease in luciferase production. The 3'UTR of TGF-beta2 was cloned behind a constitutively active firefly luciferase gene driven by the SV-40 promoter. A vector lacking a specific 3'UTR was used as a control to assay for non-specific regulation of luciferase production by experimental samples. We also designed a mutant-TGF-beta2 3'UTR where the 6 possible seed matches we identified were mutated and no longer recognized

by miR-K3 and miR-K8. Nucleotides 3-5 of the seed sequences were mutated by transversion of the nucleotides. We cloned the mutant 3'UTR into the luciferase vector and determined, by sequencing, that it was inserted properly and did not adversely affect luciferase production (data not shown). We created stable cell lines in HEK293 cells by transfection of the plasmid and selected for neomycin resistance and expression of RFP. Control, TGF-beta2 and mutant-TGF-beta2 cell lines were created and a luminometer was used to detect luciferase production in all three. KSHV activates the SV40 promoter, therefore KSHV infection of all three cell lines resulted in an increase in luciferase production and could not be used to determine if the 3'UTR of TGF-beta2 is the target of translational repression during KSHV infection (data not shown). A panel of KHSV miRNAs, vector control pSIN and mock infection were used to assay for luciferase production in all three cell lines. The same number of cells were counted and seeded for every condition tested and total protein was quantitated and used to normalize against luciferase production. Cells were lysed 48 hours post transduction and luciferase production was measured by a luminometer. With the exception of miR-K3, the miRNAs tested in the control cell line did not have significant effects on luciferase production (Fig. 4-5A). Compared to mock infection, miR-K3 greatly decreased luciferase production in the control cell line (60%)(Fig. 4-5A). This suggests there are regulatory elements within the control 3'UTR luciferase vector recognized by miR-K3 that adversely affect luciferase production.

Of the miRNAs tested in the TGF-beta2 3'UTR cell line, miR-K3 and miR-K12 had the greatest effect on silencing luciferase expression, decreasing luciferase production by 30% and 40% respectively (Fig 4-5B). A decrease in luciferase production by miR-K12 is surprising because in our initial screen of the individual miRNAs, miR-K12 had no effect on TGF-beta2 transcript levels (Fig 4-3). Interestingly, miR-K8 had little (10%) to no effect on luciferase production (Fig 4-5B) despite the presence of a perfect seed match within the TGF-beta2 3'UTR (Fig 4-4B).

The miRNAs miR-K1 and miR-K8 had no effect on luciferase production in the mutant-TGF-beta2 3'UTR cell line. For miR-K8 this is the expected result. However, as mentioned, miR-K8 also had little to no effect on luciferase production in the TGF-beta2 3'UTR cell line. The control vector pSIN, miR-K3 and miR-K12 all adversely affected luciferase production in the mutant-TGF-beta2 3'UTR cell line. It is possible that by mutating the seed sequences we enhanced the ability of miR-K3 to actively silence translation. With respect to miR-K3 and miR-K8, the 3'UTR luciferase experiments suggest that the 3'UTR of TGF-beta2 is not the target of transcriptional repression by these KSHV miRNAs. Further work is needed to determine the target of TGF-beta2 downregulation by miR-K3 and mir-K8 (see discussion).

Sponges to miR-K3 and miR-K8 together can restore TGF-beta2 levels

Inhibition of miRNAs can give insights into miRNA function and gene regulation. To verify that miR-K3 and miR-K8 downregulate TGF-beta2, we used dominant negative inhibitors called miRNA sponges. MicroRNA sponges are transcripts with repeated miRNA antisense seed sequences that can sequester miRNAs from endogenous targets. Sponges are encoded on a vector and can be expressed transiently by transfection. Sponges expressed in cultured cells serve as alternative targets for miRNAs and therefore indirectly reverse transcriptional repression of microRNA targets (170). MiRNA sponges are valuable tools for miRNA loss-of-function studies both *in vitro* and *in vivo*.

Sponges to either miR-K3 or miR-K8 and sponges that encode both miR-K3 and miR-K8 on a single sponge were encoded on GFP expressing lentiviral vectors. An empty sponge with no specific repeat sequence was used as a negative control. TIME cells were transduced with one sponge and 24 hours later they were transduced with miRNAs to either miR-K3 or miR-K8. Total RNA was harvest 48 hours post miRNA transduction and TGF-beta2 transcript levels were analyzed by quantitative real-time RT-PCR. Transcript levels of TGF-beta2 from cells transduced with both sponge and miRNA were compared to that of cells transduced with sponge only. The empty sponge control transduced with

either miR-K3 or miR-K8 results in a decrease in TGF-beta2 transcripts indicating the miRNAs function properly when co-expressed with empty sponge. Sponge directed against miR-K3 did not block TGF-beta2 downregulation by miR-K3. In fact the sponge to miR-K3 seems to potentiate the effect of miR-K3 on TGF-beta2 downregulation (Fig 4-6A). Because of this finding we are exploring alternative methods to block miR-K3 which will be discussed later. The sponge designed to sequester miR-K8 did block TGF-beta2 downreguation by miR-K8 (Fig. 4-6B). Interestingly, double sponges were able to block TGF-beta2 downregulation by both miR-K3 and miR-K8 (Fig 4-6A, B). However, neither the single sponge to miR-K3 or miR-K8 nor the double sponges were able to block TGF-beta2 downregulation during KSHV infection (data not shown). This suggests there may be another viral mechanism involved in TGF-beta2 downregulation during KSHV infection. Alternative methods to inhibit the KSHV miRNAs will be discussed further.

DISCUSSION

TGF-betas are host cytokines involved in wound healing and repair during which they aid in re-establishing cellular homeostasis and endothelial cell quiescence. They are also implicated in the late stages of angiogenesis during vessel remodeling and maturation. TGF-betas exert anti-angiogenic effects by inhibition of cellular proliferation and enhancing the formation of extracellular matrix and inhibiting extracellular matrix degradation (136, 138). TGF-beta2 is downregulated by KSHV infection and the addition of this cytokine to infected cells results in the destabilization of capillary networks. This indicates that TGF-beta2 exerts anti-angiogenic effects on KSHV infected endothelial cells when plated on a three-dimensional matrix. Therefore, during latent infection, KSHV alters TGF-beta2 at the transcriptional level in order to suppress the anti-angiogenic effects of this cytokine. MicroRNAs are small non-coding RNAs discovered for the ability to silence gene expression at the transcriptional and post-transcriptional levels. KSHV encodes 18 mature miRNAs that have been implicated in the

downregulation of host gene expression during infection. Several KSHV miRNAs are known to downregulate the anti-angiogenic molecule thrombospondin and the cellular transcription factor MAF (musculoaponeurotic fibrosarcoma oncogene homolog) is as well as the immune ligand MICB and caspase 3 have also been shown to be downregulated by KSHV miRNAs (154, 160, 164, 168). We identified two KSHV miRNAs, miR-K3 and miR-K8, that are sufficient to downregulate TGF-beta2 transcript levels.

MicroRNAs bind to target genes by base pairing of the seed sequence located in the 5'end of the miRNA sequence between nucleotides 2-9. The predominant target of miRNA binding is the 3'UTR of a target mRNA (165). Six potential seed sequences were identified in the 3'UTR of TGF-beta2, 5 seed matches to miR-K3 and 1 perfect seed match to miR-K8. A luciferase reporter system was used to determine if the mechanism of down-regulation by miR-K3 and miR-K8 is by targeting the 3'UTR of TGF-beta2. These experiments indicated that the miRNAs have non-specific effects on luciferase production. This was evidenced by the fact that addition of miR-K3 resulted in a 60% decrease in luciferase production in cells expressing the control luciferase reporter with a nonspecific 3' UTR. MiR-K3 expressed in the TGF-beta2 reporter cell line resulted in a 30% decrease in luciferase production while miR-K8 had little to no effect on luciferase expression. However, miR-K12 expression decreased luciferase 40% in the TGF-beta2 reporter cells. This result is in contrast to data indicating miR-K12 is not able to down-regulate TGF-beta2 transcript levels. Taken together, these data suggest that the miRNAs regulation of luciferase expression from the 3'UTR constructs is not specific to the 3'UTR being tested. Therefore, this data does not support the hypothesis that miR-K3 or miR-K8 targets the 3'UTR of TGF-beta2 in order to downregulate expression. It should be noted that we currently do not have a positive control for the downregulation of TGF-beta2. Little is known about the activation of TGF-beta2 and to date there are no known miRNAs, host or viral that have been shown to downregulate TGF-beta2 expression. It is possible that the 3'UTR luciferase experiments are not robust enough to detect a

downregulation of TGF-beta2 by miR-K3 and miR-K8. In order to determine direct binding of the viral miRNAs to TGF-beta2 sequences, ribonucleoprotein immunoprecipitation (RNP-IP) experiments can be performed.

MicroRNAs have also been shown to target the CDS of a target gene, and in rare cases have been reported to target the 5'UTR (169, 171). Therefore, we are exploring the hypothesis that miR-K3 and miR-K8 target the CDS of TGF-beta2 to downregulate gene expression. Eight potential seed matches have been identified in the CDS of TGF-beta2, 5 to miR-K3 and 3 to mR-K8. Luciferase reporter assays with the CDS of TGFbeta2 could give insights into the mechanism of TGF-beta2 downregulation by miR-K3 and miR-K8. It remains a possibility that miR-K3 and miR-K8 regulate TGF-beta2 expression via an indirect method by downregulating the expression of an activator of TGF-beta2. Little is known about how TGF-beta2 is regulated in endothelial cells however the transcription factor ATF-1 has been shown to bind to positive regulatory elements within the TGF-beta2 promoter (172, 173).Therefore ATF-1 may be an attractive target by which miR-K3 and miR-K8 may function to downregulate TGF-beta2 expression.

We utilized miRNA sponges to inhibit miR-K3 and miR-K8 target recognition. Preliminary results suggest that the double sponges to both miR-K3 and miR-K8 may function to block the downregulation of TGF-beta2 by these viral miRNAs. However, the single sponge designed to sequester miR-K3 had no effect on the ability of miR-K3 to downregulate TGF-beta2 and instead appears to potentiate the downregulation of TGF-beta2 by miR-K3. This finding may be explained by weak expression of the miR-K3 sponge. However it is also possible that the sponge sequence itself has adverse effects on TGF-beta2 transcription and therefore is not effective at blocking miR-K3. There are caveats to working with miRNA sponges in order for them to effectively block miRNA activity. First, in order for sponges to be effective against miRNAs they must be expressed in high amounts or at high titers within a cell and may need to be re-administered to be effective. Secondly, sponges have been reported to cause toxicity in cell

culture which may be the result of double stranded RNA or potential off target effects of the sponges. We are looking further into toxic effects of the sponges and the disruption of host keeping genes when cells are transduced with the various sponges. Therefore, we are pursuing other mechanisms of miRNA inhibition by using antagomirs to competitively inhibit miR-K3 and miR-K8. Antagomirs are synthetic antisense oligonucleotides chemically modified to resist degradation. Two types of antagomirs, 2'O methylated and locked nucleic acids (LNA), are commonly used to inhibit miRNA function. The ribose moiety of an LNA nucleotide is modified with an extra bridge that locks the ribose in a 3'-north conformation which organizes the backbone in a rigid conformation and increases the melting temperature of the oligonucleotides making LNAs stable at a variety of temperatures (174). LNAs are synthetic oligonucleotides similar to small interfering RNAs (siRNA) and therefore are easily transfected into cells *in vitro*. LNAs specific to KSHV miR-K3 and miR-K8 are commercially available and we will investigate if they are able to block the activity of miR-K3 and miR-K8 to downregulate TGF-beta2 transcripts.

We have not been able to definitively block the downregulation of TGF-beta2 by miR-K3 or miR-K8 nor have we been able to clearly identify the mechanism of downregulation by miR-K3 or miR-K8. Therefore, in order to verify our original screen of the miRNAs and to validate the involvement of the viral miRNAs in the downregulation of TGF-beta2, we are going to block the miRNA processing pathway. MicroRNAs in the nucleus are transcribed as a single multi-hairpin mRNA referred to as a pri-miRNA. The pri-miRNA is processed by the nuclear protein Drosha which cleaves the long mRNA to produce individual hairpins or pre-miRNAs. These individual pre-miRNAs are actively exported to the cytoplasm by Exportin-5 and further processed by the host protein Dicer which cleaves the hairpin to produce double stranded RNA and loads one strand of the RNA, the miRNA, onto the RISC complex which directs the action of miRNA induced gene silencing (159, 175). We have designed a short hairpin to Drosha and

created a stable knockdown TIME cell line in which to investigate the role of KSHV miRNAs in the downregulation of TGF-beta2.

We have determined that two KSHV miRNAs, miR-K3 and miR-K8, contribute to the downregulation of TGF-beta2 during latent KSHV infection of endothelial cells. However, assays designed to block miRNA function have been unable to restore TGF-beta2 transcript levels in the face of KSHV infection and have only been moderately effective against the individual miRNAs. This indicates the need for more sensitive assays and, more importantly, suggests that another viral mechanism may be involved in the downregulation of TGF-beta2 by KSHV. Therefore, we are investigating whether the other latent genes of KSHV are sufficient to downregulate TGF-beta2 expression.

KSHV is the infectious agent of KS, a highly vascularized tumor of endothelial cell origin that supports extensive neo-angiogenesis. Angiognesis is a balance of pro-angiogenic and anti-angiogenic factors. *In vitro* models of angiogenesis indicate that latent KSHV infection of endothelial cells promotes angiogenic phenotypes thereby tipping the balance (85, 98, 104, 158). TGF-betas have anti-angiogenic effects in non-disease states of angiogenesis such as wound healing and embryogenesis (136, 138). However, TGF-betas and TGF-beta signaling is deregulated in a number of tumors suggesting that TGF-betas may have pro-angiogenic effects on cancer cells (176). We have found that KSHV transcriptionally downregulates TGF-beta2, a member of the TGF-beta family of cytokines. Furthermore, we have shown that TGF-beta2 has anti-angiogenic effects on KSHV infected cells grown in a three dimensional matrix, demonstrating a phenotypic role for TGF-beta2. We have also identified a viral mechanism for the downregulation of TGF-beta2 during KSHV infection by the miRNAs, miR-K3 and miR-K8 though work on the exact mode of downregulation is ongoing. Collectively, this work uncovers a unique mechanism by which latent KSHV gene expression downregulates an anti-angiogenic host factor in order to promote angiogenic phenotypes in endothelial cells. This work supports the hypothesis that KSHV infection directly promotes angiogenesis and KS tumor formation and pathogenesis.

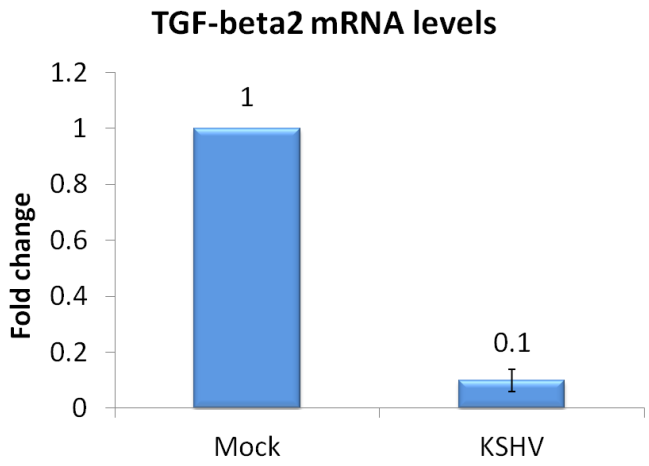

Figure 4-1: TGF-beta2 is downregulated in endothelial cells. Tert Immortalized Microvascular Endothelial (TIME) cells were mock or KSHV infected for 48 hours in order to establish latency. Cells were harvested and mRNA was isolated and analyzed by quantitative real time RT-PCR. Samples were normalized to GAPDH and reported as fold change over mock infected cells.

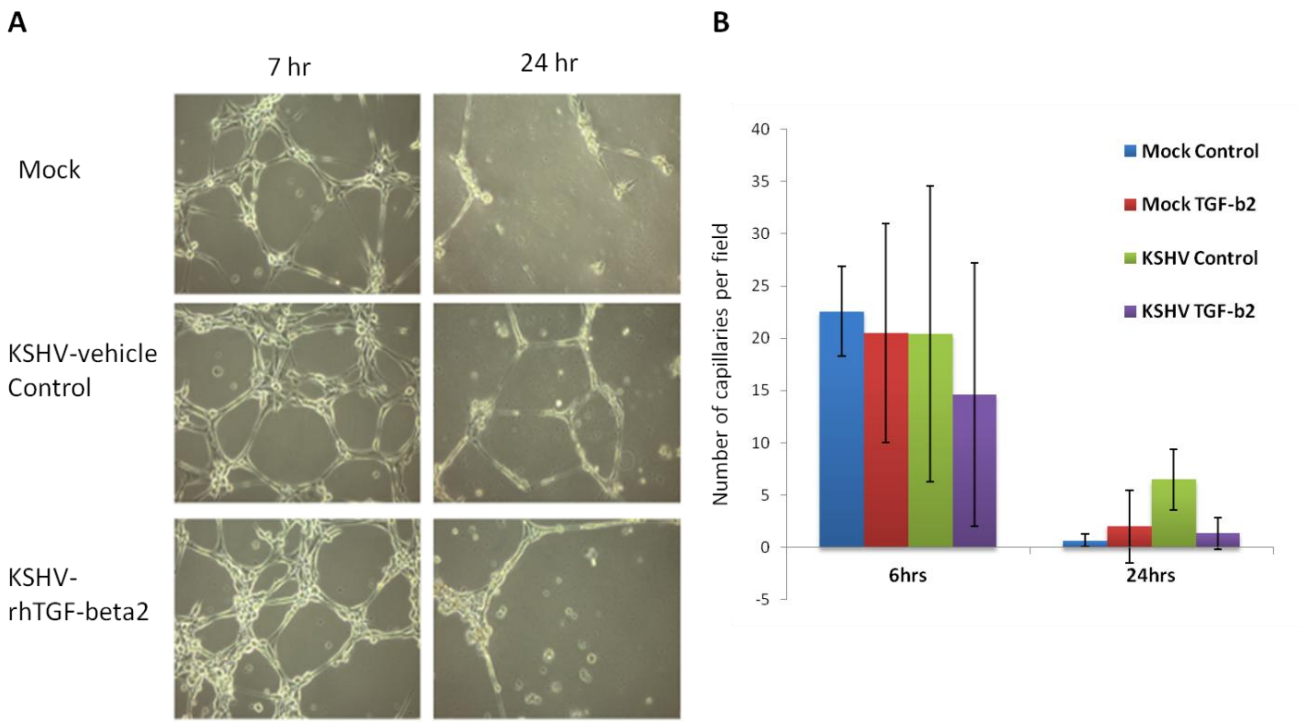

Figure 4-2: TGF-beta2 contributes to angiogenic phenotypes of KSHV infected endothelial cells. (A) TIME cells were mock or KSHV infected. 48 hpi cells were plated on Matrigel 3-dimensional matrix with either 30ug/ml of rhTGF-beta2 or vehicle control and incubated at 37^{o}C. Representative capillary morphogenesis pictures taken at 7 and 24 hours post plating are shown. (B) Quantitation of capillaries from three independent experiments similar to the one seen in A. Three fields from each experiment (9 total) were analyzed and the number of capillaries per field were counted.

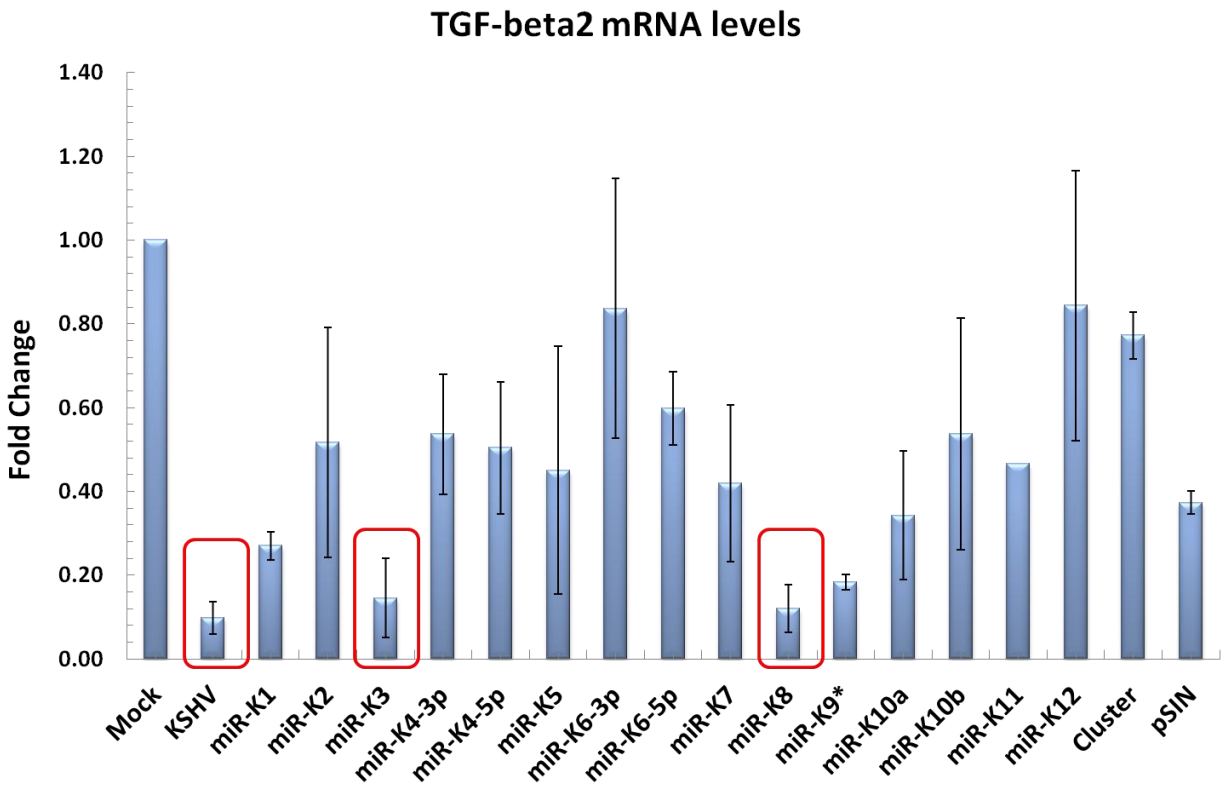

Figure 4-3: KSHV and the miRNAs, miR-K3 and miR-K8 downregulate TGF-beta2 transcript levels. TIME cells were mock or KSHV infected or transduced with individual miRNA lentiviral vectors, a lentiviral miRNA Cluster containing all miRNAs except miR-10a, miR-10b and miR-K12 or the empty lentival vector pSIN. Cells were harvested 48 hours post infection/transduction and mRNA was isolated and anlyzed by quantitative real time RT-PCR. Samples were normalized to GAPDH and reported as fold change over mock infected cells. Error bars indicate the standard errors of the means of three independent experiments.

A

Seed sequences:

miR-K3: 5′ TCA CAT TCT GAG GAC GGC AGC GA 3′

miR-K8: 5′ TAG GCG CGA CTG AG AGA GCA CG 3′

B

TGF-beta2 3'UTR: miR-K3 and miR-K8 seed matches

1 AATTCTTGGA AAAGTGGCAA GACCAAAATG ACAATGATGA TGATAATGAT GATGACGACG CAACGATGA TGCTTGTAAC AAGAAAACAT AAGAGAGCCT TGGTTCATCA GTGTTAAAAA

121 ATTTTTGAAA AGGCGGTACT AGTTCAGACA CTTTGGAAGT TTGTGTTCTG TTTGTTAAAA CTGGCATCTG ACACAAAAAA AGTTGAAGGC CTTATTCTAC ATTTCACCTA CTTTGTAAGT

241 GAGAGAGACA AGAAGCAAAT TTTTTTTAAA GAAAAAAATA AACACTGGAA GAATTTATTA GTGTTAATTA TGTGAACAAC GACAACAACA ACAACAACAA CAAACAGGAA AATCCCATTA

361 AGTGGAGTTG CTGTACGTAC CGTTCCTATC CCGCGCCTCA CTTGATTTTT CTGTATTGCT ATGCAATAGG CACCCTTCCC ATTCTTACTC TTAGAGTTAA CAGTGAGTTA TTTATTGTGT

481 GTTACTATAT AATGAACGTT TCATTGCCCT TGGAAAATAA AACAGGTGTA TAAAGTGGAG ACCAAATACT TTGCCAGAAA CTCATGGATG GCTTAAGGAA CTTGAACTCA AACGAGCCAG

601 AAAAAAAGAG GTCATATTAA TGGGATGAAA ACCCAAGTGA GTTATTATAT GACCGAGAAA GTCTGCATTA AGATAAAGAC CCTGAAAACA CATGTTATGT ATCAGCTGCC TAAGGAAGCT

721 TCTTGTAAGG TCCAAAAACT AAAAAGACTG TTAATAAAAG AAACTTTCAG TCAGAATAAG TCTGTAAGTT TTTTTTTTTC TTTTTAATTG TAAATGGTTC TTTGTCAGTT TAGTAAACCA

841 GTGAAATGTT GAAATGTTTT GACATGTACT GGTCAAACTT CAGACCTTAA AATATTGCTG TATAGCTATG CTATAGGTTT TTTCCTTTGT TTTGGTATAT GTAACCATAC CTATATTATT

961 AAAATAGATG GATATAGAAG CCAGCATAAT TGAAAACACA TCTGCAGATC TCTTTTGCAA ACTATTAAAT CAAAACATTA ACTACTTTAT GTGTAATGTG TAAATTTTTA CCATATTTTT

1081 TATATTCTGT AATAATGTCA ACTATGATTT AGATTGACTT AAATTTGGGC TCTTTTTAAT GATCACTCAC AAATGTATGT TTCTTTTAGC TGGCCAGTAC TTTTGAGTAA AGCCCCTATA

1201 GTTTGACTTG CACTACAAAT GCATTTTTTT TTTAATAACA TTTGCCCTAC TTGTGCTTTG TGTTTCTTTC ATTATTATGA CATAAGCTAC CTGGGTCCAC TTGTCTTTTC TTTTTTTTGT

1321 TTCACAGAAA AGATGGGTTC GAGTTCAGTG GTCTTCATCT TCCAAGCATC ATTACTAACC AAGTCAGACG TTAACAAATT TTTATGTTAG GAAAAGGAGG AATGTTATAG ATACATAGAA

1441 AATTGAAGTA AAATGTTTTC ATTTTAGCAA GGATTTAGGG TTCTAACTAA AACTCAGAAT CTTTATTGAG TTAAGAAAAG TTTCTCTACC TTGGTTTAAT CAATATTTTT GTAAAATCCT

1561 ATTGTTATTA CAAAGAGGAC ACTTCATAGG AAACATCTTT TTCTTTAGTC AGGTTTTTAA TATTCAGGGG GAAATTGAAA GATATATATT TTAGTCGATT TTTCAAAAGG GGAAAAAAGT

1681 CCAGGTCAGC ATAAGTCATT TTGTGTATTT CACTGAAGTT ATAAGGTTTT TATAAATGTT CTTTGAAGGG GAAAAGGCAC AAGCCAATTT TTCCTATGAT CAAAAAATTC TTTCTTTCCT

1801 CTGAGTGAGA GTTATCTATA TCTGAGGCTA AAGTTTACCT TGCTTTAATA AATAATTTGC CACATCATTG CAGAAGAGGT ATCCTCATGC TGGGGTTAAT AGAATATGTC AGTTTATCAC

1921 TTGTCGCTTA TTTAGCTTTA AAATAAAAAT TAATAGGCAA AGCAATGGAA TATTTGCAGT TTCACCTAAA GAGCAGCATA AGGAGGCGGG AATCCAAAGT GAAGTTGTTT GATATGGTCT

2041 ACTTCTTTTT TGGAATTTCC TGACCATTAA TTAAAGAATT GGATTTGCAA GTTTGAAAAC TGGAAAAGCA AGAGATGGGA TGCCATAATA GTAAACAGCC CTTGTGTTGG ATGTAACCCA

2161 ATCCCAGATT TGAGTGTGTG TTGATTATTT TTTTGTCTTC CACTTTTCTA TTATGTGTAA ATCACTTTTA TTTCTGCAGA CATTTTCCTC TCAGATAGGA TGACATTTTG TTTTGTATTA

2281 TTTTGTCTTT CCTCATGAAT GCACTGATAA TATTTTAAAT GCTCTATTTT AAGATCTCTT GAATCTGTTT TTTTTTTTTT TAATTTGGGG GTTCTGTAAG GTCTTTATTT CCCATAAGTA

2401 AATATTGCCA TGGGAGGGGG GTGGAGGTGG CAAGGAAGGG GTGAAGTGCT AGTATGCAAG TGGGCAGCAA TTATTTTTGT GTTAATCAGC AGTACAATTT GATCGTTGGC ATGGTTAAAA

2521 AATGGAATAT AAGATTAGCT GTTTTGTATT TTGATGACCA ATTACGCTGT ATTTTAACAC GATGTATGTC TGTTTTTGTG GTGCTCTAGT GGTAAATAAA TTATTTCGAT GATATGTGGA

2641 TGTCTTTTTC CTATCAGTAC CATCATCGAG TCTAGAAAAC ACCTGTGATG CAATAAGACT TCTCAAGCT GGAAAAGTCA TACCACCTTT CCGATTGCCC TCTGTGCTTT CTCCCTTAAG

2761 GACAGTCACT TCAGAAGTCA TGCTTTAAAG CACAAGAGTC AGGCCATATC CATCAAGGAT AGAAGAAATC CCTGTGCCGT CTTTTTATTC CCTTATTTAT TGCTATTTGG TAATTGTTTG

2881 AGATTTAGTT TCCATCCAGC TTGACTGCCG ACCAGAAAAA ATGCAGAGAG ATGTTTGCAC CATGCTTTGG CTTTCTGGTT CTATGTTCTG CCAACGCCAG GGCCAAAAGA ACTGGTCTAG

3001 ACAGTATCCC CTGTAGCCCC ATAACTTGGA TAGTTGCTGA GCCAGCCAGA TATAACAAGA GCCACGTGCT TTCTGGGGTT GGTTGTTTGG GATCAGCTAC TTGCCTGTCA GTTTCACTGG

3121 TACCACTGAC CACAAACAAA AAAACCCACC CTATTTCCTC CAATTTTTTT GGCTGCTACC TACAAGACCA GACTCCTCAA ACGAGTTGCC AATCTCTTAA TAAATAGGA

Figure 4-4: MiR-K3 and miR-K8 seed sequences and seed matches in identified in the TGF-beta2 3'UTR. (A) KSHV DNA sequences of miR-K3 and miR-K8. The miRNA seed sequence located between basepairs 2-8 are underlined for each sequence. (B) TGF-beta2 3'UTR DNA sequence. Five seed matches for miR-K3 are highlighted and underlined in red. 1 perfect seed match for miR-K8 is highlighted and underlined in blue.

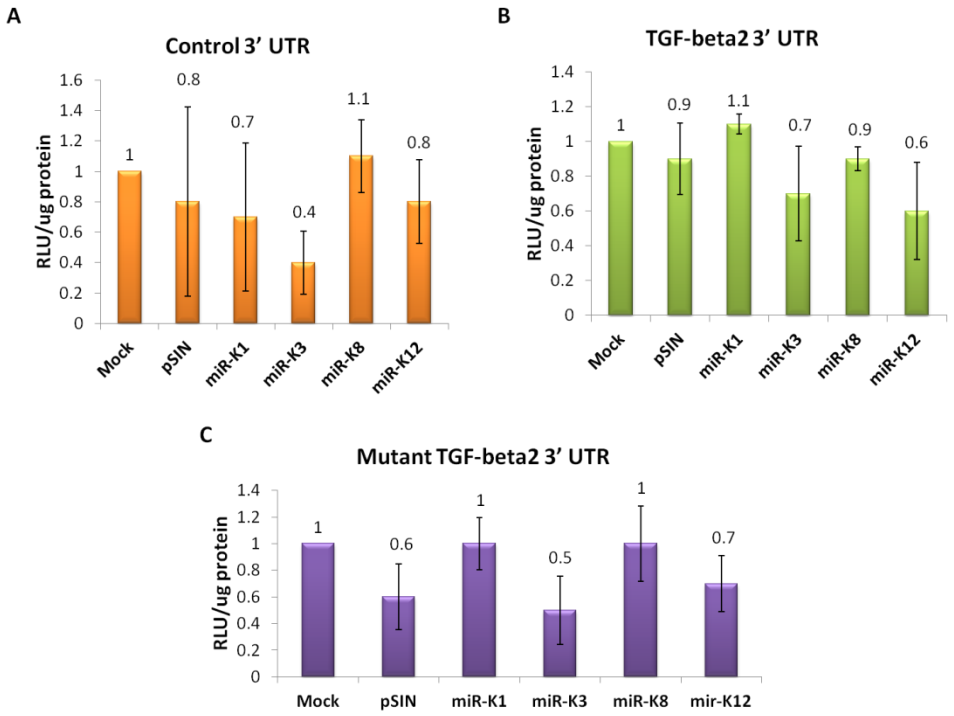

Figure 4-5: The 3'UTR of TGF-beta2 is not targeted by miR-K3 or miR-K8. HEK293 cells stably expressing constitutively active firefly luciferase fused to the indicated 3'UTRs were mock infected or transduced with the indicated miRNA lentiviral vectors or the empty control pSIN lentiviral vector. Cells were harvested 48 hours post infection/transduction and luciferase expression was measure and total protein was quantitated from each sample. Data is presented as relative luciferase units per ug of protein measured. Each sample is normalized to mock RLU/ug protein. Error bars indicate the standard errors of the means of three independent experiments.

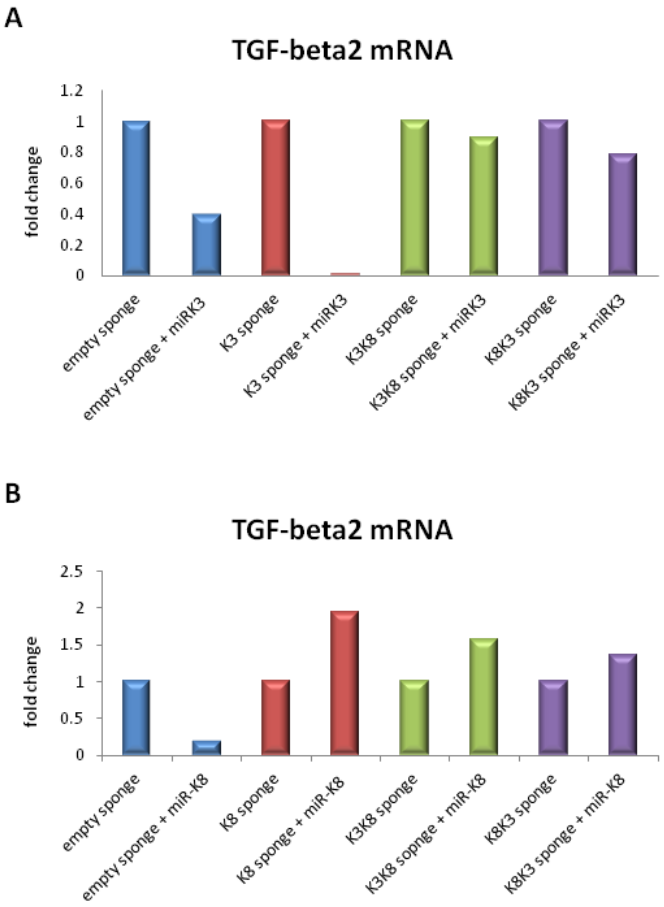

Figure 4-6: Double Sponges to miR-K3 and miR-K8 restore TGF-beta2 transcript levels. TIME cells were transduced with the indicated lentiviral sponges. 24 hours post transduction cells were transduced with the either miR-K3 or miR-K8 expressing lentivirus. 48 hours post miRNA transduction cells were harvested and mRNA was analyzed by quantitative real time RT-PCR. Samples were normalized to GAPDH and reported as fold change of sponge + miRNA over sponge alone.

CHAPTER V

Conclusions and Future Directions

SUMMARY

KSHV latent infection of endothelial cells alters host cellular pathways and gene expression to induce both lymphangiogenesis and angiogenesis. These cellular processes are tightly regulated and play a critical role in cancer and tumor development. KSHV induces lymphatic reprogramming of latently infected endothelial cells and requires signaling via the cellular receptor gp130 (49, 78, 100, 148). However, we have found that all aspects of lymphatic reprogramming and lymphangiogenesis are not controlled solely by gp130 receptor signaling. The pro-angiogenic transcription factor, Ets-1, is upregulated independently of gp130 signaling during latent KSHV infection. We defined a viral mechanism for Ets-1 upregulation by the viral gene vFLIP and describe a novel gene regulated by Ets-1 in VEGFR3. Importantly, the loss of Ets-1 expression results in the destabilization of capillary-like networks of KSHV infected endothelial cells indicating Ets-1 expression promotes angiogenic phenotypes (104). Therefore, we have identified a host transcription factor regulated by latent KSHV gene expression that plays a role in promoting aspects of both lymphangiogenesis and angiogenesis. We also found the host cytokine TGF-beta2, regulated by gp130 signaling, to be downregulated during KSHV infection and show the viral miRNAs, miR-K3 and miR-K8, are sufficient to induce transcriptional repression. TGF-beta2 was found to exert anti-angiogenic effects on capillary stability of KSHV infected endothelial cells indicating that the downregulation of this cytokine by KSHV promotes angiogenic phenotypes. This body of work shows that KSHV induced lymphatic reprogramming, lymphangiogenesis and angiogenesis are not mutually exclusive and highlights how multiple latent KSHV genes regulate the expression of multiple host factors that collectively contribute to the induction of these phenotypes which we believe to be significant to KSHV pathogenesis and KS tumor formation.

Ets-1 expression promotes lymphangiogenesis and angiogenesis

Ets-1 is upregulated during latent KSHV infection and two host genes, LANA-1 and vFLIP, have been shown to positively regulate Ets-1 expression by independent mechanisms (104, 132). This level of regulation by KSHV suggests that Ets-1 transcriptional activation may be important during latent KSHV infection. Ets-1 is pro-oncogenic, known to regulate genes involved in angiogenesis, and we showed that Ets-1 is required for the expression of the lymphatic specific gene VEGFR3 during latent KSHV infection of endothelial cells. Interestingly, Ets-1 has never been described as a regulator of lymphatic gene expression nor has a role in promoting lymphangiogenesis been described for Ets-1. We also found Ets-1 expression in primary LECs to be required for maximal VEGFR3 expression. Therefore, we have defined a novel mechanism for Ets-1 in promoting lymphatic gene expression and in KSHV induced lymphatic reprogramming of endothelial cells. The master regulator of lymphatic reprogramming, Prox1, induced by gp130 signaling during latent KSHV infection of endothelial cells, also upregulates the expression of VEGFR3. We hypothesized that Prox1 and Ets-1 cooperatively promote the expression of VEGFR3. However, promoter luciferase experiments indicated that Prox1 was unable to activate the VEGFR3 promoter with or without Ets-1 expression (data not shown). This may be due to transfection efficiency in TIME cells however when tested in HEK293 cells, we still did not detect activation of the VEGFR3 promoter with Prox1. This suggests that Prox1 and Ets-1 do not act synergistically on the VEGFR3 promoter but rather may cooperatively interact with one another. Interestingly, cooperative interaction of Ets-1 and Prox1 on VEGFR3 expression has been described in the literature however how they interact during KSHV infection to promote VEGFR3 expression remains an area of active research. Prox1 expressing TIME cells have elevated levels of Ets-1 expression suggesting that Prox1 expression may promote the upregulation of Ets-1 (data not shown). However, whether this is the case during KSHV infection remains unknown. Interestingly, knockdown of either Prox1 or Ets-1 during KSHV infection results in a loss of VEGFR3 expression suggesting that both Prox1 and Ets-1 are required for maximal

expression of VEGFR3. Understanding the interaction of Ets-1 and Prox1 and the requirement of these host genes for VEGFR3 expression during latent KSHV infection remain an area of future investigation.

Our data indicate that the viral gene vFLIP and NF-κB signaling drive the upregulation of Ets-1. It has been reported that activation of the NF-κB pathway by inflammatory stimuli activates Prox1, and together they activate the VEGFR3 promoter leading to increased receptor expression in lymphatic endothelial cells (177). Prox1 is upregulated by gp130 receptor signaling during latent KSHV infection and whether NF-κB signaling also contributes to Prox1 expression during latent KSHV infection warrants further investigation. However, analysis of the microarray indicates that known targets downstream of NF-κB signaling during KSHV infection are unaffected by the loss of gp130 expression. This suggests there is no cross-talk between genes controlled by the gp130 pathway and by the NF-κB pathway. Taken together this data suggests that Prox1 and Ets-1 expression are controlled by independent pathways during KSHV infection of endothelial cells. Therefore, KSHV appears to regulate the expression of the lymphangiogenic signaling molecule, VEGFR3 by two independent pathways.

Ets-1 is able to activate the promoters of the angiogenic and lymphangiogenic ligand receptors, VEGFR1 and VEGFR3 respectively. Knockdown of Ets-1 results in a loss of VEGFR1 expression, however, the HIFalpha subunits also play a role in the induction of VEGFR1 during KSHV infection. Ets-1 and Hif2α regulate the VEGFR1 chromatin domain to induce VEGFR1 transcription in endothelial cells and in differentiating embryonic stem cells (126). Whether this interaction is important during latent KSHV infection remains unknown and is an active area of research. VEGFR1 is a blood specific receptor responding primarily to VEGF-A. VEGFR1 signaling is pro-angiogenic and similar to VEGFR3 and VEGF-C, VEGFR1 and VEGF-A are elevated in KSHV latently infected endothelial cells. Studies with KSHV infected B cell PELs indicate that VEGF-A expression serves an anti-apoptotic role and stimulates and growth and vascular permeability of PEL cells (86, 178). This suggests that VEGFR1 signaling is important for these phenotypes in KSHV B cell lymphomas. However, the importance of VEGFR1 signaling during latent KSHV

infection of endothelial cells remains largely unknown. Our *in vitro* angiogenesis studies strongly suggest a role in VEGFR1 signaling in promoting angiogenic phenotypes of KSHV latently infected endothelial cells and continues to be an active area of research. A loss of Ets-1 expression during KSHV infection has deleterious effects on capillary stability of endothelial cells indicating that Ets-1 has pro-angiogenic functions during latent KSHV infection. We hypothesize that the loss of capillary stability is due to a loss of both VEGFR1 or VEGFR3 signaling rendering KSHV infected cells defective in receiving angiogenic stimuli.

Ets-1 has been shown to regulate the expression of multiple host factors that promote angiogenesis and lymphangiogenesis during latent KSHV infection of endothelial cells (80, 99, 104). We hypothesize there are additional host targets of Ets-1 that have yet to be described during latent KSHV infection of endothelial cells. We also showed that Ets-1 expression is upregulated in KS spindle cells of multiple KS tumor biopsies. Therefore, we hypothesize that Ets-1 expression promotes angiogenesis and lymphangiogenesis of KS spindle cells and is a significant contributor to KS tumor formation. However, there is currently no acceptable animal model in which to study KS tumor formation making the functional importance of Ets-1 expression to KSHV pathogenesis fundamentally difficult to investigate.

KSHV downregulates TGF-beta2 to promote angiogenesis

Gene expression analysis of mock and KSHV infected endothelial cells treated with either a nonspecific siRNA or siRNA against the cellular signaling receptor gp130 indicates that gp130 signaling regulates global gene expression(103). We have described how gp130 signaling is required for lymphatic reprogramming and promotes lymphangiogenesis. Microarray analysis also identified the host cytokine TGF-beta2 to be downregulated and to be controlled by gp130 signaling during latent KSHV infection. We verified that KSHV downregulates the expression of TGF-beta2 during latency and showed this cytokine is able to exert anti-angiogenic effects on capillary stability. We also described a viral

mechanism by which TGF-beta2 transcripts are downregulated by the KSHV miRNAs, miR-K3 and miR-K8. We have shown that the expression of these miRNAs is sufficient to downregulate transcript levels of TGF-beta2, however, we are still working to understand the exact mechanism by which TGF-beta2 is targeted for downregulation by miR-K3 and miR-K8. It is possible that the miRNAs do not directly target TGF-beta2 mRNA but rather act upstream to indirectly downregulate the expression of TGF-beta2. Little is known about the cellular regulation of TGF-beta2 however, according to microarray analysis pathways directed by gp130 signaling are avenues for exploration of TGF-beta2 downregulation during KSHV infection. Work is ongoing to understand the mechanism of miR-K3 and miR-K8 downregulation of TGF-beta2. Interestingly, the host anti-angiogenic molecule thrombospondin is also downregulated by KSHV viral miRNAs (168). Thrombospondin is required for the cleavage and activation of TGF-beta proteins. It has also been reported that the TGFBR2 is downregulated during KSHV infection although, we have not been able to detect a change in the expression of this receptor in our cell culture models (142). Nevertheless, it appears that TGF-betas and TGF-beta signaling is altered by several mechanisms during KSHV infection presumably in order to promote angiogenesis of latently infected endothelial cells.

As previously stated, the addition of TGF-beta2 to KSHV infected cells grown on a three dimensional matrix results in the destabilization of capillaries. We are currently working to restore TGF-beta2 expression during KSHV infection in our *in vitro* assays in order to verify the function of TGF-beta2 on angiogenic phenotypes. We are also further exploring the mechanism of capillary destabilization by TGF-beta2. TGF-betas function in the late stages of vascular maturation and remodeling by inducing apoptosis of superfluous new capillaries. We hypothesize that KSHV infected capillary destabilization by TGF-beta2 is a result of apoptosis induced by TGF-beta2 signaling. *In vitro* angiogenesis studies are currently underway using fluorescent dyes to quantitate cell death and treating with caspase inhibitors to address the mechanism of apoptosis. We suggest a model by which latent KSHV gene expression

downregulates the expression of the anti-angiogenic cytokine TGF-beta2 in order to promote cell survival and angiogenesis.

KSHV induced gp130 controlled genes and angiogenesis

We have now described a role for gp130 receptor signaling and gene regulation in promoting lymphatic reprogramming, lymphangiogenesis and angiogenic phenotypes. Additional host genes identified under gp130 receptor control are hypothesized to play roles in promoting angiogenesis and lymphangiogenesis and survival of endothelial cells. Currently, work is ongoing to validate the KSHV induced-gp130 receptor control as well as the phenotypic, pro-angiogenic effects of the following genes: *SERPINB3* (SCCA-1), *SERPINB4* (SCCA-2), and *SPP1* (Osteopontin). Both SCCA-1 and SCCA-2 have been used as tumor markers for squamous cell carcinoma (SCC) of various organs, and serum levels are correlated with clinical stage of disease (179). They have been shown to be involved with cellular migration and have anti-apoptotic functions (180). Osteopontin has been shown to induce angiogenesis by upregulating endothelial cell migration, survival and lumen formation. Furthermore, high levels of osteopontin correlate with tumor invasion, progression and metastasis in a number of tumors, including breast, gastric, lung, prostate, liver and colon cancer (181). We have chosen to take a closer look at SCCA-1, SCCA-2 and Osteopontin to determine how they may contribute to KSHV pathogenesis.

SERPINB3 and *SERPINB4* encode squamous cell carcinoma antigens 1 and 2 (SCCA-1 and SCCA-2) respectively, and are homologous genes, which are 92% identical at the nucleic acid level. SCCA-1 and SCCA-2 belong to the serpin family of serine proteinase inhibitors and are cysteine and chymotrypsin proteinase inhibitors respectively (182). Both SCCA-1 and 2 protect cancer cells from apoptosis induced by NK cells, TNFα, or chemotherapeutic drug treatment (180). In addition, SCCA-1 overexpression prevents NK cell migration and infiltration in tumor models of SCC (183). Serpins are also involved in the intercellular adhesion events, indicating that SCCAs likely take part in the malignant behaviors of

squamous cancer such as cellular invasion and metastasis (184). Interestingly, the promoters of both SCCA-1 and SCCA-2 have STAT binding sites and can be controlled by inflammatory cytokine induction of STAT-1 and STAT-6 activation (185). In several cancer cell lines, STAT3 was shown to activate SCCA-1 and SCCA-2 promoters and promote cell survival (186).

Given pro-angiogenic functions of SCCA-1 and SCCA-2, we speculate that KSHV induction of these proteins may be important for host cell survival and migration, and can contribute to KS tumor formation. The common use of both SCCA-1 and SCCA-2 serum levels as biomarkers for SCC also make their induction by KSHV interesting since they could potentially be used as biomarkers for KS during clinical prognosis and evaluation of treatment. In our microarray study, SCCA-1 and SCCA-2 were induced 4–fold and 10.3-fold by KSHV infection of TIME cells, and decreased 3.7-fold and 7.3-fold by gp130 receptor knockdown (103). We have verified SCCA-1 and SCCA-2 mRNA induction by qRT-PCR, and we have determined that SCCA-1 and SCCA-2 are regulated by the expression of STAT3 (unpublished data from VA morris, RC Wells, and M Lagunoff). We are currently working on functional assays to determine if SCCA-1 and SCCA-2 play a role in promoting the survival and migration of KSHV latently infected endothelial cells. We have created a short hairpin RNA to stably knockdown SCCA-1 gene expression in TIME cells (TIME-shSCCA-1). We are currently working to verify the hairpin knocks down expression of SCCA-1 during KSHV infection. Once we know the hairpin is functional we will determine how the knockdown of this gene effects cell survival by assaying for apoptosis and the presence of caspase 3 cleavage of KSHV latently infected endothelial cells. Preliminary unpublished data using siRNA to SCCA-1 or SCCA-2 does not support a role for these proteins in the survival of KSHV infected cells (VA Morris, RC Well, M Lagunoff). However, the stable knockdown using shRNA may have a stronger effect on knockdown, therefore this work is ongoing. We will also explore the possibility that SCCA-1 promotes cellular migration of KSHV infected cells. Transwell migration assays will be used to assay migration and invasion of mock and KSHV infected TIME and TIME-shSCCA-1 cells. Future work will also include

confirming the upregulation at the protein level by immunoblot analysis and using ELISA kits to test for SCCA-1 and SCCA-2 levels in the supernatent of KSHV-infected TIME cells. If the above experiments are positive, levels of SCCA-1 and SCCA-2 can be examined in KS tumor biopsies and in serum from KS patients.

Osteopontin is a pro-angiogenic secreted phosphoprotein, and contains domains that bind to Beta3 integrins and CD44 to mediate signaling (181). Osteopontin was previously shown to be upregulated by KSHV infection of endothelial cells (66, 187). In our microarray data, we found osteopontin induced 5.5-fold by KSHV infection and blocked 4.1-fold by gp130 receptor knockdown (103). We have found by ELISA that Osteopontin is only detected in the supernatant of latently infected endothelial cells and that signaling via gp130 and activation of the PI3K/AKT pathway is involved in the upregulation of Osteopontin (Figure 5-1). Previous work demonstrated that osteopontin did not affect KSHV-induced transformation, as measured by foci formation of d-HMVECs immortalized by human papillomavirus proteins E6 and E7 (187). However, osteopontin may be playing a role in the high degree of neo-angiogenesis observed in KS tumors. Unpublished ELISA data indicates that transfection of control siRNA disrupts the ability of KSHV infected cells to secrete functional osteopontin (KD Gutierrez and M Lagunoff). Therefore, we have created a stable knockdown cell line using a short hairpin RNA in TIME cells in order to study, *in vitro,* the contribution of osteopontin on KSHV infected endothelial cells (TIME-shSPP1). Work is ongoing to determine the effectiveness of the shRNA and to validate the use of the cell line. Once the cell line has been established, we will be able to determine the role osteopontin plays in promoting angiogenic phenotypes of KSHV latently infected endothelial cells.

In summary, we have shown that latent KSHV infection of endothelial cells leads to aberrant activation of host genes that contribute to lymphatic reprogramming and promote lymphangiogenic and angiogenic phenotypes. The KS tumor is highly vascularized and the phenotypic nature of the tumor supports a role for angiogenesis in tumor formation. KSHV induction of tumorigenesis is a complex

mechanism that is not fully understood. We have demonstrated that one host factor, Ets-1, can have multiple effects on cellular changes within KSHV latently infected endothelial cells which is indicative of the extensive crosstalk between cellular pathways and gene expression that are affected by KSHV gene expression. KSHV infection of endothelial cells induces the expression of pro-angiogenic cytokines such as VEGF-A and -C, IL-6 and Ang-2 (83, 188, 189). However, we have demonstrated that KSHV infection also downregulates the expression of anti-angiogenic cytokines such as TGF-beta2 presumably to promote angiogenesis of infected endothelial cells. Therefore, KSHV seems to tip the balance of pro- and anti-angiogenic factors in favor to angiogenesis. Angiogenesis is a tightly regulated process that involves the coordinated and temporal regulation of many host genes. By continuing to investigate angiogenic host factors altered by KSHV we can further our understanding of how KSHV infection promotes angiogenesis and KS tumor formation. In a broader context this work may uncover an attractive therapeutic target in which to treat Kaposi's Sarcoma or angiogenic maladies in general.

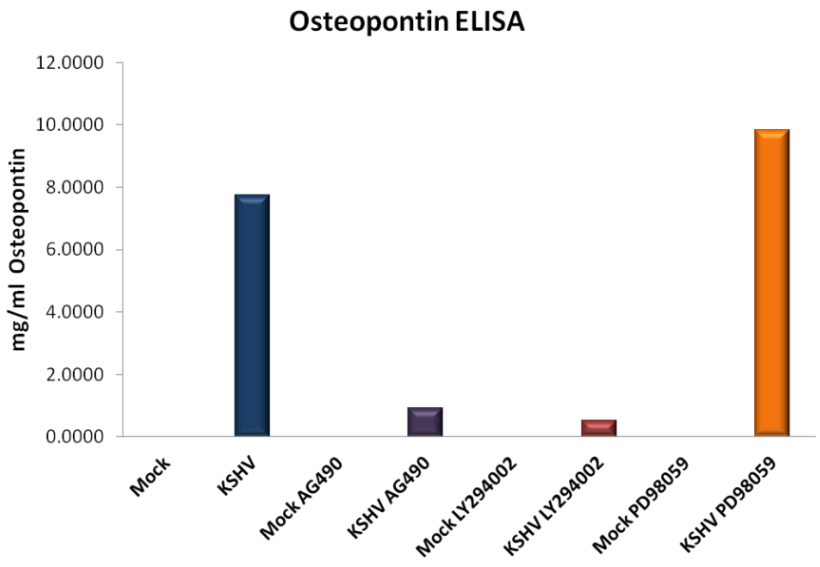

Figure 5-1: Osteopontin (SPP1) is controlled by gp130 signaling. TIME cells were mock or KSHV infected and treated with the indicated pharmacological inhibitor 16 and 40 hours post infection. AG40 (JAK2 inhibitor) was used at a concentration of 100uM, LY294002 (PI3Kinase inhibitor) was used at a concentration of 40uM, and PD98059 (MEK inhibitor) was used at a concentration of 20uM. Cellular supernatents were harvested 48 hours post infection and analyzed by ELISA.

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CURRICULUM VITAE

Kimberley Diane Gutierrez

EDUCATION:

Ph.D. in Microbiology, May 2013
University of Washington
Seattle, Washington, USA
Thesis Advisor: Michael Lagunoff

M.B.S. in Biomedical Sciences, June 2007
Midwestern University
Glendale, Arizona, USA
Thesis Advisor: Sam Katzif

B.S. in Microbiology, December 2005
Auburn University
Auburn, Alabama, USA

PUBLICATIONS:

Gutierrez KD, Morris VA, Wu D, Barcy S, Lagunoff M. 2013. Ets-1 is required for the activation of VEGFR3 during latent Kaposi's Sarcoma Herpesvirus infection of endothelial cells. J Virol. 87 (12): 6758-6768
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DiMaio TA, **Gutierrez KD,** Lagunoff M. 2011. Latent KSHV infection of endothelial cells induces integrin beta3 to activate angiogenic phenotypes. PLoS Pathog 7**:**e1002424.

PRESENTATIONS:

Poster Presentation: International Congress on Oncogenic Herpesviruses and Associated Diseases
August 1-4, 2012 Philadelphia, Pensylvannia
Title: "Ets-1 is required for the activation of VEGFR3 during latent KSHV infection of blood endothelial cells"

Oral Presentation: 16th International Workshop on Kaposi's Sarcoma Herpesvirus (KSHV) and Related Agents
June 30-July 3 2013 Puerto Vallarta, Mexico
Title: "Ets-1 is required for the activation of VEGFR3 during latent KSHV infection of blood endothelial cells"