Virus-host cell interplay in the pathogenesis of Kaposi's sarcoma herpesvirus

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ACADEMIC DISSERTATION

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To my family

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ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by their Roman numerals.

I. Varjosalo M*, Björklund M*, Cheng F, Syvänen H, Kilpinen S, Sun Z, Kallioniemi O, He W, Ojala P§ and Taipale J. §: Application of active and kinase-deficient kinome collection for identification of kinases regulating Hedgehog signaling. Cell, 2008, May2; 133(3):537-48. * equal contribution; §*shared correspondence*

II. Cheng, F., Weidner-Glunde M., Varjosalo M., Eeva-Maija Rainio, Lehtonen A., Schulzv T.F., Koskinen P.J., Taipale J., and Ojala P.M. KSHV reactivation from latency requires Pim-1 and Pim-3 kinases to inactivate the latency-associated nuclear antigen LANA. PLoS Pathogens, Mar; 5(3): e1000324, 2009.

III. Cheng F*, Pekkonen P*, Laurinavicius L,* Sugiyama N, Henderson S, Günther T,Rantanen V, Kaivanto E, Aavikko M, Sarek G, Hautaniemi S, Biberfeld P, Aaltonen L, Grundhoff A, Boshoff C, Alitalo K, Lehti K, and Ojala PM. KSHV-initiated Notch activation leads to membrane-type-1 matrix metalloproteinase-dependent lymphatic endothelial-to-mesenchymal transition. Cell Host & Microbe, 2011, Dec15; 10(6):577-590. * equal contribution

Additional unpublished material is also presented

Publication I was also used in the thesis of Ph.D. Markku Varjosalo.

ABBREVIATIONS

ADAM	a disintegrin and metalloprotease
ALDH1	aldehyde dehygrogenase 1
ALK	activin-like kinase
Asp	aspartate
BAC	Bacterial Artificial Chromosome
BECs	blood endothelial cells
BL	Burkitt's lymphoma
BM	bone marrow
BMP	bone morphogenetic protein
bp	base pair
CAFs	cancer associated fibroblast
CAMK	calcium/calmodulin-regulated kinases
CDK	cyclin-dependent kinase
CKI	cyclin-dependent kinase inhibitor
CSCs	cancer stem cells
CSF1	Colony-stimulating factor 1
CTL	Cytoxic T Lymphocytes
DE	delayed early
DMEM	Dulbecco's modified Eagle Medium
EBV	Epstein-Barr virus
ECM	extracellular matrix
ECs	endothelial cells
EGF	epidermal growth factor
EMSA	electrophoretic mobility shift assay
EMT	epithelial–mesenchymal transition
EndMT	endothelial-mesenchymal transition
EpCAM	epithelial cell adhesion molecule
FACS	fluorescence-activated cell sorting
FAP	fibroblast-activated protein
FCS	fetal calf serum
FGF	fibroblast growth factors
FSP1/S100A4	fibroblast-specific protein-1
Fzd	Frizzled
G-CSF	Granulocyte Colony-stimulating factor
GEM	gene expression microarray
GFP	green fluorescent protein
GM-CSF	granulocyte–macrophage colony-stimulating factor
GPI	glycosylphosphatidylinositol
GSK	glycogen synthase kinase
GST	Glutathione-S-transferase

HAART	Highly Active Anti-Retroviral Therapy
HAT	histone acetyltransferases
HBV	hepatitis B virus
HCC	hepatocellular carcinoma
HCS	high-content screening
HCV	hepatitis C virus
HDAC	histone deacetylase
HGF	hepatocyte growth factor
Hh	Hedgehog pathways
HHV-8	human herpesvirus type 8
HIF-1	hypoxia induced factor 1
HIV	Human immunodeficiency virus
HPCs	hematopoietic progenitor cells
HPV	human papillomavirus
HSP90	heat shock protein 90
HTLV-1	human T-cell leukemia virus
HUVEC	human umbilical vein endothelial cells
IE	immediate early
IGF1	insulin-like growth factor 1
IGFR	insulin growth factor receptor
IL-8	interleukin-8
KS	Kaposi's sarcoma
KSHV	Kaposi's sarcoma herpesvirus
LANA	latency-associated nuclear antigen
LBS	LANA binding sites
Leu	leucine
LT	latency transcript
LEC	lymphatic endothelial cells
Lef/Tcf	lymphoid enhancer factor/T cell factor
LYVE-1	lymphatic vessel hyaluronan receptor-1
MCD	multicentric Castleman disease
MCP	monocyte chemoattractant protein
MCPyV	Merkel cell polyomavirus
MDM2	murine double minute 2
MEF	mouse embryo fibroblast
miRNA	microRNA
MMP	matrix metalloproteinases
MOI	multiplicity of infection
MoMuLV	moloney murine leukemia virus
mRNA	messenger RNA
MT-MMP	membrane- bound MMP
MT1-MMP	membrane-bound type 1 MMP
NaB	sodium butyrate

NF-kB	nuclear factor kappa-B
NG2	neuron-glial antigen-2
NICD	Notch intracellular domain
NK	Natural-killer Cells
NLS	nuclear localization signal
NOD	non-obsese diabetic
NPM	nucleophosmin
N-terminal	aminoterminal
ORC	origin recognition complex
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PAN	polyadenylated nuclear
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PDGF	platelet derived growth factor
PDGFR	platelet derived growth factor receptor
PDGFR-a	platelet derived growth factor receptor alpha
PDGFR-b	platelet derived growth factor receptor beta
PECAM1	platelet/endothelial cell adhesion molecule 1
PEL	primary effusion lymphoma
PFA	paraformaldehyde
PI3K	phosphatidylinositol 3 (PI3)-kinase
PKA	protein kinase A
PKC	protein kinase C
proline	Pro
Rb	retinoblastoma
RBPJ	recombination signal binding protein for immunoglobulin kappa J region
RFP	red fluorescent protein
RNA	ribonucleic acid
RNAi	RNA interference
RTA	replication transcriptional activator
s.c.	subcutaneous
SA	signal anchor
SCID	severe combined immunodeficiency
SDF1	stromal-cell-derived factor 1
Ser	serine
sh-RNA	short hairpin RNA
SUMO	small ubiquitin-related modifier
SV40	Simian virus 40
TA	transactivation
TAM	tumor associated monocyte/macrophage
TGF-b	transforming growth factor beta
Thr	threonine

TIMPs	the tissue inhibitors of metalloproteinases
TNF-α	tumor necrosis factor alpha
TP53	tumor protein 53
TPA	12-O-tetradecanoyl phorbol-13-acetate
TR	terminal repeat
Trp	tryptophan
TSA	trichostatin A
TSP-1	thrombospondin-1
UV	ultraviolet
v-cyclin	viral cyclin
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor
v-FLIP	viral FLICE-inhibitory protein
vGPCR	viral G protein-coupled receptor
vIL-6	Viral interleukin-6
vIRF	Viral interferon regulatory factor
VSVG	vesicular stomatitis virus G protein
wt	wild type
XIAP	X-linked inhibitor of apoptosis
ZO-1	zonula occludens 1
aSMA	a-smooth-muscle actin
b-gal	b-galactosidase
2D	2-dimentional
3D	3-dimentional

ABSTRACT

Kaposi's sarcoma herpesvirus (KSHV) is the etiological agent of three types of malignancies: Kaposi's sarcoma (KS), multicentric Castleman disease (MCD), and primary effusion lymphoma (PEL). Infection by KSHV displays two different phases: latent and lytic replication phase. By using an unbiased gain-of-function human kinome cDNA screen, the work in this thesis identified two kinases, Pim-1 and -3, to be involved in KSHV reactivation. Ectopic expression of Pim-1 and Pim-3 induced viral lytic replication leading to production of progeny viruses, whereas depletion of Pim-1 and Pim-3 by RNA interference inhibited the induction of lytic reactivation. Pim-1 and -3 was shown to regulate viral reactivation by phosphorylation of LANA, which abolished the LANA-mediated repression of lytic transcription.

In this thesis project we developed a novel three-dimensional (3D) cell model to identify novel oncogenic processes involved in the KSHV-induced endothelial cells (EC) transformation. The results demonstrate that KSHV induces transcriptional reprogramming of primary lymphatic endothelial cells (LECs) to mesenchymal cells via endothelial-to-mesenchymal transition (EndMT), a process implicated in promoting tumor growth and cell invasiveness. Two viral gene products, vFLIP and vGPCR, were found to trigger Notch signaling and lead to the KSHV-induced EndMT. Our data further identifies a membrane associated matrix metalloproteinase MT1-MMP as a previously unrecognized regulator downstream of Notch to induce EndMT. 3D KSHV-infected LECs (K-LECs) transcriptome showed significant up-regulation of invasion related genes that were found co-regulated in 3D K-LECs and KS biopsies. The results further demonstrate that 3D culture provides a permissive microenvironment for continuous viral replication and persistence. To summarize, this PhD thesis greatly expands the understanding of host signaling pathways involved in KSHV reactivation and oncogenesis. Furthermore, this thesis provides novel information about cellular targets for pharmacological control in KS and other virus-associated cancers.

INTRODUCTION

KS is the most common cancer in HIV-infected untreated individuals and remains a primary cause of cancer deaths in many subequatorial African countries as a result of the AIDS pandemic. KS displays an extraordinary diversity of cell types ranging from endothelial to mesenchymal cells of unclear origin. During the last decade KS tumor cells have been thought to be of endothelial origin, and immortalized ECs infected with KSHV lose cell contact inhibition, can grow post-confluently, form foci in soft-agar and even form tumors in nude mice, which are hallmarks of cellular transformation. However, infection of primary LECs by KSHV does not cause a similar transformed phenotype when maintained in standard monolayer cell culture, but rather tend to lose viral episomes, suggesting that the tumorigenic potential of KSHV on the endothelium may be dependent on the culture microenvironment. As with other herpes viruses, infection by KSHV displays these two phases. During latency only few viral genes are expressed, while in the productive infection the virus is reactivated with initiation of extensive viral DNA replication and gene expression, resulting in production of new viral particles, contributing to progression of KS. Host signal-transduction pathways are intimately involved in the switch between latency and productive infection of herpes viruses, but they have not been rigorously addressed.

The emphasis of this PhD thesis is to illustrate the importance of virus-cell interactions in KSHV spread and viral induced oncogenesis. In the first part of the thesis, I have investigated cellular kinases regulating the switch from latency to lytic replication of KSHV by systematically screened the effect of expression of 466 human kinases on KSHV reactivation. In addition, I have examined cellular processes involved in KSHV pathogenesis by using a 3D model for KSHV-infected primary LECs to mimic important aspects of the tissue environment. The experimental part of my PhD thesis gives novel insight into molecular mechanisms involved in KSHV pathology, which can be used for developing targeted therapies to prevent or at least slow down the progression of KS in immunosuppressed patients.

REVIEW OF THE LITERATURE

1. Kinase signaling pathways

1.1. Human kinome

Since the discovery of protein phosphorylation nearly 50 years ago, protein kinases have been recognized as major players in cell signaling, accounting for ~2% of genes in the human genome (Manning et al., 2002b). By phosphorylating substrate proteins, kinases modify the activity, location and affinities of up to 30% of all cellular proteins, and direct most of the cellular processes, particularly in signal transduction and co-ordination of complex pathways. Mutations and dysregulation of protein kinases play causal roles in human disease, offering the possibility to develop agonists and antagonists of these enzymes for use in disease therapy (Hunter and Cooper, 1985).

All the mammalian protein kinases can be divided into tyrosine kinases, serine/threonine kinases, and kinases that can phosphorylate both tyrosine and serine/threonine residues. Despite these differences in substrate specificity, all known mammalian protein kinases have structurally similar kinase domains (Huse and Kuriyan, 2002; Nolen et al., 2004). The kinase domain is composed of 250-300 amino acid residues and can be divided into two subdomains, a smaller N lobe and a larger C lobe, between which is the cleft into which ATP and the substrates bind (Huse and Kuriyan, 2002; Nolen et al., 2004). The relatively strong conservation of kinase domains has allowed the computational characterization of the protein kinase complement of the human genome, and for most kinases, also the identification of critical residues required for their catalytic activity (Manning et al., 2002b). These residues include a lysine (Lys72 in PKA) in the N lobe, which is required for proper orientation of ATP, and an aspartate (Asp166 in PKA) in the catalytic loop, which interacts with the hydroxyl side chain of the substrate (Huse and Kuriyan, 2002). Mutations in these residues kill the catalytic activity of kinases (Manning et al., 2002a), but do not interfere with substrate recognition or binding to other proteins. As a result, catalytically inactive kinase can either have no activity, or dominant-negative activity due to titration of cofactors from the corresponding active kinase (Mendenhall et al., 1988).

Certain mutations in a subset of kinases (such as EGFR, c-Met, c-Kit, and Phosphatidylinositol 3 (PI3)-kinases (PI3K)) change the expression, conformation and/or stability of these kinases, leading to constitutive activation of these kinases and related signaling pathways. For instance, a key PI3-kinase gene PIK3CA is found to be mutated frequently in human cancer. Most of these mutations are heterozygous missense changes clustered in the helical region and in the catalytic domains of the gene, which affect highly conserved residues within these domains. These mutations were shown to cause constitutive activation of PI3K as well as enhanced phosphorylation of downstream

target AKT in tumor cells, which contribute to the malignant transformation, growth, and metastasis of human cancers (Lengyel et al., 2007; Samuels et al., 2005).

So far there are more than 620 protein kinase complement in the Human Genome (the human ‘‘kinome’’) identified by different genomic approaches. These kinases are classified into nine major classes with 90 families and 145 subfamilies by sequence comparison of their catalytic domains, aided by knowledge of sequence similarity and domain structure outside of the catalytic domains, known biological functions, and a similar classification in the yeast, worm, and fly kinomes (Hanks and Hunter, 1995; Manning et al., 2002a) (http://www.kinase.com/human/kinome/). Deciphering the complex network of phosphorylation-based signaling is essential in understanding fundamental cellular processes in physiological and pathological states, providing a thorough knowledge base for therapeutic intervention.

1.2. Human kinases as the therapeutic targets of cancers

Human kinases are intimately involved in cancer cell growth, proliferation, survival and evasion. Deregulation of kinase activity has emerged as a major mechanism in the development of different types of cancers (Hanahan and Weinberg, 2011). Among the most frequently mutated oncogenes and tumor suppressors, human protein kinases have become the largest class of new drug targets. In recent 10 years, there are more than 10,000 patent applications for oncogenic kinase inhibitors being filed in the United States alone, a dozen of kinase inhibitors being proved as cancer treatments, and more kinase targets being developed at the clinical or preclinical stage (Akritopoulou-Zanze and Hajduk, 2009; Zhang et al., 2009).

One such exciting advance in cancer therapy is the discovery of Bcr-Abl gene fusion and the subsequent development of imatinib mesylate, a small molecule tyrosine kinase inhibitor, to target the catalytic activity of the bcr-abl protein product in chronic myeloid leukaemia (CML) (Roychowdhury and Talpaz, 2011). This inhibitor was later used to effectively target mutant c-Kit in gastrointestinal stromal tumors (GIST) and a few other tumors, leading to multi-year increases in survival of patients. It is encouraging that this protein-kinase inhibitor is achieving unprecedented durability for complete hematologic, cytogenetic, and molecular responses and proving to be well tolerated compared to conventional chemotherapeutic treatments.

However, about half of the patients who initially benefit from imatinib treatment eventually develop drug resistance because of the acquisition of secondary mutations in the kinase domain, the activation of surrogate kinases that substitute for the drug target or the presence of activating mutations in downstream pathway components. Overcoming these resistance mechanisms may require targeting tumor cells at multiple levels by using either single drugs that inhibit multiple proteins or cocktails of several selective kinase inhibitors. Indeed, a new c-Kit inhibitor sunitinib, which also targets

VEGF receptor (VEGFR) and platelet derived growth factor receptor alpha (PDGFR-α), has proven efficacious in patients who are intolerant or refractory to imatinib.

The future efforts in salvaging patients from failure of the kinase inhibitor monotherapies is to learn how to identify larger panels of kinases and related signaling components that are implicated in cancer, how to predict the best combinations of these targets and then how to prioritize those combination and develop agents with multiple targets to overcome tumors resistant against a single-targeting agent.

1.3. Functional genomics for identification of kinome targets in virus-associated cancers

Advances in genomic technologies have opened up the field of systematic identification of novel human genes required for tumor formation and progression in mammalian cells. RNA interference (RNAi) is a RNA dependent gene silencing process in which short double-stranded RNA molecules lead to either degradation or translational arrest of target mRNAs in a cell. The selective and robust effect of RNAi on gene expression can be exploited as a research tool by transfecting siRNAs designed to target specific genes into a cell and evaluating the effect of knocking down the expression of these genes on a cellular process (Dorsett and Tuschl, 2004). The development of large-scale RNAi screens has made it possible to get an unbiased tumor signaling network by looking for genes that are crucial for tumor growth. In addition, these screens facilitate the identification of new kinase targets, as any gene that selectively blocks tumor growth when knocked down by RNAi is a candidate. As a complementary tool, the special collection of kinome cDNA library could be used to systematically screen for kinases affecting a given cellular phenotype. Because increased kinase expression often leads to gain-of-function phenotypes due to increased activity, the special collection of the kinome cDNA library could also be used to systematically screen for kinases affecting a given cellular phenotype, or to validate the kinase hits obtained from RNAi screens.

To date, accumulating evidences suggest that at least seven different human viruses--Epstein-Barr Virus (EBV), hepatitis B virus (HBV), human papillomavirus (HPV), human T-cell leukemia virus (HTLV-1), hepatitis C virus (HCV), Kaposi's sarcoma herpesvirus (KSHV) and Merkel cell polyomavirus (MCPyV) are bona fide etiologic agents of human malignancy, contributing to 15-20% of human cancers worldwide (McLaughlin-Drubin and Munger, 2008). Human tumor viruses have served as important experimental models in understanding key molecular events in multi-step tumor development and progression. RNA and DNA viruses differ in their general mechanisms of inducing tumorigenesis, partially due to the difference in their replication mode and life cycle. The studies of viral proto-oncogenes have led to the discovery of their cellular counterparts, oncogenes and relevant cellular growth-regulatory networks, whereas research on DNA tumor viruses has inspired further identification of p53 tumor suppressor and many functions of the retinoblastoma (Rb) tumor suppressor.

Given that the genome size of a virus is highly restricted to ensure packaging within an infectious structure, viruses need to target host cellular signaling pathways that control proliferation, differentiation, cell death, genomic integrity, tumor invasion and immune surveillance in cancer progression (McLaughlin-Drubin and Munger, 2008). Our understanding of how these viruses exploit cellular genes and pathways has not only contributed to our knowledge of viral pathogenesis, but also revealed much of the molecular mechanisms within the cell.

In spite of the effectiveness of antiviral regimens targeting viral infection, broad-spectrum antiviral drugs have been elusive due to the rapid evolution of viruses and low fidelity of viral proteins. A promising pharmacological strategy for treating individuals living with tumor viruses has been to simultaneously target host factors required for virus infection or replication. However, the identity of these virus associated host factors and signaling pathways remains incompletely understood (Goff, 2008; Lama and Planelles, 2007). Therefore, unbiased, genome-scale research into cellular factors associated with viral replication and pathogenesis will provide new insight into the multiple functions of cellular pathways, offering the potential to further understand virus-host interactions and identify host targets for developing anti-virus therapeutics.

With the advent of cell-based screening technologies, it has become possible to screen for cellular protein kinases required for viral infection in a given host cell during a bona fide viral life cycle, rather than in simplified *in vitro* systems. In the last few years, a series of genome-scale screens have been performed against human oncogenic viral pathogens. This has led to the identification of a large number of new host cell proteins and pathways that influence different phases of viral life cycles. This work has greatly expanded our knowledge of the interface between each virus and its host, and will undoubtedly lead to insight into the cell biological roles of many of these genes. Of particular interest is the fact that several screens have been carried out in cells infected with the same type of viruses such as HIV and HCV, allowing analysis of the reproducibility and robustness of the screening technique.

1.3.1. HIV screens

Patients with HIV infection have a severely compromised immune system and show substantially increased incidence of several cancers, primarily due to co-infection with an oncogenic DNA virus, especially EBV, KSHV and HPV (Boshoff and Weiss, 2002; Yarchoan et al., 2005). To date, four independent screens have been published identifying host factors important in different phases of the HIV lifecycle (Brass et al., 2008; König et al., 2008; Yeung et al., 2009; Zhou et al., 2008), as well as a meta-analysis further examining the combined results of three of these studies (Bushman et al., 2009).

Two of these screens were performed in HeLa cells with quite a similar strategy, although the studies used different siRNA libraries (Brass et al., 2008; Zhou et al., 2008). In the first screen, by Brass et al. the assay was based on p24 Gag expression making it sensitive

to all of the early phases of HIV replication. The late-acting host factors affecting viral assembly and egress were assayed by culturing of infected cell supernatants with fresh Tat-responsive cells and using tat mediated β-galactosidase activity as a second measure of productive infection. From the first screen, the authors identified genes inhibiting p24 Gag production that fell into classes spanning many aspects of cell biology, from nuclear pore components, autophagy, the mediator complex (an RNA-polymerase II-associated complex involved in activation of transcription) to vesicular trafficking (Brass et al., 2008). In the second screen, by Zhou et al. the authors assessed the virus infection and tat activation by following a tat-responsive β-gal reporter expression (Zhou et al., 2008). Interestingly, only 15 of the identified genes were among those also identified by Brass *et al.* in the first screen. Seven of the 15 overlapping genes were previously identified as playing a role in HIV replication, including the viral receptors CD4 and CXCR4. In addition, several components of the mediator complex identified by Brass *et al.*, as well as other components of this complex, were identified in this screen, further confirming the role of the mediator complex in HIV replication. Another set of identified genes validated by cDNA rescue were involved in Akt/NFkB signaling, although the step in the life cycle was not addressed.

A complementary approach to RNAi loss-of-function screening is to perform arrayed cDNA screening to identify gain-of-function activities. A screen by Nguyen et al. used the same HeLa cell line as Brass and Zhou, and cotransfected a library of 15 000 human cDNAs along with a tat-responsive luciferase reporter (Nguyen et al., 2007). The authors identified 315 genes increasing the tat-mediated luciferase production upon overexpression, and these were genes involved in mitochondrial biology, transcription, translation, and antiapoptotic functions. Three out of the six top candidates were found to be required for replication when siRNAs were tested, validating this approach.

1.3.2. HCV screens

The *Flaviviridae* family member Hepatitis C virus (HCV) chronically infects approximately 170 million people worldwide. HCV infection is the leading cause of chronic liver disease and associated with an increased risk of primary liver cancer. Given the importance of better anti-HCV therapies, many groups have launched genome wide screen approaches to identify cellular cofactors of the virus life cycle, with a hope to develop novel therapeutics (Berger et al., 2009; Ng et al., 2007; Randall et al., 2007; Supekova et al., 2008; Tai et al., 2009; Trotard et al., 2009).

Ng et al. screened a library of 4000 druggable genes using Huh-7 derived cells that stably maintain a HCV subgenomic replicon expressing HCV NS3- NS5B and the reporter secreted alkaline phosphatase (SEAP) (Ng et al., 2007). Nine cellular genes that potentially regulate HCV replication were identified through the screen and a re-screen, four of which are members of the TNF/LT signaling pathway, making this pathway a potential target for anti-HCV therapeutics. In addition, there were two small-scale

screens to identify cellular factors regulating HCV replicon replication, due to technical difficulties in working with the infectious virus (Berger et al., 2009; Randall et al., 2007).

To date, however, only one study has used a siRNA library covering the entire genome (Tai et al., 2009). This screen was carried out in Huh-7 cells harboring an HCV luciferase-expressing replicon, examining viral protein expression and RNA replication. 96 human genes that support HCV replication, with a significant number of them being involved in vesicle organization and biogenesis were identified through primary and secondary screens, including several previously identified putative HCV host factors such as phosphatidylinositol 4-kinase PI4KA and multiple subunits of the COPI vesicle coat complex (Gosert et al., 2003). Interestingly, Supekova et al. (Supekova et al., 2008) screened the human kinome (510 genes) for their role in HCV replicon replication using a similar assay to Tai et al. in the genome-wide screen (Tai et al., 2009). Validation of the hits derived from this screen identified three novel kinases CSK, JAK1, and VRK1 required for HCV replicon propagation, which were not identified as hits in previous screens described above.

1.4. Pim kinase family

1.4.1. Discovery of Pim kinase family

The human Pim serine/threonine kinase family belongs to the group of calcium/calmodulin-regulated kinases (CAMK), which are highly conserved through evolution in multicellular organisms. Three Pim family members Pim-1, Pim-2 and Pim-3 share significant sequence similarities and homologous structures (containing a characteristic kinase domain, an ATP anchor and an active site) and largely overlapping functions (Bachmann and Moroy, 2005; Bullock et al., 2005).

The *pim-1* gene was originally identified as a preferential proviral insertion site of the Moloney Murine Leukemia Virus in experiments designed to find new genes involved in murine T-cell lymphomas (Selten et al., 1985). In subsequent experiments, Pim-1, Myc and Gfi1 in each combination were shown to have the ability to cooperate in experimental T-cell lymphomagenesis (Allen and Berns, 1996), establishing Pim-1 as a proto-oncogene and important player in the process of malignant transformation. Further, Pim-2 was also identified as a proviral integration site associated with a rapid development of malignant T-cell lymphomas.

1.4.2. Function and regulation of Pim kinases

Pim kinases are able to phosphorylate different targets, most of which are involved in multiple cellular functions such as cell growth, differentiation and apoptosis. Several Pim-1 substrates have been identified over the recent years including nuclear adapter protein p100 (Leverson et al., 1998), T-cell receptor transcription factor NFATc (Rainio

et al., 2002), the pro-apoptotic protein Bad (Aho et al., 2004) and G1-specific cell cycle regulator Cdc25A (Losman et al., 2003).

Expression of Pim kinases can be stimulated by a large set of cytokines and regulated at transcriptional, post-transcriptional, translational and post-translational levels. Several signal transduction pathways such as the JAK/STAT axis may be important for Pim-1 expression. For instance, STAT3 activated via different cytokines or growth factors such as IFN-λ, c-Kit, SCF, GCSF and EGF, binds directly to the *pim-1* promoter at the ISFR/GAS-sequence (gamma interferon activation sequences), which leads to an up-regulation of *pim-1* gene expression. In addition, Pim-1 itself can negatively regulate the Jak/STAT pathway by binding to a group of negative regulators of STAT activity, the so-called SOCS proteins (Losman et al., 1999), which implicates the role of Pim-1 in immune responses.

1.4.3 Pim kinases and tumorigenesis

The Pim kinases are involved in a number of signaling pathways that control critical processes in hematogenesis and lymphopoiesis, which have implications in tumor development. Indeed, Pim kinases are overexpressed in various lymphomas and leukemias (Amson et al., 1989; Hoefnagel et al., 2005) as well as in prostate cancer (Dhanasekaran et al., 2001). Examples for a possible involvement of Pim-1 in human tumors are prostate cancer, oral cancer and Burkitt lymphoma (Gaidano et al., 1993).

Recent results have implicated Pim kinases in regulation of herpesviral oncogenesis. Expression levels of Pim-1 and Pim-2 are upregulated upon EBV infection and they in turn enhance the activity of the viral nuclear antigen EBNA2, suggesting roles in EBV-induced immortalization and tumorigenesis (Rainio et al., 2005). In addition, KSHV infection has been shown to enhance expression of Pim-2 in CD34+ bone marrow cells (Mikovits et al., 2001).

Pim-1-deficient mice generated by gene targeting did not show a pronounced phenotype except subtle changes in cytokine signaling. A triple knock out mouse of all three Pim genes was found to be viable and fertile but still lacks a strong phenotype save for a reduction of body size and moderate defects in growth factor signaling and T-cell proliferation (Mikkers et al., 2004). These *in vivo* results suggest the suitability of Pim kinases as drug targets in therapeutic measures.

2. Tumor microenvironment

In past decades, research on the biology of cancer has mainly focused on the tumor cells themselves. Accumulation of mutations gives the cells the ability to grow out of control, move out and metastasize at distant sites. However, the switch from a normal to malignant state is driven not just by what is happening within the tumor cell itself but also by what is happening around it. More recently tumors have gradually been regarded as complex

organs in which multiple specialized cells, components and molecules that surround the tumor area construct the ‘‘tumor microenvironment’’ influencing the course of multistep tumorigenesis. A set of cell types and components in the tumor microenvironment are described in Figure 1 as important contributors in the process of tumor progression (Lorusso and Ruegg, 2008).

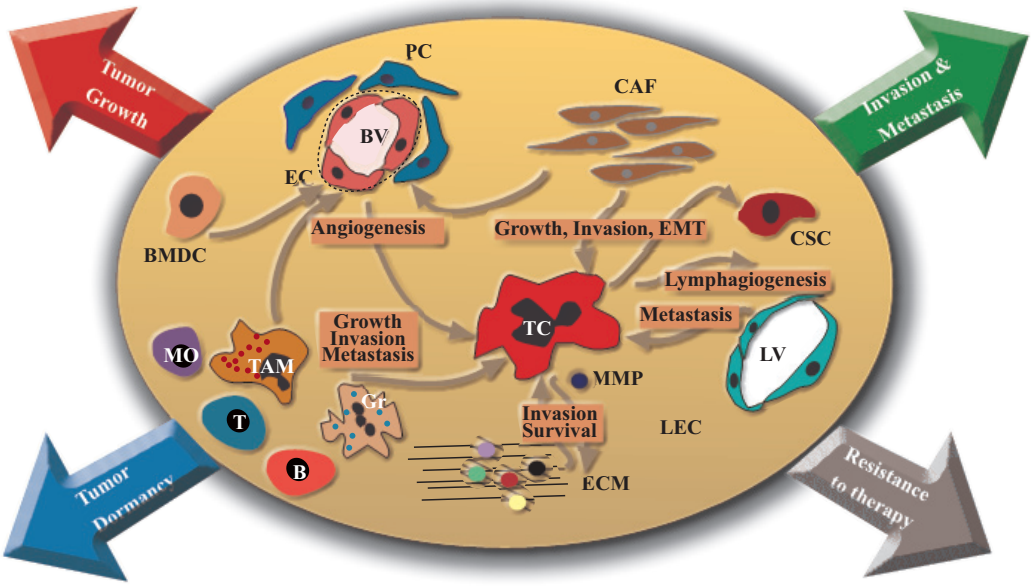

Figure 1. Heterotypic cellular interactions in Tumor microenvironment. Tumor cells orchestrate the modification of the microenvironment by attracting or activating many non-tumoral cells and in turn, tumor microenvironment promotes tumor progression by stimulating tumor growth, survival and angiogenesis, and facilitating invasion and metastasis via EMT and maintenance of CSCs. The interaction between tumor cells and microenvironment determine the outcome of tumor progression: tumor growth, tumor dormancy, tumor invasion and metastasis and resistance to therapy. Abbreviations: B, B lymphocyte; BMDC, bone marrow-derived cells; BV, blood vessel; CAF, cancer associated fibroblast; EC, endothelial cell; ECM, extracellular matrix; EMT, epithelial to mesenchymal transition; Gr, granulocyte; LEC, lymphatic endothelial cell; LV, lymphatic vessel; MMP, matrix metalloproteinases; Mo, monocyte; CSC, cancer stem cell; PC, pericyte; T, T lymphocyte; TAM, tumor associated monocyte/macrophage; TC tumor cells (Adapted and modified from Lorusso and Rüegg, 2008).

2.1. EMT/EndMT in the modulation of tumor microenvironment

2.1.1. The concept of EMT/EndMT

During embryonic development, epithelial cells must escape the structural constraints imposed by tissue architecture and adopt a phenotype more amenable to cell movement, a phenomenon known as the epithelial to mesenchymal transition (EMT). As a process

of cellular plasticity by which differentiated epithelial cells undergo a phenotypic conversion that gives rise to the matrix-producing fibroblasts/mesenchymal cells, EMT is increasingly recognized as an integral player of tissue fibrogenesis after injury and initiating invasive and metastatic behavior in cancer cells.

Under normal organ development or pathological conditions such as cancer and cardiac fibrosis, endothelial cells may undergo a special form of EMT called endothelial-to-mesenchymal transition (EndMT) (Zeisberg et al., 2007). The cells undergoing EndMT exhibit a wide range of phenotypic variability and plasticity depending on their location and function (Chi et al., 2003). Besides being a crucial contributor during vascular development and remodeling (Arciniegas et al., 2007; Kizu et al., 2009), EndMT in the tumor microenvironment was recently identified as a candidate source of cancer-associated fibroblasts (CAFs) which may be essential to facilitate tumor progression (Potenta et al., 2008; Zeisberg et al., 2008).

2.1.2. Phenotypic markers of EMT/EndMT

Compelling cell-biological and molecular changes have been observed during the EMT/EndMT program, being loosely defined into three cellular phenotypes (Boyer et al., 2000; Hay, 1995): (1) morphological changes from a cobblestone-like monolayer of epithelial/endothelial cells with an apical-basal polarity to depolarized, spindle-shaped mesenchymal cells with migratory protrusions; (2)biomarker changes, such as loss of cell–cell junction markers and certain epithelial/endothelial markers, and gain of mesenchymal markers and (3) the functional changes associated with the conversion of stationary cells to motile ones that can invade through the extracellular matrix.

In recent years, changes in the expression levels of a variety of biomarkers have been increasingly used to monitor EMT. The cadherin switch is one of the most important EMT biomarkers, where E-cadherin is switched into other types of cadherin such as N-cadherin during embryonic development and cancer progression, or OB-cadherin (OB for osteoblast) during fibrogenesis (Strutz et al., 2002; Zeisberg and Neilson, 2009). β-catenin is a cytoplasmic protein linking cadherins to the cytoskeleton, which is translocated from cell membranes to cytoplasm upon EMT, reflecting its dissociation from E-cadherin due to the cadherin switch (Brabletz et al., 1998). The tight junction protein zonula occludens 1 (ZO-1) is found to be downregulated on the cell membrane and relocalized to the cytoplasmic compartment upon EMT (Zeisberg and Neilson, 2009). Another EMT-linked process on cell surface is so called integrin switch, where various integrins are expressed on epithelial/endothelial and mesenchymal cells in a context-dependent manner (Bates and Mercurio, 2005; White and Muller, 2007).

Several cytoskeletal markers also reflect changes upon EMT, including FSP1 (a prototypical fibroblast marker belonging to Ca^{2+}-binding S100 protein family) (Iwano et al., 2002; Zeisberg and Neilson, 2009), vimentin (intermediate filament expressed in various cells, including fibroblasts, endothelial cells, cells of the hematopoietic lineages,

and glial cells) (Boyer et al., 1989; Colucci-Guyon et al., 1994; Raymond and Leong, 1989), and α-SMA (alpha smooth muscle actin, one of six actin family members expressed prominently in vascular smooth muscle cells, myofibroblasts and myoepithelial cells) (Damonte et al., 2007; Nakajima et al., 1997; Sarrio et al., 2008).

A few extracellular proteins serve as EMT markers, of which fibronectin appears to be a universal extracellular scaffold glycoprotein upregulated in various types of EMT (Duband and Thiery, 1982; Hynes et al., 1982; Zeisberg et al., 2001). Of the principal basement membrane constituents, laminin-1 is downregulated during EMT (Miner and Yurchenco, 2004; Zagris et al., 2005), whereas an increasing level of laminin-5 is associated with invasive cancers cells undergoing EMT (Carpenter et al., 2008; Giannelli et al., 2005; Marinkovich, 2007). EMT biomarkers also contain signaling molecules involved in biological processes. The number of environmental EMT stimulating factors containing growth factors, ECM constituents, proteases, and hypoxia is increasing. In addition, EMT-associated downstream factors include a panel of transcription factors, such as Twist, HMGA2, LEF1, Ets-1, FTS-1, CBF-A, KAP-1, Snail family members of zinc finger proteins (Snail1, Snail2, and Snail3), and forkhead box family (such as FOXC2), which behave as potential key regulators of various signaling pathways to control the EMT response (Zeisberg et al., 2007).

2.1.3. EMT/EndMT signaling pathways in cancer progression

In cancer cells, multiple signaling pathways, including those triggered by different members of the transforming growth factor-β (TGF-β) superfamily, Wnt, Notch, EGF, HGF, FGF, HIF, and many others induce common EMT-inducing transcription factors and in particular E-cadherin repressors associated with cell migration and invasion (Jing et al., 2011; Thiery and Sleeman, 2006; Yang and Weinberg, 2008) (see Figure 2).

The TGF-β signaling pathway has been implicated as a major EMT signaling pathway induced during cancer progression (Medjkane et al., 2009). Members of the TGF-β superfamily can activate and phosphorylate Smad2 and Smad3, which then form heteromeric complexes with Smad4 and translocate into the nucleus to control multiple EMT-inducing transcription factors through interaction with specific binding motifs in their gene regulatory regions, such as Snail, Slug, Zeb, SIP1 (Derynck and Zhang, 2003). TGF-β also directly activates various types of non-Smad signaling in certain types of tumor microenvironment, including Ras/ERK, JNK, PI3K, Par6, Cdc42 GTPases, playing important roles in TGF-β-induced EMT (Miyazono, 2009; Ozdamar et al., 2005). In addition, the TGF-β pathway is known to interact with Notch, Wnt, Hh and receptor tyrosine kinase signals to generate a complex, context dependent, and reflective network required for EMT in various tumor progression steps (Guo and Wang, 2009).

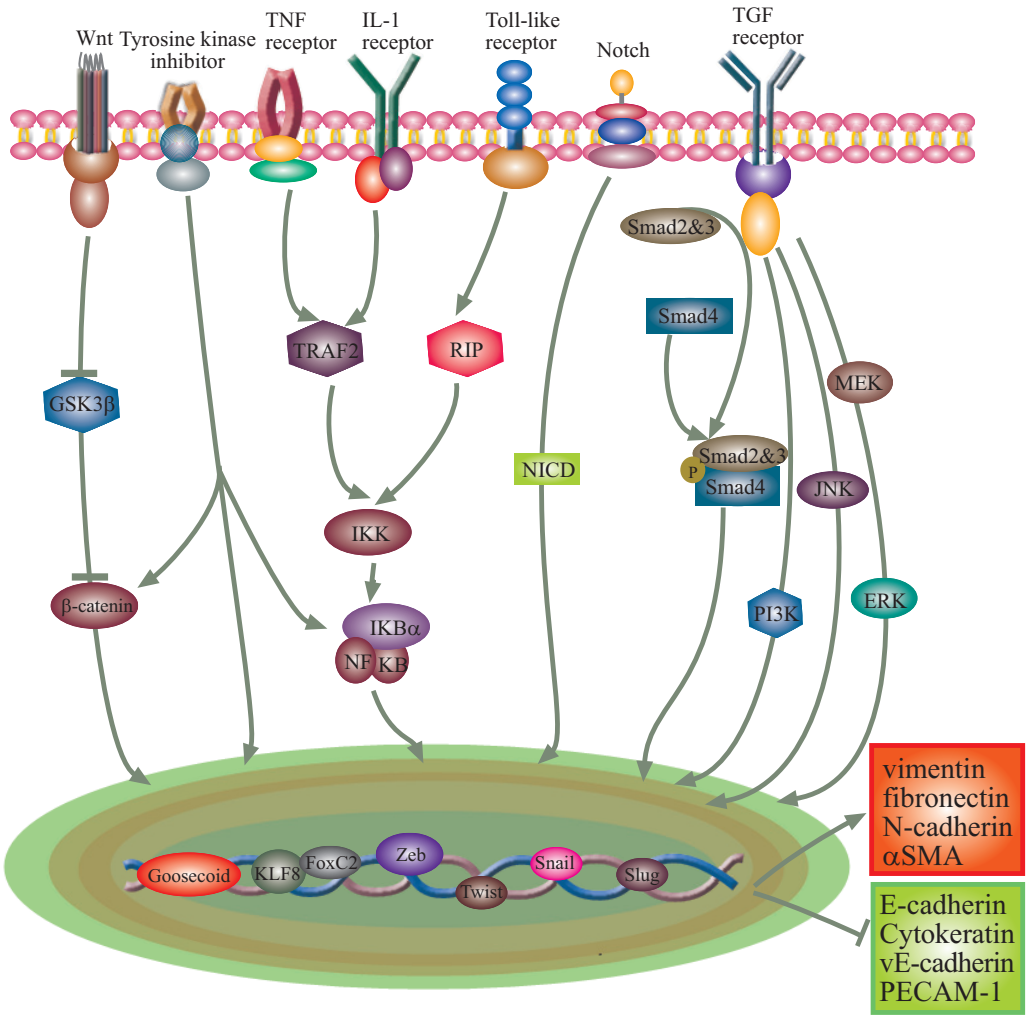

Figure 2. Signaling pathways that orchestrate EMT in tumor microenvironment. In various tumor models *in vitro* and *in vivo*, EMT-inducing signals arising from the tumor-associated stroma lead to an EMT-related signaling network including TGF-β, Notch, Wnt, NF-κB, tyrosine kinases and others. These signalling pathways appear to be responsible for the induction or functional activation of a series of EMT-inducing transcription factors such as Snail, Slug, Zeb, Twist, Goosecoid, KLF8 and FOXC2. These transcription factors therefore change the global gene expression pattern of cells with a distinct phenotype and tissue function to facilitate movement of cells, invade local tissue, and enable genetically unstable cancer cells to acquire a mesenchymal phenotype to facilitate local and systemic dissemination (Adapted and modified from Jing et al., 2011).

Both Wnt/β-caternin and Notch pathway have been implicated in modulating the EMT program during embryogenesis (Timmerman et al., 2004; Zavadil et al., 2004), and are also emerging as important regulators of EMT in carcinoma cells, as well as the maintenance of stemness properties of cancer stem cells (Malanchi et al., 2008).

In canonical/β-catenin Wnt signaling, Wnt bind to Frizzled (Fzd), leading to β-catenin accumulation within the cell nucleus, where it interacts with the lymphoid enhancer factor/T cell factor (Lef/Tcf) complex leading to targeted gene transcription, including several EMT-inducing transcription factors such as Slug, Twist1, and Goosecoid. Inhibition of Wnt signaling can block EMT transcription factors and promote epithelial differentiation.

The Notch pathway is a highly conserved cell signaling system present in most multicellular organisms. In humans, a diverse group of ligands from the Delta (-1, -3 and -4) and Serrate/Jagged families (-1 and -2) recognize the extracellular domain of four Notch receptors (Notch 1, 2, 3, 4), inducing proteolytic cleavage through a disintegrin and metalloprotease (ADAM) followed by γ-secretase cleavage of receptors, and releasing intracellular domain (NICD). Once released from the membrane receptor, NICD translocates to the nucleus where it interacts with the CSL transcription factor to activate the Notch target genes such as HES, HEY, as well as EMT transcription factors such as Snail1 and Snail2 (High et al., 2007; Leong et al., 2007; Niessen et al., 2008; Pannuti et al., 2010). A relationship between overexpression of Notch signaling and poor overall survival in breast cancer patients has been reported (Reedijk et al., 2005). Interestingly, Notch directly promotes Snail1 activation during hypoxia through the binding of its processed intracellular form to the Snail promoter and the activation of Lox2 expression by the hypoxia factor 1 (HIF-1), thereby stabilizing the Snail1 protein (Sahlgren et al., 2008). However, unlike the TGF-β and Wnt pathways, activation of the Notch signaling pathway is not conserved among all the EMT processes during the course of embryogenesis and therefore may require other coordinating signaling inputs in order to promote an EMT.

In addition, multiple tyrosine kinase receptor pathways, including FGFR, EGFR, PDGFR, IGFR, HGFR and their ligands, can induce the expression of Snail, Slug and Zeb in tumor cells via crosstalk with other major signaling pathways, leading to their rapid induction and execution of the EMT program (Grotegut et al., 2006; Thiery et al., 2009). In the tumor environment, the inflammatory cytokines (TNF-α, IL-6, LPS) and reactive oxygen species (ROS) under oxidative stress or hypoxia can induce the NFκB pathway (Huber et al., 2004), which activates the expression of potent EMT inducers (Julien et al., 2007; Strippoli et al., 2008; Wu and Zhou, 2008). Recent studies have established the role of microRNAs in regulating EMT inducers (Cano and Nieto, 2008; Gibbons et al., 2009; Huang et al., 2008; Ma et al., 2007). A regulatory cascade involving microRNAs and EMT transcriptional regulators is likely to contribute significantly to the progression of many human cancers (Burk et al., 2008).

2.2. Cancer-Associated Fibroblasts

Fibroblasts are associated with cancer cells at all stages of cancer progression, and the ‘activated’ fibroblasts within the tumor stroma have been termed cancer-associated fibroblast (CAFs), which often acquire phenotypes including the production of growth

factors, chemokines and extracellular matrix to facilitate the angiogenic recruitment of endothelial cells and pericytes into tumor stroma regions (Liotta and Kohn, 2001). CAFs are therefore a key determinant in the malignant progression of cancer and are attracting increasing attention both as recipients and as producers of pro-tumorigenic signals.

Although there are diverse subtypes of CAFs in different tumor types with specific functions, currently CAFs are mainly characterized based on morphological characteristics or the expression of a combination of different markers such as α-smooth-muscle actin (αSMA), vimentin, desmin, fibroblast-activated protein (FAP), fibroblast-specific protein-1 (FSP1/S100A4), neuron-glial antigen-2 (NG2) and PDGFbeta receptor (PDGFR-β). Recent CAF gene expression profiling analysis have identified a series of novel markers including cell surface proteins, secreted growth factors and extracellular matrix proteins (Yang et al., 2008). Experimental data indicate that cultured CAFs, due to genetic or epigenetic alterations, retain at least partially these phenotypic features during *in vitro* culture in the absence of tumor cells (De Wever et al., 2008; Garin-Chesa et al., 1990; Ronnov-Jessen et al., 1996).

Local fibroblasts or fibroblast precursors in tumor stroma regions, stimulated by the PDGF or TGF-β family members, have generally been considered as the major source of CAFs (Hagood et al., 2005; Kalluri and Zeisberg, 2006; Koumas et al., 2003). However, recent studies have suggested that CAFs can also be derived from malignant or normal epithelial cells via the EMT process (Thiery, 2003; Thiery et al., 2010). Endothelial cells undergoing EndMT were recently identified as another source of CAFs, expressing both endothelial marker (CD31) and CAF markers (αSMA or FSP1) in experimental tumor model systems (Di Tommaso et al., 2003). Besides EMT/EndMT origin, host fibroblasts or bone marrow-derived cells can be recruited into the developing tumor region as an additional source of CAFs, altering the tumor microenvironment by several means, e.g. by secreting paracrine factors or directly influencing the behavior of many different cell types within the tumor (Direkze et al., 2004; Ishii et al., 2003).

Activated CAFs can upregulate the expression of metalloproteinases (MMPs) and other extracellular matrix (ECM)-degrading proteases (Boire et al., 2005; Mackie et al., 1987; Sternlicht et al., 1999; Stetler-Stevenson et al., 1993) to allow cancer cells crossing tissue boundaries and escaping the primary tumor site. Additionally, CAFs express a range of growth factors and cytokines to stimulate tumor cell survival, proliferation, migration and invasion (Ostman and Augsten, 2009). The tumor promoting activities of CAFs have best been defined by transplantation of CAFs admixed with cancer cells into mice, and more recently by genetic and pharmacologic perturbation of their functions in tumor-prone mice (Rasanen and Vaheri, 2010).

2.3. Inflammatory and immune cells

The abundance of inflammatory and immune cells in tumor tissue was observed a long time ago, and these cells have been revealed to play conflicting roles in tumor initiation,

progression and metastasis. Although immunologists have confirmed the presence of tumor-antagonizing inflammatory and immune cells such as Cytotoxic T Lymphocytes (CTL) and Natural-killer Cells (NK), in most if not all neoplastic lesions, evidence has accumulated in the last two decades demonstrating that the regional infiltration of immune or inflammatory cells may serve to promote tumor progression (Grivennikov et al., 2006; Karin et al., 2006). The balance between conflicting tumor-antagonizing and tumor-promoting immune/inflammatory responses in tumors is likely to prove instrumental in prognosis and, quite possibly, in therapies designed to redirect these cells toward tumor destruction.

Cancer cells overexpress a variety of growth factors such as granulocyte colony-stimulating factor (G-CSF), granulocyte–macrophage colony-stimulating factor (GM-CSF), and colony-stimulating factor 1 (CSF1) that recruit haematopoietic cells such as lymphocytes, monocytes (macrophages), and neutrophils into the tumor vicinity. The list of tumor-promoting inflammatory cells now contains subtypes of 'alternatively activated' macrophages, mast cells, neutrophils, as well as T and B lymphocytes that are normally engaged in wound healing and tissue regeneration. These cells can express diverse effector molecules to sustain tumor angiogenesis, to stimulate cancer cell proliferation, to facilitate tissue invasion, and to support the metastatic dissemination and seeding of cancer cells (Coffelt et al., 2010; Egeblad et al., 2010; Mantovani, 2010; Murdoch et al., 2008; Qian and Pollard, 2010). In addition, subclasses of B and T lymphocytes may facilitate the recruitment, activation, and persistence of such tumor-promoting macrophages and neutrophils (Biswas and Mantovani, 2010; DeNardo et al., 2010; Egeblad et al., 2010). Interestingly, a variety of partially differentiated myeloid progenitors have been identified in stroma regions (Murdoch et al., 2008) with demonstrable tumor-promoting activity. Of particular interest, certain myeloid cells may help to evade immune destruction of tumors, indicated by identification of tumor-infiltrating CD11b+ Gr1+ myeloid cells capable of suppressing CTL and NK cell activity (Ostrand-Rosenberg and Sinha, 2009; Qian and Pollard, 2010).

2.4. Cancer Stem Cells

In recent years, evidence has accumulated pointing to the existence of a specific subpopulation of tumor cells possessing stem cell properties, termed cancer stem cells (CSCs). CSCs were initially identified in hematopoietic malignancies (Bonnet and Dick, 1997; Reya et al., 2001) and later in some solid tumors, in particular breast carcinomas and neuroectodermal tumors (Al-Hajj et al., 2003; Gilbertson and Rich, 2007), which share transcriptional profiles with normal stem cells in the tissue-of origin, motivating their designation as stem-like (Creighton et al., 2009; Singh and Settleman, 2010). CSCs could arise from existing stem/progenitor cells or from dedifferentiation of terminal differentiated cells into a primitive stem cell-like state. Recent research has connected the EMT transdifferentiation program with the acquisition of CSC traits. Studies indicate that immortalized human mammary epithelial cells that have undergone EMT driven

by TGF-β treatment or by forced expression of E-cadherin transcriptional repressors, such as Snail, give rise to a CD44high, CD24low CSC-like population (Mani et al., 2008; Morel et al., 2008; Singh and Settleman, 2010). These EMT-induced cancer stem cells are proposed to become migratory in the circulation and revert into a differentiated phenotype when they arrive at a suitable distant organ, seeding the bulk of secondary tumor mass (Brabletz et al., 2005).

2.5. Angiogenesis and lymphangiogenesis

In order to meet increasing needs of oxygen and nutrients as well as efficiently remove metabolic wastes, normally quiescent vasculature gets angiogenically activated to continuously sprout new vessels during tumor growth (Hanahan and Folkman, 1996). New blood-vessel formation, maturation and recruitment of perivascular cells will continue as long as the tumor grows, and the blood vessels specifically feed hypoxic and necrotic areas of the tumor to provide it with essential nutrients and oxygen. Eventually, the sustained angiogenesis leads to a high number of leaky blood vessels infiltrating the tumor tissue, promoting not only tumor growth, but also progression from a premalignant to a malignant and invasive tumor phenotype (Mueller and Fusenig, 2004).

A compelling variety of evidence indicates that angiogenesis is orchestrated by countervailing factors that either activate or inhibit this process (Baeriswyl and Christofori, 2009; Bergers and Benjamin, 2003). The stimulatory angiogenic regulators are mostly receptor tyrosine kinase ligands (Carmeliet and Jain, 2000), such as VEGF, fibroblast growth factors (FGF), PDGF and epidermal growth factor (EGF) (Baeriswyl and Christofori, 2009). Among these signaling pathways, VEGF ligands (VEGF-A, VEGF-C and VEGF-D) are well known to regulate the growth of new blood vessels via three receptor tyrosine kinases (VEGFR1-3) at multiple levels (Hanahan and Folkman, 1996).

In contrast to capillary blood vessels, lymphatic capillaries are lined by a single layer of terminal differentiated LECs, which have poorly developed junctions with frequent large inter-endothelial gaps. A number of lymphatic endothelium-specific markers have been discovered including the prospero-related homeodomain transcription factor Prox1, the membrane glycoprotein podoplanin, vascular endothelial growth factor receptor-3 (VEGFR-3), and lymphatic vessel hyaluronan receptor-1 (LYVE-1). However, LECs also express blood endothelium markers such as von Willebrand factor (vWF) and CD31 (platelet/endothelial cell adhesion molecule 1, PECAM1) (Dejana et al., 2009). The main function of lymphatic vasculature is to drain interstitial fluid from tissues and returns it to the blood circulation. Lymphatic vessels are also an essential component of the immune system, and play important roles in the pathogenesis of lymphoedema, cancer and a number of immune/ inflammatory diseases (Schulte-Merker et al., 2011).

Similar to angiogenesis, lymphangiogenesis is *de novo* lymphatic formation from pre-existing lymphatic vessels. Lymphangiogenesis can be induced by VEGF-C/D

and their receptor VEGFR-3. The VEGF-C/D coreceptor neuropilin-2 (Nrp2) and the Eph4 tyrosine kinase ligand ephrinB2 are required for efficient sprouting of lymphatic capillaries (Tammela and Alitalo, 2010). Although VEGF-C and VEGF-D also activate VEGFR-2 and thus induce angiogenesis, another VEGFR-2 activator, VEGF-A, cannot substitute for VEGF-C/D in promoting efficient lymphatic sprouting unless the Notch pathway activity is inhibited (Alitalo et al., 2005; Ridgway et al., 2006; Zheng et al., 2011). Tumor lymphangiogenesis is thought to be correlated with the formation of metastasis in regional lymph nodes. A number of reports have recently described a significant link between the levels of the lymphangiogenic factor VEGF-C in primary tumors and lymph node metastases. Larger lymphatics have been found surrounding the VEGF-C overexpressing tumors, which may be sufficient to increase metastasis, although the respective roles of lymphangiogenesis and lymphatic hyperplasia in tumor dissemination need to be explored in the future (Mumprecht and Detmar, 2009; Pepper, 2001; Stacker and Achen, 2008).

2.6. Matrix metalloproteinases

Matrix metalloproteinases (MMPs) are a family of zinc-dependent enzymes that were initially identified as the ECM cleaver but have subsequently been found to closely associate with many changes in the microenvironment during tumor progression (Kessenbrock et al., 2010). Based on the differential domain structure, MMPs can be principally divided into secreted (MMP-1, -2, -3, -7, -8, -9, -10, -11, -12, -13, -19,-20, -21, -22, -27, -28) and membrane-anchored proteinases (MT1-MMP to MT6-MMP, also called MMP-14, -15,-16, -17, -23, -24, -25, respectively) (Kessenbrock et al., 2010). The activity of MMPs depends on the local balance between them and their physiological inhibitors. The most important physiological inhibitors of MMP activity are tissue inhibitors of metalloproteinases (TIMPs), which are also commonly expressed in tumor sites (Deryugina and Quigley, 2006), forming 1:1 stochiometric complexes with active MMPs and thus inhibiting their proteolytic activity. The different types of stromal cells produce a specific set of proteinases and proteinase inhibitors, which are released into the extracellular space and create diverse protease activities in the tumor microenvironment (Ardi et al., 2007).

Almost all stages of tumor progression and metastasis are modulated by MMPs and other extracellular proteinases, mainly provided by non-malignant, infiltrating stromal cells such as neutrophils, macrophages, fibroblasts or endothelial cells (Kessenbrock et al., 2010). During tumor invasion, cancer cell intravasation into blood vessels and the egress of metastatic tumor cells into the circulation, MMPs remodel the composition of the ECM by degrading the basement membrane and other ECM proteins, or downregulate cell adhesion molecules, such as CD44 or E-cadherin (Radisky, 2005; Yu and Stamenkovic, 2000), thereby acting to support tumor progression. Elevated levels of MMPs in the tumor microenvironment can directly induce EMT in epithelial cells (Billottet et al., 2008; Radisky et al., 2005). Furthermore, EMT can generate activated stromal-like cells

that drive cancer progression via further MMP production. Studies of MMP localization in human tumors have shown that tumor progression and poor prognosis are associated with stromal expression of MMP-1, MMP-7, and MMP-12 (Finak et al., 2008), and with fibroblast-specific production of MMP-9, MMP-11, and MT1-MMP (Del Casar et al., 2009; Vizoso et al., 2007). Although extracellular proteolysis is mostly implicated in cancer promotion, MMPs also exhibit tumor-suppressing effects in several circumstances. For instance, angiogenesis can be triggered by the release of pro-angiogenic factors such as VEGF, which is mainly cleaved by MMP9 to release the active product (Bergers et al., 2000), suggesting a beneficial effect of MMP inhibitors on tumor angiogenesis. However, in other cancer models, MMP-9 provides anti-angiogenic signals, resulting in increased tumor growth in MMP-9-deficient mice (Hamano et al., 2003). This illustrates that the same MMP can have opposing effects in different tumor types and underscores a careful evaluation of MMP inhibitors for each specific kind of cancer.

Similar to other MT-MMPs, proMT1-MMP (64 kDa) is converted to a catalytically active MT1-MMP enzyme (55 kDa) by proteolytic cleavage by furin in the trans-Golgi network prior to its arrival at the plasma membrane (Mazzone et al., 2004; Yana and Weiss, 2000). Activation of the secreted MMPs is mediated by a cell-surface complex that consists of a homodimer of MT1-MMP as well as a single molecule of tissue inhibitor of metalloproteinases-2 (TIMP-2; a natural inhibitor of MMPs) (Itoh et al., 2001; Strongin et al., 1995). In addition, MT1-MMP also directly cleaves ECM components including collagen, gelatin, laminins 1 and 5, fibronectin, vitronectin, aggrecan and fibrin, and cell-surface proteins such as CD44, αV integrins and syndecan 1 (Overall and Dean, 2006). MT1-MMP promotes tumor progression and metastasis (Deryugina and Quigley, 2006; Hofmann et al., 2005) as well as tumor growth and local invasion in a confined three-dimensional (3D) ECM environment (Hotary et al., 2003; Lehti et al., 2009). Intriguingly, MT1-MMP was shown to accumulate at the invasive front of tumors (Hofmann et al., 2003; Ueno et al., 1997) and to be enriched at invadopodia, facilitating matrix degradation (Bowden et al., 1999; Buccione et al., 2004; Clark and Weaver, 2008; Gimona et al., 2008). Indeed, poor prognosis of EMT-driven tumor progression is associated with fibroblast-specific production of MT1-MMP (Vizoso et al., 2007). Furthermore, MT1-MMP contributes to tumor angiogenesis via various mechanisms, including activation of αvβ3-integrin (which protects endothelial cells from apoptosis), fibrinolytic activity, MMP-2 activation and the transcriptional regulation of VEGF expression (Mu et al., 2002; Tatti et al., 2008). MT1-MMP has also been identified as a novel proteolytic modifier of PDGFR-β signaling pathway in regulating vessel wall architecture (Lehti et al., 2005).

3. Kaposi's sarcoma herpesvirus (KSHV)

Gammaherpesviriae family consists of two subfamilies called Lymphocryptovirus (e.g., EBV) and Rhadinovirus (e.g., KSHV) (Cesarman et al., 1995a). Kaposi's

sarcoma herpesvirus (KSHV), also known as human herpesvirus-8 (HHV-8), was identified in 1994 from Kaposi's sarcoma biopsies through representational difference analysis (Chang et al., 1994). KSHV is a double-stranded DNA virus, representing an etiological agent of three types of malignancies: Kaposi's sarcoma (KS), Multicentric Castelman Disease (MCD), and B cell primary effusion lymphoma (PEL) (Ablashi et al., 2002).

3.1. KSHV-associated malignancies

Most of sub-Saharan Africa regions have more than 50% overall seroprevalence rates of KSHV infection, followed by the Mediterranean region with an intermediate seroprevalence rate of 10–30%. The KSHV seroprevalence in northern Europe, Asia and the United States is usually quite low, with less than 10% rates (Mesri et al., 2010).

3.1.1. Kaposi's sarcoma (KS)

In 1872, the Hungarian doctor Moritz Kaposi found a new type of skin neoplasms consisting of red and purple plaques or nodules described as the term "sarcoma", indicating the similarities between this disease and traditional mesenchymal tumors. According to epidemiologic distinctions, KS has been classified into four different types. Classic KS is usually an indolent disorder in Mediterranean elderly men, with no link to HIV infection (DiGiovanna and Safai, 1981). The lesions progress slowly and are generally confined to the skin, rarely threatening life. Endemic KS is a more aggressive disease than classic KS, affecting HIV negative sub-Saharan Africans (Bayley, 1984; Stein et al., 1994; Taylor et al., 1971). Iatrogenic KS involves immunosuppressed patients undergoing solid-organ transplantation (Cattani et al., 2000; Farge, 1993; Farge et al., 1999; Margolius et al., 1994; Parravicini et al., 1997; Qunibi et al., 1998; Shepherd et al., 1997). The most common type of KS is AIDS-associated KS, also called "epidemic" KS, which is a highly aggressive and disseminated cutaneous malignancy. The early stage of KS lesions on the skin is often the first and most common symptom in AIDS patients, especially amongst the the homosexual population. These lesions can spread to large areas of the body surface, sometimes in a symmetrical fashion. Advanced complications involve the oral mucosa and visceral organs including the gastrointestinal tract and lungs, leading to life-threatening gastrointestinal bleeding or respiratory failure (Beral et al., 1990; Biggar and Rabkin, 1996; Hermans et al., 1998).

KS lesions are composed of a remarkable diversity of cell types, which are different from histologically monotonous clonal outgrowths of a single cell type in most cancers (Grayson and Pantanowitz, 2008; Herndier and Ganem, 2001). Early-stage patch KS lesions are composed of elongated, spindle-shaped cells accompanied by an infiltration of inflammatory cells (T and B cells, monocytes). Prior to establishment of recognizable tumor mass, abundant neovascularity has been prominent at this stage, making the lesions reddish to the naked eye. As the lesions progress to the plaque stage, the vascular

process expands through the skin to form intense red or even violacerous color. With the continuous proliferation of spindle cells, late nodular stage KS is composed of prominent multiple layers of spindle cells and slit-like neovascular spaces, accompanied again by inflammatory cells (Ganem, 2010).

Spindle cells are the primary infected cell type in KS lesions and are considered to be the tumor cells of KS (Boshoff et al., 1995). These cells bear many markers of the endothelial lineage, including CD31, CD34, CD36, and factor XIII (Rutgers et al., 1986). However, the endothelial origin of these cells is not conclusive due to the heterogeneous pattern of marker expression, such as those of smooth muscle cells and fibroblasts, by the spindle cells, suggesting a potential mesenchymal origin of these cells (Ganem, 2010). Lymphatic makers (e.g., podoplanin and LYVE-1) as well as the lymphangiogenetic factor VEGF-C and its receptor VEGFR-3 were found to be expressed on spindle cells, indicating that they could be derived from a lymphatic rather than vascular endothelium (Marchio et al., 1999; Skobe et al., 1999; Weninger et al., 1999). Interestingly, global gene expression analyses have demonstrated that the KS gene expression signature resembles more that of LECs than blood endothelial cells (BECs). KSHV can drive BECs to shift their gene expression towards a lymphatic lineage, while infected LECs alter transcriptional patterns toward vascular endothelium, indicating a role of transcriptional reprogramming to explain the pleiotropy of marker expression in spindle cells (Carroll et al., 2004; Hong et al., 2004; Wang et al., 2004a).

Another histological complexity of KS is the occurrence of multifocal lesions, which often arise independently (multicentricity) from a primary lesion (Brooks, 1986). Molecular analysis of multiple KS lesions confirms the genomic differences of KSHV in different lesions, suggesting that they arise from independent infection events (Duprez et al., 2007). In addition, the inflammatory microenvironment constitutes an important part of KS histology. Spindle cells in KS lesions produce cytokines (IFN-γ, TNF-α, IL-1, IL-6), chemokines (MCP-1, IL-8), angiogenic factors (FGF, VEGF, PDGF), and display upregulation of TGF-β, PDGF-β, CCR5 as well as their receptors, which provide proinflammatory factors, proangiogenic factors, growth factors and other substances necessary for spindle cell survival and proliferation (Mesri et al., 2010).

3.1.2. Primary effusion lymphoma (PEL) and other KSHV-induced disorders

After the discovery of KSHV in 1994, PEL was recognized as a unique type of diffuse large B-cell lymphoma commonly found in AIDS associated KS patients as well as HIV negative organ-transplantation patients (Ansari et al., 1996; Carbone et al., 1996; Cesarman et al., 1995a; Nador et al., 1996). This type of malignant lymphoma is caused by KSHV, and in most cases, the lymphoma cells are co-infected with EBV, suggesting that EBV could be an important pathogenic co-factor (Cesarman et al., 1995b; Nador et al., 1996). As a rare, aggressive (fast-growing) type of non-Hodgkin lymphoma, PEL tumors consist of a malignant counterpart of B-cells that have matured but have

not undergone terminal plasma cell differentiation (Carbone et al., 1997; Gaidano et al., 1996). PEL is also called "body cavity lymphoma" since the majority of cases arise in body cavities, such as the peritoneal, pericardial or pleural space to form malignant effusions without associated solid tumor masses. During the advanced stages of PEL, accumulation of the effusions in certain body cavities cause serious symptoms such as dyspnea (due to pleural or pericardial effusion) or abdominal swelling (due to peritoneal effusion), with the majority of the patients dying within a few months (Boulanger et al., 2003; Boulanger et al., 2005; Boulanger et al., 2004; Waddington and Aboulafia, 2004).

MCD is a very rare disorder characterized by hyperproliferation of certain types of lymphocytes in multiple lymphatic tissues throughout the body. More than 50% of MCD cases are associated with KSHV, while the remaining KSHV negative cases are in hyaline-vascular form of unknown cause (Casper, 2005; Dupin et al., 1999; Gessain et al., 1996; Soulier et al., 1995). MCD progresses rapidly and is difficult to diagnose. Even in the case of a lymph-node biopsy a conclusive diagnosis remains problematic. The most common symptoms of MCD are high fever, anemia, weight loss, low white blood cell counts, lymphadenopathy, and dyspnea, causing high fatality of the patients (Oksenhendler et al., 1996).

3.2. KSHV life cycle

The linear, double-stranded KSHV DNA of approximately 165 kilobases (kb) in the viral capsid circularizes into a closed episome in the nucleus of the host cell upon infection. 140 kb of this DNA contains at least 87 open reading frames (ORFs), flanked on each side by multiple 801-base pair (bp) terminal repeat units (TR) (Wen and Damania, 2010) (see Figure 3). As with other herpesviruses, infection by KSHV displays two life cycle phases known as latency and lytic replication. During latency KSHV persists as extrachromosomal episomal DNA with only few viral genes expressed, while during lytic replication the virus is reactivated with initiation of extensive viral DNA replication and gene expression, resulting in production of new viral particles and contributing to KSHV pathobiology (Mesri et al., 2010; Miller et al., 1997; Renne et al., 1996).

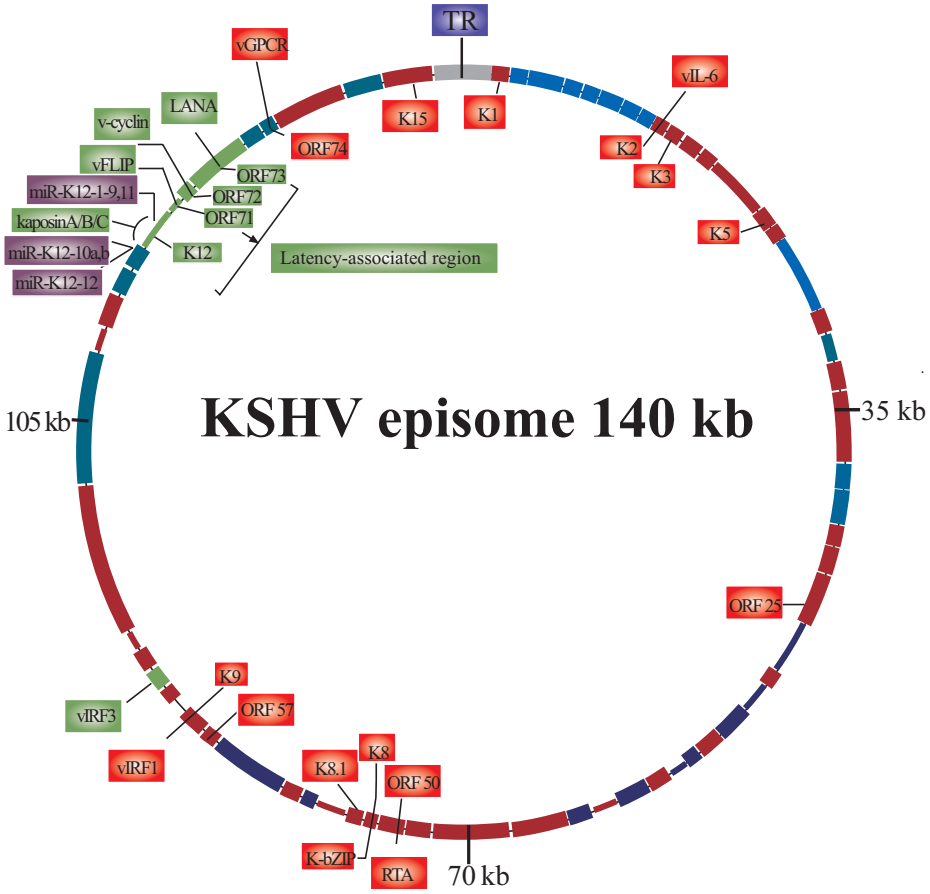

Figure 3. The genome of KSHV with indicated latent and lytic genes. KSHV genome exists as an episome (double-stranded circular DNA) within the host nucleus and it encodes 87 ORFs as well as at least 17 microRNAs, 14 of which are co-expressed as a cluster. Putative latent ORFs and encoded proteins are indicated in green boxes, microRNA clusters are indicated in purple boxes, and lytic genes/encoded proteins mentioned in this thesis are indicated in red boxes. TR (blue box) denotes terminal repeats of the genome (Adapted and Modified from Wen and Damania 2010).

3.2.1. Latency and lytic cycle

During latent infection, KSHV expresses only a minority of the nearly 100 genes in the genome (Zhong et al., 1996). Among these are genes encoding latency-associated nuclear antigen (LANA), viral cyclin (v-cyclin), and anti-apoptotic viral FLICE-inhibitory protein (vFLIP), which are translated from a common polycistronic transcriptional unit called the latency transcript (LT) cluster (Dittmer et al., 1998; Sarid et al., 1998; Talbot et al., 1999). Viral interleukin-6 (vIL-6), which was regarded as a lytic gene, has recently been demonstrated as a bone fide latent protein in primary effusion lymphoma (PEL) cells, supporting PEL growth and survival in an autocrine manner (Chen et al., 2009). In addition, other later genes expressed in the latent phase include Kaposin (Muralidhar

et al., 1998; Sadler et al., 1999), viral interferon regulatory factor 3 (vIRF3, LANA2) (Lubyova and Pitha, 2000) and a recently discoved microRNA cluster (See also in Figure 3) (Cai et al., 2005; Pfeffer et al., 2005; Samols et al., 2005).

The switch from latent infection to lytic replication is called viral reactivation. Several chemicals such as protein kinase C agonists, histone deacetylase (HDAC) inhibitors, phorbol esters, DNA methyltransferase inhibitors or physiological stimuli such as hypoxia can trigger the reactivation program, after which the viral lytic genes start to be expressed in an orderly pattern (immediate early/IE, delayed early/DE and late/L) based on their temporal expression kinetics, leading to the production of infectious virions (See also in Figure 3) (Chen et al., 2001; Davis et al., 2001; Miller et al., 1997; Renne et al., 1996; Vieira et al., 2001). Expression of immediate early (IE) lytic genes is activated upon primary infection or reactivation from latency. Delayed early (DE) genes are expressed after the IE, but prior to viral DNA synthesis, while late lytic genes, whose expression is inhibited by phosphonoacetic acid or ganciclovir, usually appear after 30 hours post-induction (Sun et al., 1999; Zhu and Yuan, 2003). The full execution of the lytic program results in additional replication of viral DNA, transcription of viral genes, translation of viral gene products, assembly and release of viral particles, causing the death of host cells (Lukac et al., 1999; Lukac et al., 1998; Sun et al., 1998).

3.2.2. LANA and latency maintenance

LANA encoded by the open reading frame 73 (ORF73) of the KSHV genome is expressed in all KSHV-infected cells (Gao et al., 1996a; Kedes et al., 1997) and is a major protein expressed in KSHV latency. It is a protein of 1,162 amino acids, 222 to 234 kDa in molecular size, containing an N-terminal domain rich in serine, threonine, proline, and basic residues, a highly polymorphic internal repeat domain and a C-terminal domain abundant in charged and hydrophobic residues (Gao et al., 1999; Zhang et al., 2000). As a multifunctional protein, LANA can promote cell survival and regulates cell cycle progression by modulating various cellular targets, including p53, Rb, and Wnt pathways (An et al., 2005; Friborg et al., 1999; Fujimuro et al., 2003).

During latency, LANA tethers the viral episomal DNA to the cellular mitotic chromosomes, appearing as typical nuclear spots upon indirect immunofluorescence with LANA-specific antibodies (Gao et al., 1996a; Gao et al., 1996b; Kedes et al., 1996). Previous studies have implied that the C-terminus of LANA binds specifically to sequences within the terminal repeat (TR) regions (Garber et al., 2001; Grundhoff and Ganem, 2003; Hu et al., 2002) of the KSHV episomal genome (Schwam et al., 2000; Verma et al., 2006; Viejo-Borbolla et al., 2005). Additionally, Both N- and C-terminal LANA domains bind to host chromosomes, tethering episomes to the nucleosome during mitosis and interphase through interaction with core histones H2A and H2B (Barbera et al., 2004; Barbera et al., 2006; Krithivas et al., 2002; Piolot et al., 2001; Shinohara et al., 2002). Of note, LANA not only tethers the KSHV episome to the host chromosomes but also actively cooperates with a panel of chromosome-binding and origin recognition

complex (ORCs) proteins to facilitate viral episomal replication (Stedman et al., 2004; Stedman et al., 2008; Verma et al., 2006). In addition to its role in episomal maintenance and segregation, LANA also acts as a transcriptional activator or repressor to modulate cellular and viral gene expression, helping to keep the virus in its latency cycle.

3.2.3. RTA and lytic reactivation

The best characterized IE lytic gene is RTA (replication and transcription activator, a homologue of EBV RTA, also called ORF50), which serves as the molecular switch for the viral life cycle (Lukac et al., 1998; Sun et al., 1998). During latency, RTA expression is repressed through epigenetic modifications. It has been suggested that DNA methylation and acetylation may regulate the transcription of the ORF50 gene through chromatin remodelling (Lu et al., 2003). Hypermethylation of the RTA promoter can suppress RTA protein expression as well as recruit transcriptional repressors and HDACs in the promoter region (Chen et al., 2001). In addition, KSHV latent protein LANA can inhibit RTA transcription by suppressing its promoter (Lan et al., 2005a; Lan et al., 2004) or influencing related transcription factors (Lan et al., 2005b; Verma et al., 2004). For instance, by directly interacting with RBPJ (Recombining binding protein for immunoglobulin kappa J region), LANA may not only recruit additional corepressors to suppress the transcription of the RTA gene, but also repress RTA autoactivation activity by competing with RTA in RBPJ-binding (Lan et al., 2005b; Verma et al., 2004).

Several lines of evidence demonstrate that RTA is both sufficient and necessary to mediate the switch from latency to lytic replication of KSHV. Ectopic expression of RTA in latently infected cells can induce the expression of downstream lytic proteins and encapsidated viral DNA, suggesting that RTA alone is capable of initiating reactivation and driving the whole viral lytic cycle cascade (Gradoville et al., 2000; Lukac et al., 1999; Sun et al., 1998). Complete deletion of RTA or expression of a dominant-negative RTA mutant suppresses viral reactivation, further supporting the necessity of RTA in viral lytic reactivation (Lukac et al., 1999; Xu et al., 2005).

As the earliest lytic protein, RTA is able to transactivate a variety of downstream lytic genes to complete lytic replication cascade. The demonstrated RTA downstream targets include IE genes such as K3 (Chang et al., 2005a), K5 (Haque et al., 2000) and ORF45 (Zhu et al., 1999), DE genes such as K1 (Bowser et al., 2002; Bowser et al., 2006), K2/vIL-6 (Deng et al., 2002), K9/vIRF-1 (Ueda et al., 2002), K8 (Lukac et al., 1999), K15 (Wong and Damania, 2006), ORF57 (Lukac et al., 1999), *ORF59*(Chan and Chandran, 2000) ORF74/vGPCR (Kirshner et al., 1999) and others, as well as late (L) lytic genes such as K8.1 (Chandran et al., 1998) and *ORF25* (Ascherl et al., 1999). The activation of these downstream genes is mediated through two distinctive strategies: RTA either directly recognizes RTA-responsive elements in the promoters of its target genes, or it interacts with cellular or viral factors already recruited to the promoters (Chang et al., 2005a; Chang et al., 2005d; Song et al., 2003). As a potent transcriptional activator, RTA protein can also activate the ORF50 gene itself, representing a critical way for

KSHV to efficiently respond to environmental stimuli and maximally activate the virus lytic cycle. A 3-kb fragment of RTA promoter is shown to be highly responsive to the lytic inducing chemical 12-O-tetradecanoyl-phorbol-13-acetate (TPA), sodium byturate (NaB) as well as RTA itself (Deng et al., 2000; Gradoville et al., 2000). Upon a luciferase reporter assay, the RTA response element (RRE) on the RTA promoter was mapped to an octamer-binding site, bound by cellular Oct1 protein in an electrophoretic mobility shift assay (EMSA) (Sakakibara et al., 2001). RBPJ, a known cellular partner of RTA (Liang et al., 2002), is also involved in the autoactivation of RTA promoter. Chang et al. have shown that the RTA promoter with multiple RBPJ-binding sites was autoactivated at a higher level than that lacking RBPJ-binding sites (Chang et al., 2005a). These lines of evidence suggested that autostimulation of the RTA promoter is mediated by the RTA protein and at least two cellular proteins, RBPJ and Oct-1.

3.2.4. Involvement of host cellular signaling pathways in KSHV reactivation

A variety of cellular signaling pathways have been shown to regulate KSHV reactivation. As discussed before, RBPJ, the primary target of Notch signaling, mediates RTA-dependent activation of KSHV lytic genes via binding to multiple RBPJ-binding sites on the RTA promoter and on multiple RTA target promoters, such as ORF57, TK, K14/vGPCR, RTA, K3, K5, vIL-6, and vMIP-1 (Liang et al., 2002). Indeed, in an RBPJ-knockout cell line (Liang and Ganem, 2003), KSHV can still establish latency but RTA-induced lytic reactivation is completely abolished, probably due to the defective expression of multiple lytic genes that depend on RBPJ for activation by RTA. Three MAPK pathways (MEK/ERK, MEK/JNK, and p38 MAP kinase pathways) have been shown to mediate the latent-lytic switch (Cohen et al., 2006; Ford et al., 2006; Pan et al., 2006) through activation of the transcriptional factor AP-1, which associates with the RTA promoter, leading to expression of RTA protein, and to activation of the lytic program (Xie et al., 2008). Moreover, dopamine receptors were reported to be associated with MAPK pathways to detect and transmit favorable stress signals for viral reactivation (Lee et al., 2008). Recently a functional genome-wide cDNA library (containing twenty-six thousand mammalian genes) screen was conducted to systematically evaluate the cellular signals regulating KSHV life cycle through a luciferase reporter system. In the screen, the Raf/MEK/ERK/Ets-1 pathway was identified to mediate Ras-induced KSHV reactivation (Yu et al., 2007b).

The role of the NF-κB pathway in KSHV lytic replication remains controversial. In PEL cells, inhibition of NF-κB led to KSHV reactivation and the expression of lytic proteins (Brown et al., 2003), however, it was reported that NF-κB induction was required for the production of KSHV infectious virions in PEL cells induced for reactivation by TPA (Sgarbanti et al., 2004). Recently, PI3K-Akt pathway has been identified as a negative regulator as inhibition of this pathway enhanced KSHV lytic replication and facilitated reactivation from latency (Peng et al., 2010).

Several host factors have been found to regulate the latent-lytic switch via remodeling of the RTA promoter. For instance, the chromatin-remodeling complex SWI/SNF and the TRAP/mediator coactivator can be recruited downstream of the RTA promoter, which is essential for RTA-dependent viral gene expression (Gwack et al., 2003). Furthermore, two transcription factors, the CCAAT/enhancer-binding protein-α (C/EBPα) (Landschulz et al., 1988; Wang et al., 2003) and the X-box binding protein 1 (Wilson et al., 2007; Yu et al., 2007a) have been shown to transactivate the RTA promoter and enhance lytic reactivation.

3.2.5. Chemical and physiological inducers of KSHV reactivation

Viral reactivation can be induced in cell culture by the addition of a number of chemical agents, providing valuable tools for studying cellular pathways that may be involved in KSHV reactivation. As the RTA promoter is associated with histone deacetylases in latent PEL cells, treatment with inhibitors of histone deacetylase (HDAC) such as NaB (Boffa et al., 1978), or trichostatin A (TSA) can reverse the acetylation process of the RTA promoter and therefore induce KSHV reactivation (Lu et al., 2003). An histone acetyltransferases (HAT) inducer, TPA (Masumi et al., 1999) can also affect the acetylation status of the RTA promoter by activating a variety of transcriptional factors and enhancing their DNA-binding activity (Renne et al., 1996; Sarid et al., 1998; Wang et al., 2004b). Following the initial activation by NaB, RTA can be recruited to its responsive elements through direct interaction with the Notch signaling pathway transcription factor RBPJ (Chang et al., 2005a; Chang et al., 2005b; Liang and Ganem, 2004) while TPA can activate the calcium-dependent calcineurin signaling pathway as well as the protein kinase C (PKC) δ pathway, inducing KSHV reactivation from latency (Deutsch et al., 2004; Zoeteweij et al., 2001). In addition, activation of the Ap-1 pathway has also been suggested to be involved in TPA-induced KSHV reactivation (Wang et al., 2004b). Recently, it was reported that Ras/Raf/MEK/ERK/Ets-1, JNK and p38 MAPK pathways also mediate TPA-induced KSHV reactivation from latency (Xie et al., 2008; Yu et al., 2007b). Induction of KSHV lytic replication has been reported in response to a variety of other chemicals such as ionomycin (Lukac et al., 1999) and 5-azacytidine (Chen et al., 2001) via chromosomal modulation and enhancement of viral release.

The immune and inflammatory systems, consisting of a variety of cellular elements and humoral factors, are the initial host defense against viral pathogens. Several cytokines such as IL-6 (Chang et al., 2000; Chatterjee et al., 2002), IFN-γ (Blackbourn et al., 2000; Chang et al., 2000; Mercader et al., 2000; Monini et al., 1999) and oncostatin M (Mercader et al., 2000) have been shown to induce KSHV reactivation, but the underlying mechanisms remain undefined. Several neuron transmitters associated with stress responses, such as epinephrine and norepinephrine, could efficiently reactivate KSHV from latency via β-adrenergic activation of the protein kinase A (PKA) signaling pathway (Chang et al., 2005c). The cellular IRF-7 was shown to be a negative regulator of KSHV lytic replication by competing with RTA for binding to the RTA-responsive element in the ORF57 promoter (Wang et al., 2005) and in turn RTA, by using its own

E3-ubiquitin ligase activity, targets IRF-7 for proteasome-mediated degradation (Yu et al., 2005). Hypoxia stress, which is usually a physiological stimulus rich in the KS tumor microenvironment, has also been found to reactivate KSHV, probably via cytokines such as IL-6 (Haque et al., 2003). Moreover, when cells are co-infected with KSHV and HIV, the HIV Tat protein may induce KSHV replication and increase KSHV viral load via JAK/STAT signaling (Zeng et al., 2007).

3.2.6. Implications of lytic replication in KSHV pathogenesis

Although persistent KSHV infected cells are predominantly latent (Reed et al., 1998; Staskus et al., 1999; Staskus et al., 1997; Zhong et al., 1996), lytic replication has been suggested to play a key role in the development and progression of KSHV-induced malignancies. KSHV lytic genes such as vGPCR, vIL-6, vBcl-2, K1 and vIRF1 have been shown to have oncogenic potential *in vitro* and/or *in vivo* (Mesri et al., 2010). Indeed, the viral lytic replication is associated with KS malignancy as higher KSHV viral loads are usually detected in higher grade of clinical KS (Ganem, 2010). Moreover, another KSHV malignancy, MCD, is characterized by a periodic KSHV lytic replication with high viremia in the peripheral blood (Katano et al., 2000; Oksenhendler et al., 2000). Therefore, an anti-viral treatment designed to abolish KSHV lytic replication may represent a therapeutic option in KSHV-induced malignancies.

So far, a couple of anti-herpetic DNA synthesis inhibitors have been developed against KSHV associated malignancies (Naesens and De Clercq, 2001). These inhibitors, either via phosphorylation by the herpesvirus thymidine kinase and by UL97 phosphotransferase (aciclovir and ganciclovir) or via an alternative mode of action (Cidofovir and Foscarnet), are able to incorporate a nucleoside analogue into a growing viral DNA and thereby terminate the elongation of DNA synthesis (Mercorelli et al., 2008). A series of reports revealed promising therapeutic outcomes in PEL patients treated with ganciclovir or cidofovir alone or in combination with chemotherapy or Highly Active Anti-Retroviral Therapy (HAART) (Crum-Cianflone et al., 2006; Hocqueloux et al., 2001; Luppi et al., 2005; Pastore et al., 2000). Similar effects were also achieved in MCD patients treated with ganciclovir (Casper et al., 2004; Valencia et al., 2005), but not with cidofovir (Berezne et al., 2004). For those patients either intolerable or refractory to standard chemotherapies, intracavitary administration of cidofovir together with adjuvant IFN-α may turn out to be an alternative solution (Boulanger et al., 2003).

3.3. KSHV encodes several proteins with transforming capacity

KSHV is strongly associated with KS development since the KSHV DNA is found in spindle cell region in all KS lesions. These spindle cells are highly proliferative and thought to be the cells to drive KS pathogenesis (Boshoff et al., 1995; Collandre et al., 1995; Dupin, 1995). Moreover, KSHV infection precedes KS development and the seroprevalence of viruses predicts the KS risk as well as mirrors the frequency of clinical KS, implying that KSHV is the etiological agent necessary for KS tumorigenesis (Gao

et al., 1996b; Kedes et al., 1996; Martin et al., 1998; Simpson et al., 1996; Smith et al., 1999). So far, several KSHV encoded proteins, both latent and lytic, have shown their transforming capacity in cell culture and/or in mice.

3.3.1. LANA

In addition to its role in episomal maintenance, LANA is one of the most important KSHV proteins contributing to tumorigenesis. For example, LANA can physically associate with the tumor suppressors Rb as well as the bromodomain-containing protein RING3/Brd2, thereby inhibiting apoptosis, deregulating the cell cycle and enhancing cell proliferation (Denis et al., 2000; Friborg et al., 1999; Guo et al., 2000; Platt et al., 1999; Radkov et al., 2000; Viejo-Borbolla et al., 2005). However, overexpression of LANA alone fails to initiate anchorage-independent growth of NIH3T3 cells, demonstrating the importance of other viral genes and factors for KSHV-induced malignancies (Watanabe et al., 2003). LANA can also cooperate with H-Ras to transform primary rat embryo fibroblasts, or deregulate oncogene CCND1 and Myc expression via glycogen synthase kinase (GSK)-3β/β-catenin signaling pathway (Boshoff, 2003; Fujimuro and Hayward, 2003; Radkov et al., 2000). It also interacts with Sp1 transcription factor to mediate telomerase expression (Verma et al., 2004).

LANA has been found to interact with p53, inhibit p53 transcriptional activity and repress p53-dependent apoptosis (Friborg et al., 1999). LANA could form a complex with p53 and mouse double minute protein 2 (MDM2), regulating ubiquitin E3-ligase activity of MDM2 towards p53 (Chen et al., 2010; Sarek et al., 2007). Suppression of p53 activity by LANA has also been linked with genomic instability (Si and Robertson, 2006). Moreover, enhanced multinucleation, abnormal establishment of centrosomes, and creation of mitotic bridges are reported in mammalian cells with a stable expression of LANA (Si and Robertson, 2006). Finally, transgenic mice expressing LANA developed follicular hyperplasia with increased germinal centers in the spleen as well as lymphomas, further indicating its role in early stage of KS associated tumor development (Fakhari et al., 2006).

3.3.2. vFLIP

The viral FLICE (Fas-associated death-domain like IL-1β-convertase enzyme) inhibitory protein (vFLIP) is a latent KSHV protein, which can inhibit Fas-mediated apoptosis through binding and prevention of procaspase-8 maturation, thus providing a survival advantage for infected cells (Belanger et al., 2001; Djerbi et al., 1999). Additionally, vFLIP, in cooperation with cellular FLIP through their inhibitory interaction with the E2-like enzyme, Atg3, effectively blocked cell death with autophagy, induced by rapamycin, an mTOR inhibitor and an effective anti-tumour drug against KS and PEL (Lee et al., 2009). Furthermore, vFLIP expression was shown to inhibit anoikis (detachment induced apoptosis) in primary dermal microvascular endothelial cells, partly by inducing the secretion of paracrine survival factors (Efklidou et al., 2008). Similar to cellular FLIPs,

vFLIP also associates with the Iκκ complex and the heat shock protein 90 (HSP90) to induce NF-κB survival signaling (Chaudhary et al., 1999; Field et al., 2003). The enhanced NF-κB signaling may be important for the transforming and oncogenic potential of vFLIP as demonstrated in Rat-1 fibroblast transformation assays and growth of tumors in nude mice (Sun et al., 2003). A recent study showed that vFLIP cooperates with c-Myc to elevate NF-κB activity, to decrease in apoptosis and to promote lymphoma in vFLIP/iMyc(Eμ) double transgenic mice, confirming its oncogenic capability *in vivo* (Ahmad et al., 2010). Another recent report demonstrates that vFLIP increases levels of Notch ligand JAG1 through an NF-kB dependent mechanism. Notch activation elicits significant suppression of the expression of cell cycle components in adjacent uninfected cells and may provide a growth advantage to KSHV infected cells over uninfected surrounding cells (Emuss et al., 2009).

3.3.3. v-cyclin

The latent KSHV protein viral cyclin (v-cyclin) is a functional cyclin D homolog with an approximate molecular weight of 29 kDa. The v-cyclin preferably binds to and activates cyclin-dependent kinase 6 (CDK6) and only weakly associates with CDK2, CDK3, CDK4, CDK5, and CDK9 (Chang and Li, 2008; Godden-Kent et al., 1997; Li et al., 1997; Platt et al., 2000). The v-cyclin-CDK kinase complex phosphorylates a broad range of substrates including histone H1 and Rb (Godden-Kent et al., 1997; Li et al., 1997), p27KIP1 (Ellis et al., 1999; Mann et al., 1999; Swanton et al., 1997), p21CIP1 (Jarviluoma et al., 2006), Id-2 and Cdc25a (Mann et al., 1999), Cdc6 and ORC1 (Laman et al., 2001), Bcl-2 (Ojala et al., 2000) and NPM (Cuomo et al., 2008; Sarek et al., 2010).

V-cyclin-CDK6 is regulating cell cycle progression by phosphorylating and inactivating the cell-cycle checkpoint protein Rb (Chang et al., 1996; Godden-Kent et al., 1997). In addition, v-cyclin-CDK6 overcomes cell cycle arrest by phosphorylating and degrading cyclin-dependent kinase inhibitors (CKI) such as p27KIP1, p21CIP1 and p16INK4 (Jarviluoma et al., 2006; Sarek et al., 2006; Swanton et al., 1997). Similar to many other oncogenes, v-cyclin can also lead to p53-dependent apoptosis in the presence of high levels of CDK6 (Ojala et al., 1999; Ojala et al., 2000), or stress signals (Verschuren et al., 2002). To promote apoptosis, v-cyclin-CDK6 phosphorylates and inactivates the cellular anti-apoptotic protein Bcl-2 (Ojala et al., 2000). Centrosome amplification and aneuploidy are frequently observed in v-cyclin expressing cells (Koopal et al., 2007; Verschuren et al., 2002). Additionally, v-cyclin causes a DNA damage response and senescence in endothelial cells (Koopal et al., 2007). In animal model studies, transgenic mice expressing v-cyclin in LECs under control of the VEGFR-3 promoter suffered from pleural fluid accumulation due to dysfunction of lymphatic fluid absorption and lived no more than 6 months (Sugaya et al., 2005). Another Eμ-v-cyclin transgenic mouse model (targeted expression of v-cyclin to the B and T lymphocyte compartment via the Eμ-promoter/enhancer) developed T and B cell lymphomas in the thymus or spleen, with accelerated lymphoma formation in $p53^{-/-}$ background (Verschuren et al., 2004; Verschuren et al., 2002).

3.3.4. vGPCR

KSHV immediate-early lytic protein Viral G-protein-coupled receptor (vGPCR) is a constitutively active homologue of the human angiogenic IL-8 receptors CXCR1 and CXCR2 (Ahuja and Murphy, 1993; Arvanitakis et al., 1997; Cesarman et al., 1996; Guo et al., 1997), which increases endothelial cell survival and proliferation by activating MAPK, PI3K, NF-κB and p38 MAP pathways (Schwarz and Murphy, 2001; Smit et al., 2002; Sodhi et al., 2000). vGPCR was shown to have potent oncogenic capabilities *in vitro*, as evidenced by directly transforming murine NIH3T3 cells and immortalizing human umbilical vein endothelial cells (HUVEC) with autocrine activation of VEGFR2 and downstream Akt signaling pathway (Bais et al., 1998; Bais et al., 2003; Couty et al., 2001; Jensen et al., 2005; Montaner et al., 2001). Moreover, as a homolog of the chemokine receptor, vGPCR also directly enhances expression of a variety of other cytokines and growth factors including IL-4 and GM-CSF (Pati et al., 2003), IL-6 and TNF-α (Schwarz and Murphy, 2001), Gro-α (Montaner et al., 2004), and CCL-2 (Choi and Nicholas), which are rich in tumor microenvironment and facilitate tumor cell proliferation, angiogenesis, and inflammation. In addition, vGPCR is capable of Notch signaling pathway activation by modulating the expression of Notch ligand DLL4 in LECs (Emuss et al., 2009).

vGPCR transgenic mice developed KS-like multicentric, angioproliferative lesions with surface markers and cytokine profiles resembling those of KS, suggesting that a self-perpetuating autocrine/paracrine loop induced by vGPCR might contribute to KS and other KSHV-driven malignancies (Guo et al., 1997; Yang et al., 2000). This scenario could explain why only a small group of tumor cells express vGPCR in vGPCR-driven KS-like tumors in mice. Likewise, this obvious potential to affect the tumor microenvironment is a good clue to understand that only a relatively minor subset of cells in tumor regions are KSHV infected and even fewer express lytic genes such as vGPCR (Dupin et al., 1999; Linderoth et al., 1999; Staskus et al., 1997; Teruya-Feldstein et al., 1998). Importantly, the mouse bone marrow endothelial-lineage cells haboring a KSHV Bacterial Artificial Chromosome (BAC) form KS-like, angioproliferative tumors in mice. In these vascularized sarcoma lesions, the spindle cells express the KSHV hallmark protein LANA and the LEC marker /podoplanin, overexpress VEGF and Angiopoietin ligands and receptors, and display KSHV and host transcriptomes reminiscent of KS. However, the inhibition of vGPCR in tumors inhibited angiogenicity and tumorigenicity, supporting the important role of vGPCR in KSHV-dependent KS (Mutlu et al., 2007).

In addition, a few other KSHV proteins including vIL-6, K1, vIRF1, K15 and Kaposin A/B have shown transforming potential in different assays. Most likely the co-operation between different KSHV-encoded genes is required for tumorigenesis (Mesri et al., 2010). A summary of KSHV viral proteins involved in KSHV pathogenesis is shown in Figure 4.

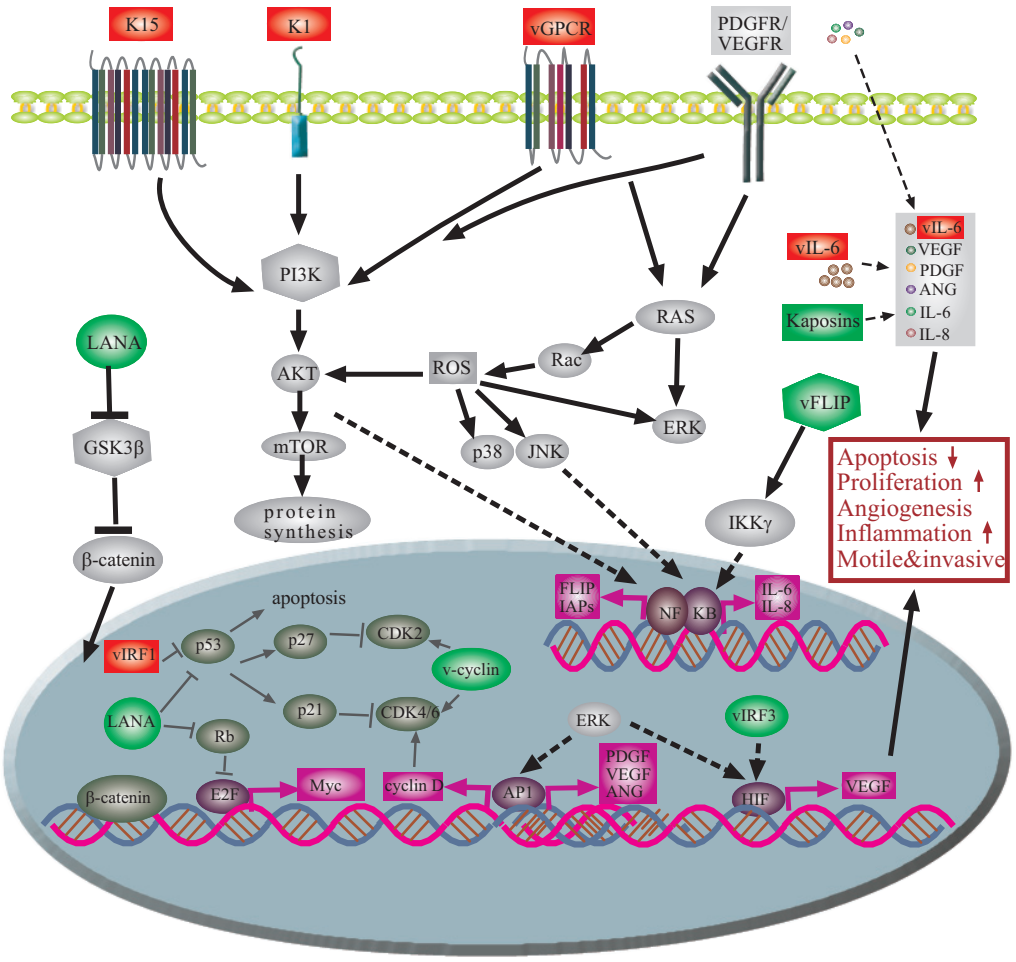

Figure 4. Latent and lytic viral proteins involved in KSHV pathogenesis. In infected cells, expression of KSHV latent proteins (such as LANA, vFLIP, v-cyclin and vIRF3; shown in green) and lytic proteins (such as vGPCR, K1, vIL-6, K15 and vIRF1; shown in red) pirate host signaling pathways, leading to the expression and secretion of a variety of cellular factors through autocrine or paracrine mechanisms. These provide infected cells a survival advantage, together with enhanced angiogenesis, inflammation and invasive potentials in the context of KSHV-induced tumors. CDK, cyclin-dependent kinase; GSK3β, glycogen synthase kinase 3β; HIF, hypoxia-inducible factor; IAPs, inhibitor of apoptosis proteins; NF-κB, nuclear factor-κB; PKC, protein kinase C; ROS, reactive oxygen species (Adapted and modified from Mesri, Cesarman and Boshoff, 2010).

AIMS OF THE STUDY

The switch from latency to productive viral replication (reactivation) is essential in the KSHV viral life cycle, and recent clinico-epidemiological studies support the importance of lytic replication in the development and progression of KS. The first aim of the thesis was to identify novel cellular kinases that are involved in KSHV reactivation from latency and the production of progeny virus particles.

As infection of primary LECs by KSHV does not readily lead to transformation of the cells, the second aim of this work was focused on gaining a deep understanding of cellular events involved in the KSHV-induced EC transformation by using a 3D cell model better mimicking the *in vivo* tumor microenvironment.

MATERIALS AND METHODS

The materials and methods of this study are listed below, for most of them detailed descriptions can be found in the original publications here referred to by Roman numerals.

Table 1. Cell lines used in this study

Cell line	Description	Source or reference	Used in
U2OS	Human osteosarcoma cell line; KSHV-/EBV-	ATCC	I,II, III
BC-3	Human primary effusion lymphoma cell line (PEL); KSHV+/EBV-	Dr. Ethel Cesarman (Arvanitakis et al., 1996)	II
BCBL-1	Human primary effusion lymphoma cell line heterozygous for the M246I mutation in one of the copies of the TP53 gene (PEL); KSHV+/EBV-	A. Moses; NIH AIDS Research (Cat# 3233 from McGrath and Ganem)	II, III
BJAB	B-lymphoblastoid cell line	Dr. J. Salonen	II
DG-75	Burkitt lymphoma cell line; KSHV-/EBV-	ATCC	II
EA.hy926	Endothelial cell line derived from fusing human umbilical vein endothelial cells with the human lung carcinoma cell line A594; KSHV-/EBV-; wt p53	Dr. Kari Alitalo (Edgell et al., 1983)	II
Vero			I, II,III
LEC	Primary juvenile foreskin lymphatic endothelial cells	PromoCell	III
HEK-293	Human Embryonic Kidney 293 cells	ATCC	I,II,III
rKSHV-Vero	Vero cells latently infected with a recombinant GFP-expressing KSHV (rKSHV.219)	(Miller et al., 2007)	I,II,III
BEC	Primary juvenile foreskin blood endothelial cells	PromoCell	III
293-FT	Human Embryonic Kidney 293 cells with T antigen	Invitrogen	III

Table 2. Expression vectors used in this study

Vector	Description	Source or reference	Used in
Lentiviral vectors			
psin	psin-MCS backbone	Dr. Chris Boshoff (Emuss et al., 2009)	III
pSin-vFLIP	Targets vFLIP	Dr. Chris Boshoff (Emuss et al., 2009)	III
pSin-vGPCR	Targets vGPCR	Dr. Chris Boshoff (Emuss et al., 2009)	III
pLenti6/V5-DEST-GFP	Encodes GFP	Dr. Juergen Haas	III
pLenti6/V5-DEST-LANA	Encodes LANA	Dr. Juergen Haas	III
Retroviral vectors			
pMX		Dr. Kari Alitalo	III
pMX-MT1-MMP	Targets MT1-MMP	Dr. Kaisa Lehti	III
pBMN	Encodes GFP	Dr. Emmy Verschuren	III
v-cyclin-pBMN (KpBMN)	Encodes v-cyclin	Dr. Emmy Verschuren	III
Mammalian expression vectors			
pcDNA.3-LANA	Encodes full length of LANA	(Viejo-Borbolla et al., 2003)	II
pcDNA.3-LANA SS205/206RR	Encodes full length of LANA with serine to arginine (SS205/206RR) mutations	(Viejo-Borbolla et al., 2003)	II
pCMV6- Pim2	Encodes full length of human Pim2		I
pcDNA3.1/V5-HisC -Pim1	Encodes full length of human Pim1		I, II
pcDNA3.1/V5-HisC -Pim1KD	Encodes full length of human Pim1 with K67M mutation		I, II
PcDNA3.1-V5-LacZ-Pim2	Encodes full length of human Pim2		I, II
pcDNA3.1/V5-HisC -Pim2KD	Encodes full length of human Pim2 with K61M mutation		I, II
pcDNA3.1/V5-HisC -Pim3	Encodes full length of human Pim3		I, II
pcDNA3.1/V5-HisC -Pim3KD	Encodes full length of human Pim3 with K69M mutation		I, II

Vector	Description	Source or reference	Used in
pcDNA3.1/V5-HisC	Empty vector with V5 and His tagging and LacZ selection		I, II
pGL2-ORF50p	pGL2-ORF50p backbone containing the RTA promoter (3 kb sequence upstream of the RTA translation initiation codon)	(Deng et al., 2000)	II
pGL3-7xTR	the luciferase reporter containing seven terminal repeats (TR) of KSHV genome, pGL3-7xTR	(Garber et al., 2001)	II
pRL-CMV-luc	the luciferase reporter control that constitutively expresses Renilla luciferase		II
E.coli expression vectors			
pGEX-6P-1-GST-Pim-1	The GST-tagged Pim-1 generated by PCR subcloning into the pGEX-6P-1 vector		II
pGEX-6P-1-GST-Pim-1KD	The GST-tagged Pim-1KD constructs generated by PCR subcloning into the pGEX-6P-1 vector		II
pGEX-2T-GST	pGEX-2T plasmid used to produce GST in control samples	GE Healthcare	II
pGEX4T1-GST-C-LANA	The GST-tagged carboxy-terminal LANA (GST-C-LANA; amino acids 972-1162)	(Viejo-Borbolla et al., 2003)	II
pGEX4T1-GST-N-LANA	The GST-tagged amino-terminal LANA (GST-N-LANA; amino acids 1-340)	(Viejo-Borbolla et al., 2003)	II
pGEX4T1-GST-N6LANA	The GST-tagged truncated amino-terminal LANA (GST-N6-LANA; amino acids 100-340)		II
pGEX4T1-GST-N11ANA	The GST-tagged truncated amino-terminal LANA (GST-N11-LANA; amino acids 24-100)		II
pGEX4T1-GST-N17-LANA	The GST-tagged truncated amino-terminal LANA (GST-N17-LANA; amino acids 75-200)		II

Table 3. Primary antibodies used in this study

Antibody	Description	Source or reference	Used in
α-SMA (1A4)	Cy-3 conjugated mouse monoclonal antibody against human α-smooth muscle actin (α-SMA)	Sigma	III
fibronectin	Mouse antibody against human fibronectin	Dr. Antti Vaheri	III
K8.1 A/B	Mouse monoclonal antibody against K8.1	ABI Biotechnologies	I, II
LANA	Rabbit polyclonal antibody against LANA	(Sharma-Walia et al.)	III
LANA (HHV8-ORF73)	Rat monoclonal antibody against LANA	ABI Biotechnologies	I, II,III
LYVE-1	Rabbit polyclonal antibody against LYVE-1	Dr. Pirjo Laakkonen	III
MT1-MMP	Mouse monoclonal antibody against MT1-MMP	Chemicon	III
N-cadherin	Rabbit polyclonal antibody against N-cadherin	BD Biosciences	III
ORF59	Mouse monoclonal antibody against ORF59	(Chan et al., 1998)	I, II, III
PDGFR-α	Mouse monoclonal antibody against PDGFR-α	Santa Cruz	III
PDGFR-β	Mouse monoclonal antibody against PDGFR-β	Santa Cruz	III
PECAM-1	Mouse monoclonal antibody against human PECAM-1	Dako	III
Pim-1 (12H8)	Mouse monoclonal antibody against Pim-1	Santa Cruz	I, II
Pim-2	Mouse monoclonal antibody against Pim-2	Atlas	I, II
Pim-3 (C-term)	Rabbit polyclonal antibody against Pim-3	Abgent	II
pTyK(4G10)	Mouse monoclonal anti-phosphotyrosine antibody	Millipore	III
podoplanin	affinity-purified rabbit polyclonal antibody against human podoplanin	(Breiteneder-Geleff et al., 1999)	III
transgelin	Mouse monoclonal antibody against transgelin	Abcam	III
Tubulin (5H1)	Mouse monoclonal antibody against tubulin	BD Biosciences-Pharmingen	I, II,III
VE-cadherin	Mouse antibody against human fibronectin	BD Biosciences	III
VEGFR3 (9D9)	Rat monoclonal antibody against VEGFR3	Dr. Kari Alitalo	III
vimentin (V9)	Mouse monoclonal antibody against human vimentin	Dako	III
V5	Mouse monoclonal antibody against V5 tag peptide	Invitrogen	I, II
anti-ZO-1	Rabbit polyclonal antibody against ZO-1	Zymed	III
Mouse IgG (sc-2025)	Mouse antibody isotype	Santa Cruz	III
Rabbit IgG (sc-2027)	Rabbit antibody isotype	Santa Cruz	III
Rat IgG (sc-2026)	Rat antibody isotype	Santa Cruz	III

Table 4. Chemicals used in this study

chemical	Working concentration	Source or reference	Used in
SU16f	100nM	Tocris Bioscience	III
GM6001	10μM	Calbiochem	III
TIMP1	50nM	R&D Systems	III
TIMP2	50nM	R&D Systems	III
DAPT	5 μM	Sigma	III
SB431542	10μM	Sigma	III
TGF-β blocking antibody	15μg/ml	R&D Systems	III
nocodazole	100ng/ml	Sigma	III
cytochalasin D	1μM	Sigma	III
Dll4-Fc	10μg/ml; Signal peptide and extracellular domain of recombinant human DLL4 (Delta-like protein 4; Delta4) (aa 1-529) are fused at the C-terminus to the Fc portion of human IgG)	(Zheng et al., 2011)	III
recombinant TGF-β1	1ng/ml	R&D Systems	III
PDGF-AA	25ng/ml	R&D Systems	III
PDGF-BB	25ng/ml	R&D Systems	III
PMA	20ng/mL12-O-tetradecanoyl phorbol-13-acetate (or called TPA)	Sigma	II, III
ionomycin	500ng/ml	Sigma	II, III
NaB	1.35mM	Sigma	I, II
thrombin	2 U/ml	Sigma	III
aprotinin	400μg/ml	Sigma	III
VEGF-A	50ng/ml	R&D Systems	III
VEGF-C	50ng/ml	R&D Systems	III

Table 5. Methods used in this study

Methods	Used in
Chromatin Immunoprecipitation (ChIP-on-ChIP)	III
Cytocentrifugation	I, II, III
DNA extraction	I, II, III
Gene knockdown by RNA interference	II, III
Generation spheroids in 3 dimentional (3D) matrix	III
High content microscopy imaging	I, II
Immunofluorescence microscopy	I, II, III
Immunohistochemistry	III
Immunoprecipitation	I, II
Invasion assay	III
In vitro kinase assay	I, II
Lenti- and retroviral production	III
Luciferase reporter assay	II
Mammalian cell culture	I, II, III
Microarray Processing	III
Protein purification	II
Production of wt and recombinant KSHV virions	I, II,III
Quantitative image analysis	II, III
Quantitative real-time PCR	II, III
Recombinant protein production in bacteria	II
RNA extraction and Reverse transcription	II, III
SDS-PAGE and immunoblotting	I, II, III
Statistical methods	I, II, III
Transduction of mammalian cells	I, II, III
Transfection of cells	I, II, III
Ultracentrifugation	I, II, III
Virus infection of mammalian cells	I, II, III
Virus titer determination	I, II, III

Cell culture

PEL cell lines as well as KSHV-negative BJAB cell line were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) (Invitrogen), 100 U/ml penicillin G, and 100 µg/ml streptomycin. HEK-293, Vero, U2OS human osteosarcoma, EA.hy926 endothelial cells and WI-38 cells (ATCC) were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FCS, 100 U/ml penicillin G, and 100 µg/ml streptomycin. Primary juvenile foreskin LEC and BEC cells were cultured in the basal EC medium plus supplement pack (PromoCell), in the presence of 5% human serum, 25µg/mL amphotericin B, and 25µg/mL gentamycin. All cells were cultured in a humidified 5% CO_2 atmosphere at 37°C.

Virus reactivation assay

Viral reactivation was addressed in Vero and EA.hy926 cells stably infected with recombinant KSHV (rKSHV.219) (Vieira and O'Hearn, 2004). The rKSHV.219 is a double-reporter virus, which expresses the green fluorescent protein (GFP) from a constitutively active EF-1-promoter, and can be induced to express the red fluorescent protein (RFP) from the RTA-responsive KSHV lytic promoter for polyadenylated nuclear RNA (PAN). To address the effect of Pim kinases or their mutants on reactivation, rKSHV.219-Vero and -EA.hy926 cells were seeded on 6- or 24-well dishes and transiently transfected with the empty vector pcDNA3.1 or vectors expressing wt or kinase-dead Pim family members. In brief, $2x10^5$ rKSHV.219-Vero and -EA.hy926 cells per well were plated on a 6-well plate. On the following day, 1µg aliquots of DNA samples were mixed with serum-free medium containing 3µl FuGENE HD (Roche, Nutley, NJ) according to the manufacturer's protocol. One day after transfection, the medium was changed into fresh DMEM medium. To monitor transfection efficiency, pcDNA/DsRed plasmid was transfected into duplicate wells and the number of dsRed-positive cells was determined by fluorescence microscopy. The typical efficiency of transfection was between 15-20%. After 48 h, cells were treated for 2 h with a low-titer baculovirus (BacK50) expressing the KSHV lytic activator ORF 50/RTA. This triggered basal reactivation but led to a low level of RFP induction (2.5% in rKSHV-Vero, and 1.9% in rKSHV- EA.hy926) over the non-treated control (0.4% in rKSHV-Vero, and 0.05% in rKSHV-EA.hy926). Cells transfected with the control vector and treated as above served as negative controls, while positive controls were prepared by treating these cells for 2 h with a high-titer BacK50, followed by replacement with media containing 1.25 mM NaB (Sigma) to induce maximal reactivation (25% in rKSHV.219-Vero and 13% in rKSHV.219-EA.hy926 cells). 30 h after baculovirus infection, cells were fixed with 4% paraformaldehyde (PFA) and RFP expression was monitored using an automated high-content fluorescence microscope Arrayscan 4.5 (Cellomics). To measure completion of the lytic cascade and production of progeny virions, naïve target cells (U2OS) were infected 72 h later with 500 µl of the supernatant from transfected/reactivated cells in the presence of 8 ug/ml polybrene to enhance the infectivity. Plates were spin-transduced by centrifugation at 1,050 g for 30 minutes at room temperature and returned to 37°C, 5 %

CO2 for 2 h, after which the supernatant was replaced with complete medium. At 72 h after infection, target U2OS cells were fixed by 4% PFA and GFP intensity was analyzed by Cellomics Arrayscan 4.5.

Purification of the Glutathione-S-transferase (GST)-fusion proteins

GST-fusion proteins were produced in E. coli BL21 (GST-Pim-1 and -Pim-1KD) or DH5α cells (the GST-LANA proteins). Briefly, bacteria were grown at 30ºC for the GST-Pim-1, -Pim-1KD, and for all the GST-N-LANA forms (full length and truncations), or at 25ºC for the GST-C-LANA. The proteins were induced with 200 nM IPTG for the GST-Pim-1, -Pim-1KD, 100 µM for all the GST-N-LANA forms for 6 h, or with 100 µM for the GST-C-LANA for 16 h before the cells were harvested. The bacteria were lysed in PBS containing 0.5 % Triton X-100 and protease inhibitors (Complete Mini EDTA-free, Roche) and sonicated. Solubilized GST-fusion proteins were bound to glutathione sepharose beads (GE Healthcare) and eluted with buffer containing 30 mM glutathione in 75 mM Tris, pH 8.0. To cleave off the GST-tag from the Pim-kinases, PreScission protease was used according to the manufacturer´s instructions (GE Heatlhcare).

Immunoprecipitation and kinase assay

To induce KSHV lytic replication, BC-3, BCBL-1 and control BJAB cells were treated with either 20 ng/ml TPA or 2 mM NaB for 48 h prior to lysis into the ELB lysis buffer (150 mM NaCl; 50 mM HEPES, pH 7.4; 0.1% Igepal; 5 mM EDTA; 2 mM DTT; 1 mM phenylmethylsulfonyl fluoride [PMSF]; 2 µg/ml leupeptin; 2 µg/ml pepstatin; and 1.5 µg/ml aprotinin). 600 µg of the whole-cell extracts were incubated with anti-Pim-1 or -3 antibodies for 2 h at +4°C. Immunocomplexes were coupled to protein-A or G Sepharose beads for an additional 1.5 hour at 4°C and washed 3 times with the lysis buffer followed by one wash with the kinase buffer (20 mM Tris-HCl, pH 7.5; 50 mM KCl; 7.5 mM $MgCl_2$; 1 mM DTT; 25 mM ß-glycerophosphate; leupeptin 2 µg/ml; pepstatin 2 µg/ml; and aprotinin 1.5 µg/ml). Complexes bound to protein-A/G beads were either directly subjected to a kinase reaction towards co-precipitated proteins or were supplemented with exogenous GST-fusion proteins as substrates. For *in vitro* kinase assays, wild-type and kinase-dead Pim-1 proteins produced in bacteria as GST-fusion proteins were purified and cleaved by Precission protease (GE Healthcare). Kinase reactions were performed in the presence of 2 µCi (0.074 MBq) of [^{32}P] adenosine triphosphate (ATP) for 30 minutes at 30°C and stopped by boiling in Laemmli buffer for 10 min. Phosphorylated proteins were resolved in 8% SDS-PAGE, transferred onto nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany) and analysed by autoradiography and Western blotting when needed. Equal loading of proteins was confirmed with Colloidal Coomassie or Ponceau staining.

Luciferase reporter assays

The luciferase reporter plasmids were transfected with FuGENE HD (Roche, Nutley, NJ) into HEK-293 cells according to the manufacturer's protocol. All transfections were

performed with equal amounts of DNA by normalization with empty vector. The pRL-CMV plasmid that constitutively expresses Renilla luciferase was included as an internal control. Relative luciferase activities were calculated by dividing the normalized firefly luciferase activity of each reporter by that of pGL3 or pGL2 plasmid in transfected cells. 24 h or 48 h after the transfection, cells were harvested, washed in PBS and lysed into the cell lysis buffer provided by the manufacturer of the Dual-Luciferase Reporter Assay System kit (Promega). 100 µl of the cell lysate was used for the reporter assay using a DCR-1 luminometer (DIGENE, Gaithersburg, MD). An aliquot of the cell lysate was used for Western blotting to ensure equal expression of the transfected cDNAs.

Short-term replication assay

$4x10^5$ 293 cells were plated per one well of a 6 well plate. On the next day cells were transfected with pGTR4 or pGTR4:73 plasmids (Grundhoff and Ganem, 2003), both of which contain four KSHV terminal repeats (TR) and GFP coding sequence. pGTR4:73 additionally encodes the KSHV LANA protein. Where indicated cells were also transfected with plasmids expressing Pim proteins or their respective kinase dead mutants. In addition each sample contains as well the pEGFP-C1 (Clonetech) plasmid, which is used as an internal control, since it does not replicate, but can be detected with the same probe as the pGTR4/pGTR4:73 plasmids. At 24h post transfection, cells were split in half to stimulate replication. 48h later the cells were harvested in 300µl lysis buffer (10 mM Tris-HCl, 10 mM EDTA, 0.6% SDS) per sample. The chromosomal DNA was precipitated overnight with 0.85 M NaCl. Subsequently, an aliquot was removed for the analysis of protein expression by Western blotting and the episomal DNA was purified using phenol-chlorophorm extraction. DNA was precipitated with ethanol and dissolved in 20µl of water. 90% of DNA was digested for al least 48h with 40U of MfeI (NEB) and 40U DpnI (NEB) and the remaining 10% with 10U MfeI only. The MfeI/DpnI digestion reveals the efficiency of replication, while the single MfeI digestion is used to estimate the amount of input DNA. Subsequently half of each digest was loaded on a 0.8% agarasoe gel, separated and transferred to a nitrocellulose membrane by Southern blotting. A sequence coding for GFP, labelled with alkaline phosphatase was used as a probe to detect the bands of interest (GE Healthcare/Amersham Biosciences).

KSHV production, titer determination and infection of LECs

GFP-expressing rKSHV.219 was produced as described above. Wild type (wt) KSHV was produced from BCBL-1 cells induced with 20ng/mL PMA. The supernatant was collected after three days by ultracentrifugation (SW28.1, 21,000 rpm at 4^oC for 2 h), and resuspended in TNE buffer (150mM NaCl, 10mM Tris pH 8, 2mM EDTA, pH 8). The virus titers were determined by infecting naïve U2OS cells with serial dilutions of the concentrated virus preparation and infected cells were identified by staining for LANA at 48 h post infection. Typically we obtained virus titers of $1x10^6$ infectious viral particles per ml. For infection, the LECs were split at a density of $2.5x10^5$ cells per a 6-well, and spin-infected with a multiplicity of infection (MOI) of ~3 in serum free medium with

8μg/ml polybrene. The efficiency of infection in the K-LEC cultures with wt KSHV was between 20-40% by LANA staining or 30-50% with recombinant rKSHV.219 by GFP expression in the K-LECs. To address the requirement of infectious virus, virus was inactivated by UV light (UV-KSHV). To this end, an aliquot of freshly produced KSHV was treated by 3000 mJ of UV-C light using UV Stratalinker 1800 (Stratagene) as a light source, and the loss of infectivity was confirmed by measuring the virus titer.

Generation of LEC spheroids

Confluent monolayers of spindling K-LECs or uninfected control LECs were seeded into 0.5% agarose pre-coated, nonadherent round-bottom 96-well plates at 4000 cells per well. After 16 h to 24 h incubation at 37°C, the formed spheroids were harvested and embedded into the fibrin gel consisting of plasminogen-free human fibrinogen (final concentration 3mg/ml; Calbiochem) and human thrombin (final concentration 2 U/ml; Sigma) in 50 μl Hank's Balanced Salt Solution supplemented with 400μg/ml aprotinin (Sigma). The gels were cast onto the bottom of 24-well plates and incubated for 1 h at 37°C to allow complete gelling followed by addition of EC culture medium.

Quantification of spheroid sprouting

To quantify the sprouting of the spheroids the number of sprouts per spheroid was determined from phase contrast images acquired with Zeiss Axiovert 200 epifluorescence microscope (five to ten spheroids were quantified per condition). The sprouts and the spheroid body boundaries were depicted on the images using the Inkscape software [http://www.inkscape.org]. The vector graphic data was first rendered with Inkscape, and then analyzed in a pipeline created in the Anduril framework (Ovaska et al., 2010). This pipeline searches the sprout lines and counts them, gives the total length of the sprouts and the spheroid body area. Sprouting is defined as the total length of the sprouts normalized to the spheroid body area.

Invasion assay

Collagen invasion was assessed essentially as previously described (Hotary et al., 2000). Collagen gels were cast into the upper chamber of Falcon cell culture inserts (BD Biosciences) with 8 μm pore membranes in 24-well cell culture plates and allowed to form a gel at +37°C for 1 h. LECs (2x105 cells/insert) were added on top of the gel, and EC medium supplemented with recombinant human HGF (25ng/ml, R&D systems) was added to the lower chamber. The cells were cultured at 37°C for 7-14 days replenished with complete media every second/third day. The gels were then fixed in 3% PFA, dehydrated and embedded into paraffin. Invading cells were quantified from H&E stained paraffin sections using a Leica DM LB microscope.

Microarray processing

RNA from biological triplicates of K-LEC spheroids and LEC control spheroids was collected on day three using the RNeasy Mini kit (Qiagen). RNA integrity and quantity was assessed using RNA 6000 Nano chips (Agilent 2100 bioanalyzer). Affymetrix Hg-U133 plus 2.0 oligonucleotide arrays containing more than 45,000 human gene probes were used for the hybridizations according to standard procedures (Affymetrix), and analyzed essentially as previously described (Wang et al., 2004a). Genome annotations of the array data were taken from the Bioconductor repository package '*hgu133plus2.db*' (Bioconductor 2.6 CDF and probe annotations. http://www.bioconductor.org/packages/release/data/annotation/). Enrichment of gene sets within the K-LEC spheroids over the control LEC spheroids data was determined using gene set enrichment analysis (GSEA) software from the Broad Institute.

RESULTS AND DISCUSSION

1. Identification of Pim-1 as a novel kinase regulating KSHV viral reactivation (I)

1.1. A large-scale human cDNA kinome screen

The association of KSHV with a human neoplasm and the large number of cellular counterparts in the KSHV genome make it of tremendous interest to determine the role of virus-host interactions in KSHV pathogenesis. The switch from latency to lytic replication is an elementary decision in the viral life cycle and holds substantial importance for the herpesviral tumorigenesis (Grundhoff and Ganem, 2004; Song et al., 2004). Multiple host signal-transduction pathways have been suggested to mediate the switch between KSHV latency and productive infection, but they have not been rigorously addressed. Therefore we set to investigate which cellular kinases would be involved in the reactivation of the KSHV lytic replication cycle and the production of virus particles (I).

To allow an unbiased genome-wide analysis of the cross-talk between cellular kinase pathways and KSHV reactivation, I developed and optimized a gain-of-function screen utilizing a novel expression library for 488 human protein kinase cDNAs (Fig. 1 in I). In the screen setup, a monkey kidney cell line, Vero cells stably infected with a recombinant KSHV (rKSHV.219 (Vieira and O'Hearn, 2004)) were used, which express the green fluorescent protein (GFP) from the constitutively active EF-1-promoter and can be induced to express the red fluorescent protein (RFP) from the KSHV early lytic promoter for polyadenylated nuclear RNA (PAN), which is the most abundant transcript made during the lytic phase (Vieira and O'Hearn, 2004).

In the screen, individual cDNAs of the genome-wide protein-kinase collection were transfected into rKSHV.219-Vero cells. At 48 hours post transfection of the kinases, cells were infected with a low-titer recombinant baculovirus encoding RTA (BacK50), the KSHV replication and transcription activator, for 2 hours to induce basal reactivation. Priming with exogenous RTA is needed as the latency program in the infected Vero cells is very tight, and a basal expression of RTA can enhance the activation of viral lytic cascade by cellular protein kinases. Induction of RFP expression was monitored by an automated high-content microscope (Cellomics ArrayScan HCS instrumentation) 30 hours after the baculovirus infection. For positive controls, maximal viral reactivation was achieved by treatment with high-titer RTA-encoding baculovirus and NaB, a potent reactivation enhancer due to its histone deacetylase inhibitor function.

Analysis of the RFP quantitation data allowed identification of 66 cDNA hits, which increased viral reactivation more than 4-fold above the basal level. In statistics, the Z-factor and Screening Window are proposed to be key measures of the quality or power of a high-throughput screening (HTS) assay. The Z-factor and Screening Window are

defined in terms of four parameters: the sample means and sample standard deviations of both the positive (p) and negative (n) controls (μp, σp, and μn, σn). Given these values, the Z-factor and Screening Window are defined as: Z factor=1-3(σp+σn)/|μp-μn|; Screening Window=(|μp-μn|-3(σp+σn))/σn. The Z-Factor between 0.3-1 and the screening window >2 suggest the response in a particular assay is large enough to warrant further attention (Zhang et al., 1999). In the primary KSHV cDNA kinome screening assay, the calculated Z-factor and Screening Window were 0.534 and 4.595, respectively, confirming the assay to be robust and reproducible.

1.2. Exogenous expression of the Pim-1 kinase induces KSHV reactivation

The hits (kinase cDNAs) of the primary HCS-screen were thereafter individually transfected to rKSHV.219-Vero cells for a secondary screen assay perfomed in 24-well plates, followed by priming of the lytic cycle with RTA-expressing baculovirus infection, and the phenotypes were characterized further by high-content microscopy as above.

In the secondary rescreen, a new step was added to the assay to address if the kinases identified in the first screen were able to induce the full lytic cascade resulting in the production of progeny virions. To this end, duplicates of the transfections were prepared, and one of them was fixed and analyzed for the RFP expression already at about 30 hours post lytic induction as an indication of activity of the lytic PAN promoter, the assay used in the primary screen. The second well of the duplicates was analysed at 48 hours after the transfection. Then the supernatants were harvested, centrifuged, and filtered to exclude the transfer of GFP-expressing cells from the transfection plate and finally replica-plated on human osteosarcoma (U2OS) cells. U2OS cells were chosen as target cells due to their good susceptibility to KSHV infection, and suitable morphology for automated microcopy (our unpublished results). The target cells were quantified for GFP expression after 48 hours as an indication of infectious virions in the supernatant. This approach allowed differentiation of cDNAs inducing lytic reactivation at the early transcriptional level from those affecting later stages of viral life cycle (e.g. assembly, egress). 10 out of 66 hits from the primary screen were validated in the rescreen, and processed to an even larger format rescreen (6-well format). These screening steps led to identification of Pim-1 kinase as a novel kinase involved in KSHV reactivation (Fig. 3A in I). The outline of the high-content screening (HCS) workflow is depicted in Figure 5.

In this large-scale human kinome screen, we chose to use the gain-of-function strategy. Although RNAi technology allows for large-scale loss-of-function assays to be performed, the limitations of this technology need to be acknowledged and mitigated. This owes to the potential for RNAi screen to result in ‘‘off-target effects,’’ or the silencing of untargeted mRNAs, along with variability both in siRNA efficacy and protein half-lives that will ultimately result in different silencing levels of proteins. Therefore the gain-of-function approach can be used as a complementary tool to identify genes that are not expressed in the cell type tested, act redundantly, or activate the pathways analyzed under pathological conditions. In addition, the best models for

different cellular signaling events especially in the context of viral infections may not be derived from cells of human origin. In this KSHV reactivation screen, the monkey Vero cell line was used as the screen cell model, which would limit the utility of human siRNA libraries. However, such restriction does not limit the use of human cDNA tools in cells from other mammalian species.

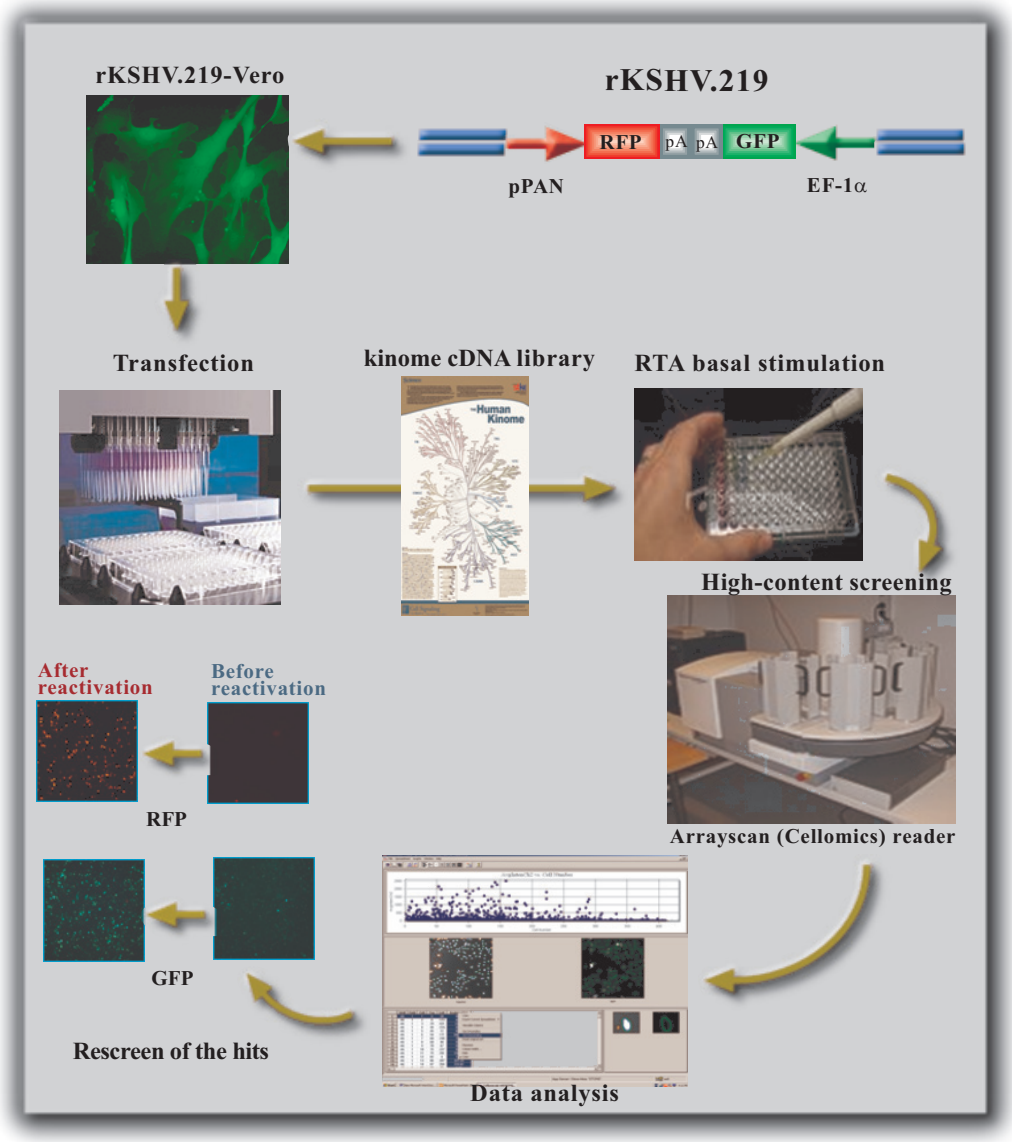

Figure 5. Workflow of the human kinome screen for KSHV reactivation. Individual 466 kinases from the ORF kinome library were transfected in rKSHV.219-Vero cells for 48 hours. The transfected cells were treated with the RTA encoding baculovirus to induce basal reactivation, and the RFP expression was monitored by Cellomics ArrayScan HCS instrumentation 30 hours later. The hits obtained from data analysis were used for the secondary rescreen in large format data.

1.3. Validation of Pim-1 as a kinase inducing KSHV reactivation

Ectopic expression of protein kinases may induce artificial effects due to scaffolding or other nonenzymatic functions of kinases. To exclude this possibility, we took advantage of our human kinome cDNA library in which a corresponding set of catalytically inactive kinases has been produced based on structurally similar kinase domains and critical catalytic residues shared by all kinases. These inactive kinase mutants lead to loss-of-function effects of corresponding kinases and can be used to test whether the kinase activity is required for the observed phenotypes. To further validate the screen, the above reactivation assay was repeated with mutant cDNAs of the Pim-1, the ATP binding site of which was disrupted by a single point mutation (K67M) (Fig. 3 in I). This Pim-1KD mutant was not able to induce RFP expression in rKSHV.219-Vero cells, excluding the possibility of possible kinase scaffolding side-effect.

Tumor cells in KS lesions express endothelial cell markers and are considered to be of endothelial cell origin. Indeed primary endothelial cells are susceptible for infection by KSHV *in vitro*. To further validate Pim-1 kinase in a biologically relevant cell model for KSHV infection, we repeated the KSHV reactivation assay in a human hybrid endothelial cell line, EA.hy926 cells, which were latently infected with rKSHV.219. The data revealed that Pim-1 also induced RFP expression in rKSHV.219-EA.hy926 cells, although the reactivation level by Pim-1 was stronger in Vero than in EA.hy926 cells, which is in accordance with the ability to obtain higher maximal reactivation efficiency in these cells by BacK50 infection and NaB induction (Fig. 3 in I and Fig. 1A in II).

2. Pim kinases are required for the viral reactivation (I, II)

2.1. Ectopic expression of Pim-2 and Pim-3 induces KSHV reactivation

In the above gain-of-function screen utilizing a novel expression library for human protein kinase cDNAs, 480 individual, ectopically expressed human kinases were used to examine their abilities to induce KSHV reactivation. Pim-1 was identified through the primary screen and secondary rescreen as a novel kinase involved in KSHV reactivation. As the Pim oncogenic serine/threonine kinase family includes three members, Pim-1, Pim-2 and Pim-3, sharing significant sequence similarities and largely overlapping functions (Bachmann and Moroy, 2005; Bullock et al., 2005), we decided to examine the roles of all three Pim kinase family members in viral reactivation.

To this end, V5-tagged Pim-1, -2 or -3 cDNAs and an empty vector control were transiently transfected into latently infected rKSHV.219-Vero or rKSHV.219- EA.hy926 endothelial cells, which represent a biologically relevant KSHV infection model. After 48 h, basal reactivation was induced by BacK50 infection and RFP expression was monitored 30 h later. The analysis revealed that all three Pim kinases induced RFP expression to more than 3-fold compared to basal reactivation level (vector control, 2.5% RFP expression

in rKSHV.219-Vero, and 1.9% in rKSHV.219- EA.hy926), with Pim-1 as the strongest inducer (4- to 7-fold) in both cell lines (Fig. 1A and 1D in II).

To study where these three Pim kinases were sufficient to trigger the complete lytic replication cascade, expression of a late lytic protein K8.1 was analyzed in rKSHV.219-Vero and -EA.hy926 cell lines transfected with the Pim kinases. In addition, the production of progeny virions in the supernatant of the transfected rKSHV.219-Vero or -EA.hy926 cells was measured by monitoring GFP expression in U2OS cells infected with the medium as described in Materials and Methods. All three wild type (wt) Pim kinases induced upregulation of K8.1 expression in rKSHV.219-Vero and -EA.hy926 cell lines, as well as GFP expression in U2OS target cells (Fig. 1B-F in II), confirming the activation of the full lytic program.

To further assess whether kinase activity is required for the induction of viral reactivation, we performed the above described reactivation assays using mutant cDNAs of the Pim kinases, the ATP binding site of which was disrupted by a single point mutation (K67M in Pim-1, K61M in Pim-2, and K69M in Pim-3), rendering the kinases inactive (Fig. 1E in I). The individual Pim kinase-deficient (KD) mutants failed to induce either RFP or K8.1 expression in both rKSHV.219-Vero and -EA.hy926 cells (Fig. 1A, 1C, 1D and 1F in II). Accordingly, expression of the Pim-1KD, -2KD and –3KD in both cell lines led to a clear reduction (maximally 10-fold) in GFP expression in target U2OS cells treated with rKSHV.219-Vero or -EA.hy926 cell supernantants (Fig. 1B and 1E in II), suggesting that the viral lytic reactivation as well as production of infectious virions were dependent on the Pim kinase activity. Furthermore, ectopic expression of the irrelevant kinases CDK7 or LKB1 did not induce any significant reactivation or GFP expression in the U2OS target cells over the negative control (Fig. S1A and S1B in II), excluding a possible kinase scaffolding side-effect.

2.2. Pim-1 and -3 are required in viral reactivation of KSHV-infected endothelial and B cells

To rule out the possibility that viral reactivation by Pim kinases was only observed as a result of over-expression, the selective and robust effect of RNAi on gene expression can be exploited as a research tool to evaluate the effect of knocking down the expression of these genes on viral reactivation. To this end, we silenced expression of Pim-1, -2 and -3 individually or simultaneously by transfecting siRNA oligonucleotides specific for Pim-1, -2 or -3, or with a nontargeting control siRNA into rKSHV.219-EA.hy926 cells. The silencing efficiency was evaluated 4 days after siRNA transfection and the efficient depletion of Pim-1 (85%), Pim-2 (90%) and Pim-3 (80%) expression was confirmed by immunoblotting (Fig. 2A in II). 48 h after siRNA transfection, the cells were subjected to maximal reactivation by BacK50 infection and NaB stimulation (RTA+NaB) and the silencing effect on viral reactivation was examined by immunoblotting with K8.1 antibody as well as by measuring the induction of RFP, and expression of GFP in target U2OS cells infected with the supernatant from reactivated/silenced rKSHV.219-EA.

hy926 cells. Interestingly, depletion of Pim-1 and -3 expressions almost completely inhibited induction of RFP and K8.1 in rKSHV.219-EA.hy926 cells or GFP expression in U2OS cells, while simultaneous knockdown of Pim-1 and Pim-3 had a synergistic effect on inhibition of viral reactivation as observed by a greater reduction in RFP, GFP and K8.1 expression (Fig. 2 in II). However, Pim-2 silencing had very little effect on KSHV reactivation (Fig. 2 in II), suggesting that expression of Pim-1 and -3, but not Pim-2, is necessary for KSHV lytic reactivation under the reactivation conditions used here.

Next we assessed the expression levels of endogenous Pim-1, -2 and -3 upon chemical reactivation of naturally KSHV-infected B cells to gain more insight into the biological relevance of Pim kinase-mediated KSHV reactivation. To this end, two PEL cell lines, BC-3 and BCBL-1 cells were first synchronized to S phase by serum starvation for 24 h, and then cultured in the presence of serum for 16 h to increase the reactivation efficiency. The synchronized cells were then treated with 20ng/ml TPA for 48 h and the late lytic marker K8.1 level was analyzed by immunoblotting to confirm efficient reactivation. Intriguingly, expression of Pim-1 and -3 was clearly up-regulated in reactivated PEL cells, whereas the expression of Pim-2 remained unchanged (Fig. 3 and S3 in II). It has been reported that Pim kinases are constitutively active whenever they are expressed, due to an unusual structure and conformation of the hinge region near the ATP-binding pocket (Bullock et al., 2005). Therefore the increase in expression levels of Pim-1 and Pim-3 upon KSHV reactivation in PEL cells would correlate with their activity.

Similar to rKSHV.219-EA.hy926 cells, Pim-1 and -3 were also required for the PEL cells reactivation indicated by K8.1 level after transient silencing of Pim-1 or -3 by using siRNAs (Fig. 3B in II). In accordance with the findings above, depletion of Pim-2 had no effect on induction of K8.1 (Fig. 3B in II). Interestingly, although depletion of Pim-2 expression had no effect on viral reactivation in PEL cells, it seemed to induce a post-translational modification on the Pim-1 protein, indicated by the mobility change of the Pim-1 band in the Western blot.

A previous study has suggested that Pim-2 is upregulated upon another herpesvisus, EBV infection and it enhances the activity of EBV viral nuclear antigen EBNA2 (Rainio et al., 2005). Another report indicates the upregulation of Pim-2 expression in KSHV infected CD34+ bone marrow cells (Mikovits et al., 2001). Therefore it is surprising that Pim-1 and Pim-3 seemed to co-operate in viral reactivation, whereas Pim-2 was not essential for KSHV reactivation either in *de novo* infected ECs or in the naturally infected PEL cells. It is possible that KSHV-infected cells require a threshold level of active Pim kinases to reactivate, which is beyond the endogenous Pim-2 level, and thus its depletion does not affect reactivation. However, the total Pim kinase activity may fall below the required threshold after efficient Pim-1 or -3 silencing, leading to inhibition of viral reactivation upon depletion of either of them.

2.3. Pim-1 and -3 counteract LANA-mediated suppression of lytic replication

2.3.1. Pim-1 and -3 interact with and phosphorylate LANA

LANA can act as a transcriptional activator or repressor to modulate viral gene expression and LANA-mediated transcriptional control helps to keep the virus in its latency cycle (Lan et al., 2005b; Verma et al., 2004). Pim-1 was shown to bind and phosphorylate KSHV LANA protein upon co-expression of Pim-1 and LANA *in vitro* (Bajaj et al., 2006). These data led us to investigate the interaction of Pim-1/-3 with LANA and Pim-1/-3 mediated LANA phosphorylation in KSHV infected cells. To address this question, whole cell extracts of untreated and TPA-treated PEL cells were immunoprecipitated with with anti-Pim-1, -Pim-3, or control IgG antibodies and subjected to an *in vitro* kinase assay on co-precipitated proteins. LANA was shown to be co-precipitated with both Pim-1 and Pim-3 only in the reactivated cells, but not in latent cells (Fig. 3B in I; Fig. 3C in II). There was no association of LANA in the Pim-1 immunoprecipitates from the more concentrated, untreated latent BC-3 extracts, confirming that the interaction requires induction of reactivation, and is not solely a result of increase in the Pim protein levels (Fig. S3 in II).

Moreover, we found that upon TPA or NaB stimulation, the LANA bands co-immunoprecipitated with Pim-1 or -3 were phosphorylated in the autoradiograph from the IP-kinase assay, suggesting that LANA was a substrate for Pim-1 and -3 in the reactivated cells (Fig. 3B in I; Fig. 3C in II). In accordance with the Pim silencing result, we did not observe interaction or phosphorylation of LANA in the Pim-2 immunoprecipitates either in latent or reactivated PEL cells. Collectively, these data suggest that LANA was phosphorylated by Pim-1 and -3, but not Pim-2 kinase in the reactivated PEL cells.

2.3.2. Pim-1 and Pim-3 phosphorylate LANA at the N-terminus on serines 205 and 206

Both N- and C-terminal LANA domains tether episomes to the host chromosomes during mitosis and interphase through interaction with core histones H2A and H2B. In addition, LANA also actively cooperates with a panel of chromosome-binding and origin recognition complex (ORCs) proteins to facilitate viral episomal replication (Stedman et al., 2004; Stedman et al., 2008; Verma et al., 2006). Of note, the C-terminus of LANA binds specifically to sequences within the terminal repeat (TR) regions of the KSHV episomal genome (Schwam et al., 2000; Verma et al., 2006; Viejo-Borbolla et al., 2005). To determine whether the N-terminus or C-terminus of LANA is phosphorylated by Pim-1 and -3, 293 cells were transfected with expression vectors for V5-tagged wt or mutant Pim-1 and -3, and phosphorylation of LANA was analyzed by immunoprecipitation with anti-V5 antibody followed by an *in vitro* kinase assay, using recombinant N-terminal (GST-N-LANA) and C-terminal (GST-C-LANA) LANA as substrates. Both Pim-1 and -3 phosphorylated GST-N-LANA while no phosphorylation was observed on GST-C-LANA or with Pim-1KD or Pim-3KD (Fig. 4A in II), confirming that LANA phosphorylation by Pim-1 and -3 *in vitro* occur on its N-terminus.

In addition, we were able to compete the phosphorylation signal on the 200 kDa LANA protein by adding GST-N-LANA (which contains the Pim phosphosites) into the cell extracts used for the IP-kinase assay, whereas addition of GST-C-LANA (with no Pim target sites) or GST only had no effect on the phosphorylation of the 200kDa band (Fig. 4D in II). This confirmed that the 200 kDa phosphoprotein is indeed LANA, and not another protein, such as a cellular transcription factor recruited by LANA and phosphorylated by Pim.

To further map the site of Pim-1 and -3 phosphorylation on the LANA N-terminus, truncated versions of the GST-N-LANA were used as substrates in the *in vitro* kinase assay as described above. A LANA fragment between amino acids 200 and 340 was demonstrated to carry the critical residues for phosphorylation by Pim-1 and -3, and the further analysis of site-specific LANA phosphomutants via kinase assay and mass spectrometry revealed that serines 205 and 206 on the LANA N-terminus are the specific residues phosphorylated by Pim-1 and Pim-3 (Fig. 4 in II and data not shown). When these two critical serines were mutated into arginines (SS205/206RR), the LANA mutant failed to be phosphorylated by either Pim-1 or -3, consistent with a previous report (Bajaj et al., 2006).

To further rule out the possibility that LANA is phosphorylated by a protein that associates with Pim kinase rather than with the Pim kinase itself, we mixed purified recombinant Pim-1 kinase (wt and kinase-deficient) with recombinant, purified GST-LANA, and were able to show specific, direct phosphorylation of the N-terminal LANA by Pim-1 (Fig. 4E in II), demonstrating Pim-1 as a *bona fide* kinase responsible for the phosphorylation of LANA.

2.3.3. Phosphorylation of LANA counteracts its ability to inhibit transcription from the terminal repeat region

Next we wanted to address the role of LANA phosphorylation by Pim kinases in the viral reactivation. LANA can specifically bind to the LANA binding sites 1 and 2 (LBS1 and LBS2) within the TR region of the KSHV genome, and binding of LANA to the TR region confers transcriptional silencing of a subset of lytic genes in the vicinity of the TR (Garber et al., 2002; Verma et al., 2006). This prompted us to examine whether phosphorylation of LANA by Pim-1 and Pim-3 would affect KSHV TR transcription. By using a luciferase reporter system consisting of multimerized TR repeats (pGL3-7xTR), we detected a 10-fold transcriptional repression of the TR-containing reporters in the presence of LANA in 293 cells. Ectopic expression of Pim-1 or -3 counteracted the LANA-mediated transcriptional repression of the TR-containing reporters in a dose-dependent manner. However, in the absence of LANA, expression of Pim-1 or -3 did not influence transcription of the reporters, suggesting that Pim-1 or -3 enhanced the transcription from reporters in an indirect manner (Fig. 5A in II).

To obtain direct evidence that phosphorylation of LANA plays a key role in the de-repression of TR transcription, a phosphosite mutant of LANA (SS205/206RR) with

Pim-1 or Pim-3 and the pGL3-7xTR luciferase reporter construct were co-transfected, and the samples were subjected to luciferase reporter assays 48 h after transfection. The LANA phosphosite mutant induced similar repression of luciferase activity as the wt LANA, which was not relieved by the co-expression of Pim-1 and -3, confirming that phosphorylation of these serine residues is required to relieve the LANA-mediated inhibition of lytic gene transcription from the TR region (Fig. 5C in II). Interestingly, LANA showed affinity for two other regions of the KSHV genome, albeit to a lesser degree than the TR repeats (Cotter and Robertson, 1999), suggesting that alternate elements of the KSHV genome are possibly targeted by LANA to maintain viral episomes in latency.

Other studies support the role of phosphorylations of LANA on LANA-dependent transcription or DNA replication. The interaction between a nuclear protein RING3 and LANA results in phosphorylation of Ser/Thr residues within the C terminus of LANA in *in vitro* assays (Platt et al., 1999). It is possible that RING3 itself or an unidentified serine/threonine protein kinase recruited into the LANA/ RING3 complex is responsible for this phosphorylation, contributing to interaction of LANA with local heterochromatin and its functional activities (Viejo-Borbolla et al., 2005). The EBV latent viral protein EBNA-1 clearly plays a role in the persistence and segregation of the latent viral episome that is formally analogous to that of KSHV LANA. Interestingly, EBNA1 amino acids 387 to 641 are phosphorylated *in vitro* by CDK1-, -2-, -5-, and -7/cyclin complexes and S393A mutation of EBNA-1 abrogates phosphorylation. This phosphorylation may have a specific effect on EBNA1-dependent transcription or EBV episome replication (Kang et al., 2011). Further investigation of the responsible kinase(s) for multiple phosphorylations of KSHV LANA or its homologues may be necessary to understand the process more fully in the years to come.

2.3.4. Pim phosphorylation on LANA counteracts its ability to repress RTA autoactivation

Besides inhibiting the TR-dependent viral gene transcription, LANA can down-modulate the auto-activatation of RTA on its own viral promoter (Deng et al., 2000; Lan et al., 2004). We observed that the RTA responsive lytic gene K8.1 was induced by Pim-1 and -3 in KSHV-infected cells (Fig. 1C and 1F in II). However, RTA promoter is not located in the proximity of the TR region in the KSHV genome. To provide further support for the potential effect of Pim phosphorylation on LANA's ability to repress RTA responsive genes, luciferase assay using a RTA-Luc reporter (pGL2-ORF50p) was performed to measure the RTA autoactivation level. Expression of LANA led to a partial repression of the RTA-Luc reporter activity, which correlated with previous reports (Deng et al., 2000; Lan et al., 2004). Upon coexpression of wt Pim-1 or -3 together with RTA, Pim-1 and -3 were both able to de-repress the RTA-Luc activity, suggesting that that expression of Pim-1 and -3 kinases can antagonize the ability of LANA to inhibit autoactivation of the RTA promoter. Interestingly, in the absence of the Pim kinases, LANA phosphosite mutant was able to repress the RTA-luciferase activity but to a lesser extent than the wt LANA, and co-expression of Pim-1 and -3 could not reverse this repression (Fig.

5D in II). While the LANA-induced inhibition of transcription from the TR region depends on direct binding of LANA to DNA at LBS1 and LBS2, the suppression of RTA autoactivation occurs through direct binding of LANA to RTA. Therefore the different mechanisms behind the repression of the TR region and the RTA promoter region by LANA might explain this variation of LANA-mediated inhibition of transcription of TR-Luc and RTA-Luc reporters.

Other known gammaherpesviruses including rhesus rhadinovirus (RRV), herpesvirus saimiri (HVS), and murine herpesvirus 68 (MHV-68) also encode LANA and RTA homologues. RRV LANA, like KSHV LANA, represses the transactivation ability of RRV RTA, and this repression is reversed by treatment with the histone deacetylase inhibitor TSA (DeWire and Damania, 2005). Similarly, the LANA homologue in HVS can repress the expression of the HVS RTA (Schafer et al., 2003). These findings support our results and suggest that the counterbalance of the latent transactivator LANA and the lytic transactivator RTA may be a common and evolutionarily conserved mechanism among the gammaherpesviruses. This eventually defines a critical control point between viral persistence latently and lytic replication and in turn results in a profound difference in overall viral load and pathogenesis within the infected host.

2.3.5. Pim kinases have a moderate effect on LANA-dependent latent replication

In addition to its viral episome tethering function, LANA is also necessary for latent replication of the genome (Grundhoff and Ganem, 2003). By using a mini-replicon system containing the minimal requirements for KSHV latent replication (Grundhoff and Ganem, 2003; Hu et al., 2002; Viejo-Borbolla et al., 2005) we tested the effect of Pim kinases on LANA-dependent episome replication. Our results indicate that all Pim kinases had a moderate inhibitory effect on minireplicon replication (Figure 6).

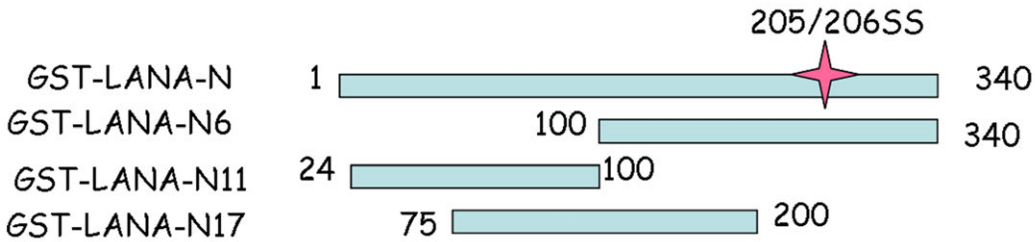

Figure 6. Ectopic expression of Pim proteins moderately reduces the efficiency of episome replication. (A) V5-tagged Pim1/2/3 expressing cDNAs were transfected together with pGTR4:73 plasmid (-), the pGTR4 vector alone (negative control), or (+) the pGTR4:73 vector also encoding for LANA (positive control). The top panel shows episomal DNA linearized by the MfeI enzyme and resistant to the DpnI digestion. DpnI cuts only DNA with bacterial methylation pattern and therefore allows differentiation between transfected DNA of bacterial origin and the newly synthesized (replicated) DNA in the 293 cells. The bottom panel shows total episomal DNA (input) linearized with the MfeI enzyme. The pEGFP plasmid does not replicate and therefore can be used as an input control. (B) Western blot showing expression of LANA and Pim proteins.

Interestingly, this effect did not require the kinase activity of Pim-1/-3 as replication was reduced also upon co-expression of the kinase deficient forms. Non-specific, over-expression related effects were ruled out by transfecting an expression vector coding for an irrelevant protein, which did not affect latent replication (data not shown). This suggests that inhibition of LANA-dependent latent replication is due to binding of Pim kinases to LANA rather than targeting LANA by phosphorylation and is most likely based on a mechanism distinct from the one involved in Pim-mediated lytic reactivation.

2.4. Pim kinases as potential novel targets for treatment of KSHV-associated malignancies

In the previous experiments, chemicals (TPA and NaB) were used to induce KSHV lytic reactivation. However, a number of immune and inflammatory factors such as IL-6 (Chang et al., 2000; Chatterjee et al., 2002), IFN-γ, and oncostatin M in KS and PEL tumor microenvironment have been shown to induce KSHV reactivation (Ensoli and Sirianni, 1998; Ensoli et al., 2001). Very interestingly, expression of Pim-1 is induced by a number of cytokines, such as IFN-α, IFN-γ, IL-3, and IL-6 (Bachmann and Moroy, 2005), which are associated with KSHV associated malignancies and suggested to modulate KSHV reactivation. It is therefore plausible that the presence of a variety of cytokines in the microenvironment of KSHV-associated tumors can lead to induction of Pim kinases, and therefore lead to viral reactivation in the affected tissues.

To address if physiological inducers of Pim kinases would induce KSHV reactivation, the expression levels of endogenous Pim-1 in PEL cells upon cytokine stimulation were analyzed. 3 h after IFN-γ stimulation in BC-3 cells, expression of Pim-1 was already found to be moderately up-regulated and was maintained at a high level at 16 h of IFN-γ treatment, which correlated with the elevated expression of two lytic transcripts ORF50/RTA (immediate-early transcript) and ORF57 (delayed-early transcript) in a quantitative real-time PCR analysis 6 hours post IFN-γ treatment (Fig. 6A and 6D in II), implying that cytokine treatment lead to expression of viral lytic genes. Silencing of Pim-1 expression by siRNA led to a significant decrease of both of the lytic transcripts (Fig. 6C and 6D in II), demonstrating the involvement of Pim-1 in activation of ORF50 and ORF57 lytic gene transcription in IFN-γ treated PEL cells.

To further address if Pim-1 phosphorylated LANA in IFN-γ stimulated PEL cells, we performed Pim-1 IP-kinase assay to compare LANA phosphorylation in untreated, IFN-γ- or TPA-treated BC-3 cells. In accordance with the induction of lytic gene expression, LANA was co-precipitated with Pim-1 and phosphorylated by Pim-1 upon IFN-γ treatment of BC-3 cells (Fig. 6B in II), further supporting the physiological role for Pim-1 in KSHV reactivation. The role of Pim-1 and Pim-3 in the regulation of KSHV reactivation is summarized in Figure 7.

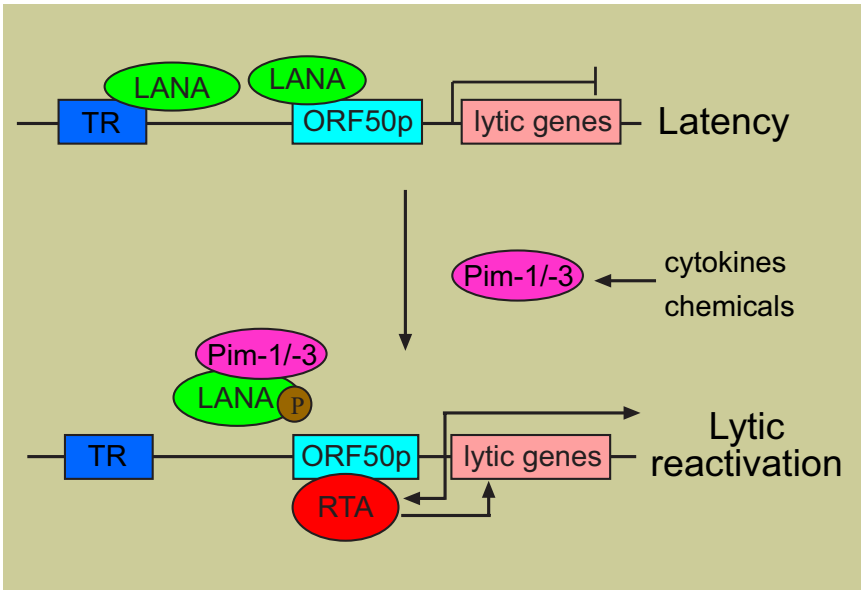

Figure 7. Pim-1 and Pim-3 as novel regulators of the KSHV lytic reactivation. This schematic model depicts the role of Pim-1 and Pim-3 in the regulation of KSHV reactivation. In latency, binding of LANA to the LANA binding sites 1 and 2 (LBS 1 and LBS2) at the terminal repeat (TR) region represses transcription from the TR region. In addition, LANA can also repress transcription from the RTA promoter and inhibit RTA to autoactivate its own promoter, which is is essential to maintain the transcriptional repression of viral lytic genes. Upon stimulation by cytokines, TPA or NaB, Pim-1 and -3 are up-regulated,and they interact with and phosphorylate LANA. The phosphorylation by LANA counteracts the LANA–mediated inhibition of transcription from the TR region and drives its dissociation from the RTA-promoter (ORF50p), leading to viral reactivation.

As majority of latent infected cells are insensitive to anti-herpes drugs, in spite of major improvements in the treatment of KSHV-associated malignancies, overall survival of patients remains poor. The switch from latency to lytic replication is a critical decision in the KSHV viral life cycle and a sustained low level of reactivation contributes to tumor progression by producing new virions as well as providing favoring tumor microenvironment rich in inflammatory and angiogenic cytokines (Grundhoff and Ganem, 2004; Song et al., 2004). Therefore cellular factors and signaling pathways involved in lytic reactivation would represent promising targets in sensitizing tumor cells to the anti-viral therapy (Brown et al., 2005; Curreli et al., 2005; Klass and Offermann, 2005). Our data established Pim-1 and -3 as key factors in KSHV reactivation and inhibition of either Pim-1 or -3 results in a significant decrease in lytic replication. As animals lacking all three Pim genes are viable with a mild phenotype (Mikkers et al., 2004), small-molecule inhibitors targeting Pim-1 and -3 might be tolerated without significant side effects. The unpublished results confirmed the efficacy of a specific Pim inhibitor to suppress viral lytic replication indicated by the expression of two lytic markers K8.1 and ORF59 (Figure 8). Therefore, targeting Pim kinases could be a promising approach for therapeutic intervention of KSHV associated tumors.

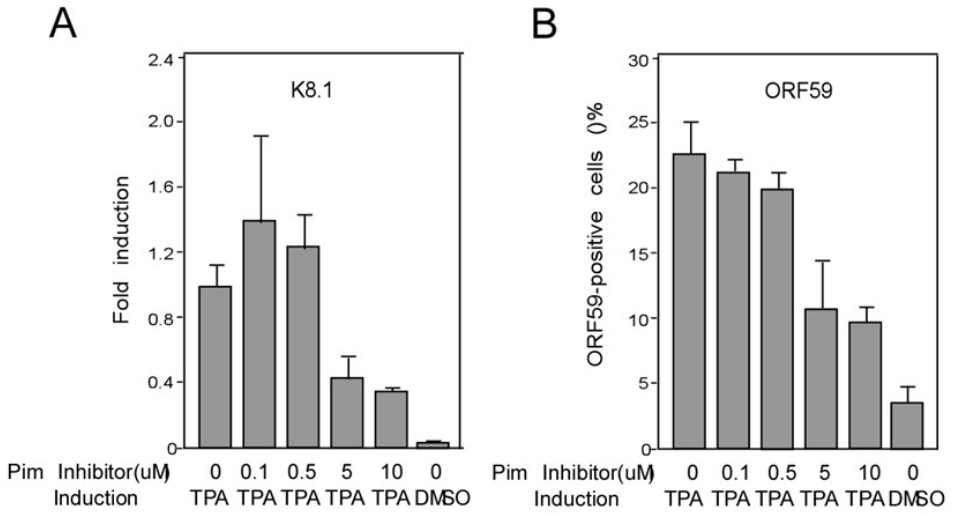

Figure 8. KSHV lytic replication is suppressed by a specific Pim inhibitor. BC-3 cells were pre-treated of with different concentrations of the Pim inhibitor for six hours followed by co-treatment with TPA (20ng/ml) or vehicle (DMSO) alone for an additional 24 hours. (A) The expression of lytic gene K8.1 was detected by qRT-PCR and (B) the expression of late lytic protein ORF 59 was detected by immunofluorescence.

3. KSHV reprograms LEC cells into a mesenchymal cell type through endothelial-to-mesenchymal transition (III)

KS is an angiogenic tumor, which consists of proliferating cells with spindle-like morphology that form irregular microvascular channels (Boshoff et al., 1995). The origin of the spindle cells in KS lesions is still debated as they are composed of quite heterogenic populations of cells expressing markers of endothelial cells (Dupin et al., 1999; Kaaya et al., 1995), smooth muscle cells and fibroblasts (Kaaya et al., 1995; Sturzl et al., 1995; Weich et al., 1991; Werner et al., 1990). The prevailing view is that KS is derived from endothelial cells (EC) (Ganem, 2010) and thus various types of ECs infected with KSHV represent a relevant model system to study pathways involved in KSHV tumorigenesis as well as the role of viral genes in virus-induced cell transformation. (McAllister and Moses, 2007).

Mouse bone marrow endothelial-lineage cells transfected with KSHV Bacterial Artificial Chromosome (mECK36) are able to form KS-like tumors in nude mice. This KSHV malignancy is reversible (Mutlu et al., 2007). When these mECK36 cells lost the KSHV episome, they lost the tumorigenic potential *in vivo*. Intriguingly, mECK36 did not form colonies in soft agar assay *in vitro*, suggesting that the tumorigenic potential of KSHV on the endothelial precursors is dependent on the tissue microenvironment (Mutlu et al., 2007). In cell culture system, primary ECs are susceptible for infection by KSHV. However, primary ECs infected with wild-type KSHV do not readily acquire

properties of transformed cells, and tend to lose the viral episomal genomes upon culturing (Lagunoff et al., 2002). Moreover, KSHV evokes anti-proliferative, rather than proliferative responses in cultured ECs (Koopal et al., 2007), making aspects of KS tumorigenesis difficult to study in a 2D system.

3.1. KSHV induces extensive sprouting of LECs in 3D

To mimic the physiological microenvironment for the KSHV-infected ECs, we sought to identify here novel cellular pathways and virus-host interactions involved in KSHV pathogenesis by developing a cross-linked 3-dimensional (3D) organotypic system (Korff and Augustin, 1998) (Rowe and Weiss, 2008). Since spindle cells in KS tumors are positive for both BEC and LEC markers, and global gene expression analyses have demonstrated that the KS gene expression signature resembles more that of the infected LECs than BECs (Carroll et al., 2004; Hong et al., 2004; Wang et al., 2004a), we chose primary LECs as the cell model to study.

Upon infection with KSHV, primary LECs in culture adopted a spindle-cell like morphology. Based on the propensity of ECs to form spheroids under non-adherent conditions (Korff and Augustin, 1998), we prepared spheroids from KSHV-infected LECs (K-LECs) and uninfected control LECs seven to ten days after infection to provide a more physiological environment for the cells. KSHV induced a dramatic outgrowth of fibroblast-like cells in the LEC spheroids while minimal sprouting was observed in uninfected control LECs (Fig. 2A in III). These sprouts were progressing with time, and their morphology was sharp fibroblast-like, different from capillary BEC sprouts. In addition, the KSHV major latent antigen LANA was expressed in most of the sprouting cells (Fig. 3D in III).

To test if viruses are required for extensive sprouting of LECs, KSHV was inactivated by UV light and the loss of infectivity was confirmed by measuring the virus titer. As expected, minimal sprouting was observed in the UV-K-LEC spheroids, demonstrating that the K-LEC sprouting required infectious virus (Fig. 3A in III). Furthermore, to examine the correlation of the extent of sprouting with the virus infection, GFP expression was monitored in spheroids prepared from a 1:3 mixture of LECs infected with the GFP-expressing recombinant rKSHV.219 (Vieira and O'Hearn, 2004) and control LECs. Confocal analysis revealed that the majority of sprouting cells are KSHV virus infected by counting the numbers of green sprouts, suggesting that infected cells are predominantly contributing to the sprouting (Fig. 3C in III).

3.2. KSHV induces endothelial-to-mesenchymal transition in LEC spheroids

Because of the fibroblast-like morphology of KLEC sprouts, we were interested in defining the differentiation status of infected cells in sprout formation. To this end, the immunofluoresence staining of a series of antibodies against lymphatic endothelial and mesenchymal markers was applied in the 3D system. Expression of endothelial

markers PECAM-1, VE-cadherin as well as lymphatic markers VEGFR3, podoplanin and LYVE-1 was remarkably downregulated both in K-LEC spheroids as well as in 2D K-LEC cultures, which is in accordance with previous reports showing KSHV-induced downregulation of EC markers (Mansouri et al., 2006; Mansouri et al., 2008; Qian et al., 2008; Wang et al., 2004a). Unexpectedly, expression of several mesenchymal proteins (αSMA, transgelin, N-cadherin, fibronectin, and fibrous vimentin) was strongly induced in the sprouts of the K-LEC spheroids (Fig. 2B in III), which was further confirmed by qRT-PCR with a panel of additional mesenchymal markers (*TAGLN*, *VIM*, *CDH2*,*CNN1*, *PDGFRB*, *COL1A1*, and *S100A4*) induced in the K-LEC spheroids, but not in parental 2D K-LEC cultures (Figure 2C in III), suggesting that the 3D environment triggered K-LEC to differentiate into a new, mesenchymal cell type via EndMT (Potenta et al., 2008). During EndMT process, cytoskeleton rearrangement is a prerequisite for polarized ECs transforming into non-polarized and motile mesenchymal cells. Indeed we found that nocodazole, which disrupts microtubules organization, was able to block the mesenchymal reprogramming, suggesting that a functional cytoskeletal network is necessary for the K-LEC mesenchymal reprogramming in 3D (Fig. S4B in III).

3.3. The Notch pathway regulates the KSHV-induced reprogramming of LECs

3.3.1. Notch, but not TGF-β, initiates the KSHV-induced EndMT

TGF-β and Notch pathways have been demonstrated to activate EndMT (Arciniegas et al., 2007; Medici et al., 2010; Noseda et al., 2004; Potenta et al., 2008; Timmerman et al., 2004). Interestingly, KSHV infection alone did not significantly induce any of the TGF- β targets in primary LECs (Fig. S3B in III). To further elucidate if the KSHV-induced EndMT is induced in response to TGF-β activation, the K-LEC and control LEC spheroids were treated with the inhibitor of TGF-β downstream kinases ALK4, -5, -7 (SB431542) or a TGF-β blocking antibody (anti-TGF-β). Surprisingly, inhibition of TGF-β signaling by SB431542 or anti-TGF-β antibody had no effect on sprouting of the K-LEC spheroids or induction of mesenchymal transcripts (Fig. S3A, S3C in III and data not shown), indicating that TGF-β signaling is not the EndMT inducer in KLEC spheroids.

In comparison, inhibition of the Notch pathway by gamma-secretase inhibitor (DAPT) or a soluble inhibitor of Dll4/Notch signaling (Dll4-Fc) dramatically reduced the number of sprouts in the K-LEC spheroids compared to control LEC spheroids. Transcripts for mesenchymal markers were decreased in the Notch inhibition, accordingly (Fig. 3, S3 in III and data not shown). As Notch has been shown to regulate viral lytic infection via functional interaction with RBPJ (Chang et al., 2005a; Liang et al., 2002; Liang and Ganem, 2003; Liang and Ganem, 2004), we also addressed if Notch inhibition influences the viral gene expression in 3D K-LECs. qRT-PCR results suggested that DAPT treatment led to a decrease in a panel of early and late lytic genes (ORF50, vIRF-2, ORF25, and K8.1) without significant effect on latent LANA transcripts (Fig. S7D in III), confirming the role of Notch in viral lytic replication.

3.3.2. vGPCR and vFLIP are engaged in the reprogramming of LECs

Besides that Notch is involved in KSHV lytic replication, recent reports demonstrated engagement of Notch signaling in LECs by two KSHV gene products vFLIP and vGPCR (Emuss et al., 2009; Liu et al., 2009). This prompted us to investigate if these viral genes are involved in the KSHV-induced EndMT. Lentivirus-mediated gain-of-function assay demonstrated that these two viral genes induced Notch downstream targets and mesenchymal markers in 3D, which was inhibited by DAPT treatment (Fig. 4A-D in III). Silencing of vFLIP and vGPCR expression individually or simultaneously in K-LECs by siRNAs reduced sprouting of the K-LEC spheroids for 30-50%, together with the reduction of αSMA and fibronectin positive sprouts in the corresponding K-LEC spheroids (Fig. 4F-G in III), confirming that vFLIP and vGPCR coregulate EndMT in the context of the whole virus infection.

Notch 1-4 are reported to be expressed in KS tumors (Curry et al., 2005; Liu et al., 2010) and consistent with previous reports (Emuss et al., 2009), we confirmed that a KSHV latency protein (vFLIP) and a lytic protein (vGPCR) activate Notch signaling pathway already in 2D. However, our results suggested that the transition into mesenchymal phenotype requires both a microenvironment provided by the cross-linked 3D fibrin matrix, and intact microtubule dynamics to support the ingression into the 3D matrix. The data is distinct with the recent report that mural cell markers were induced in 2D cultured HPV-16 E6/E7-immortalized LECs stably infected by rKSHV.219 (Liu et al., 2010). This could be due to the sensitizing effect of ectopic E6 and E7, as overexpression of these genes is implicated in EMT induction (Chamulitrat et al., 2009; Hellner et al., 2009).

3.3.3. KSHV induced EndMT and VEGF-stimulated (lymph)angiogenesis represent distinct biological processes

The 3D fibrin system has been broadly used in angiogenesis research to study EC sprouting. It has been reported that in 3D Notch inhibition synergizes with VEGF to promote angiogenic sprouting of HUVECs (BECs) (Ridgway et al., 2006) and lymphangiogenesis of LECs (Zheng et al., 2011). To clarify the Notch involvement and consequences of VEGF stimulations in our K-LEC 3D system, 50ng/ml VEGF-A or VEGF-C were added to treat K-LEC spheroids. Compared to unstimulated parental spheroids, VEGF-A or -C stimulated spheroids did not induce outgrowth of the mesenchymal-like sprouts rather than showing extensive capillary-like sprouts. These capillary sprouting cells maintained the expression of strong EC marker (PECAM) and did not express mesenchymal markers (αSMA, fibronectin) (Fig. 3G in III), indicating that the KSHV-induced mesenchymal reprogramming of LECs in 3D is a unique process induced and regulated by different biological stimuli than VEGF-A/C. As Notch signaling has been implicated in the regulation of cell fate decisions in different tissues and organs, our results suggest that Notch may be a critical player determinant for the (lymph)angiogenic vs. mesenchymal fate of LECs.

3.4. Notch activation cooperates with MT1-MMP and PDGFR-β in the KSHV-EndMT

3.4.1. KSHV-induced EndMT endows primary LECs with invasive properties

To obtain a more comprehensive picture of the properties of 3D reprogrammed K-LECs we compared the gene expression microarray (GEM) profiles of K-LEC spheroids to control LEC spheroids three days after embedding to fibrin. 3D K-LEC transcriptome analysis revealed significant up-regulation of invasion related genes (Fig. 5A-B in III), many of which were found to be also upregulated in KS biopsies (Fig. S5A-B in III) (Wang et al., 2004a). These results are correlated to the known role of EndMT in cancer cell migration and invasion. In addition, certain invasive markers such as N-cadherin and fibronection were deregulated in 3D K-LECs (Fig. 2B in III), and the tight-junction protein ZO-1 was also found to relocalize from the cell-cell contacts to more dispersed, cytoplasmic localization in the sprouts of K-LEC spheroids (Fig. 2D in III), further suggesting that the K-LECs may acquire invasive potential upon reprogramming.

3.4.2. MT1-MMP represents the major MMP involved in mesenchymal reprogramming of LECs

Next we assessed the role of MMPs, the key enzymes in cancer cell invasion, in the K-LEC spheroid sprouting. To examine whether soluble or membrane-associated MMPs are required for sprouting, we used a broad-spectrum MMP inhibitor GM6001 and two tissue inhibitors of metalloproteinases TIMP1 and TIMP2. TIMP1 targets the secreted, soluble MMPs, including MMP1, -2 and -9, whereas TIMP2 targets both soluble and membrane bound MMPs (MT-MMPs) (Baker et al., 2002). While the treatment with TIMP1 had no effect on the K-LEC spheroids, GM6001 and TIMP2 dramatically inhibited sprouting, suggesting the involvement of MT-MMPs in the sprouting. Accordingly, a strong up-regulation of *MMP14* (gene for MT1-MMP) was observed in 3D K-LECs as well as in 2D cultures. The invasive properties of K-LEC cells were validated in an MT1-MMP-dependent functional invasion assay through a cross-linked collagen matrix (Rowe and Weiss, 2008; Sabeh et al., 2004) (Fig. 5D in III), further supporting that KSHV-induced EndMT leads to MT1-MMP-dependent invasiveness.

Ectopic expression of MT1-MMP on the LEC revealed an induction of extensive outgrowth of sprouts with αSMA expression in LEC spheroids together with a panel of mesenchymal genes (Fig. 6C in III), whereas depletion of MT1-MMP led to a clear reduction of the sprouting of the K-LEC spheroids (Fig. 5F in III), further indicating that MT1-MMP is required for efficient sprouting of the K-LEC spheroids in 3D fibrin, and may represent the major MMP involved. Although primary LECs normally express very low levels of membrane bound MMPs (Petrova et al., 2002), our results demonstrated that KSHV induces a substantial up-regulation of MT1-MMP in the infected LECs, resulting in activities allowing the cells to invade 3D fibrin and collagen. This is remarkably distinct from the reported MT1-MMP expression pattern during angiogenesis where it is confined largely to the tip endothelial cell of the sprouting neovessels (Yana et al., 2007).

3.4.3. A novel Notch-MT1-MMP is an important mediator of the KSHV-induced reprogramming of LECs

To define the interplay of Notch and MT1-MMP in the KSHV-induced reprogramming and invasion, we examined the EndMT markers in the K-LEC spheroids after MT1-MMP silencing or Notch inhibition. Depletion of MT1-MMP had only a minor effect on expression of Notch target genes *HES1* and *HEY1*, while the treatment of K-LECs with DAPT or Dll4-Fc abolished the induction of MT1-MMP both in 2D (data not shown) and 3D (Fig. 6A-B in III), indicating Notch pathway as a previously unrecognized upstream regulator of MT1-MMP that was necessary to trigger the EndMT in 3D. The data further demonstrates that MT1-MMP is sufficient to provide LECs with a mechanism to undergo EndMT in 3D. Analogous to our MT1-MMP overexpressing LECs, MT1-MMP has been shown to be sufficient to induce an invasive EMT program in MCF7 cells in 3D environment, although the cells retain their epithelial morphology in 2D culture (Ota et al., 2009). While the mechanisms by which MT1-MMP can exert such global effects on LEC remain to be established, it is known that the cell invasion and growth promoting effects of MT1-MMP are associated to its ability to degrade downstream substrates including collagens, fibrin, laminin, fibronectin and other basement membrane and interstitial matrix components or to hydrolyze growth factors or other growth and invasion modifying substrates in the pericellular spaces. It is also possible that genes involved in local ECM rigidity, cell shape and tension could be altered by MT1-MMP in a direct or indirect manner (Rowe and Weiss, 2008).

An interesting finding during MT1-MMP depletion in KLEC is that *PDGFRB* gene, a known effector/regulator of EMT (Jechlinger et al., 2006) and cell invasion in fibrin (Lehti et al., 2005), was also downregulated in K-LEC spheroids. This concured with an earlier report linking Notch 4-mediated up-regulation of PDGFR to EndMT (Noseda et al., 2004). Moreover, MT1-MMP has been found to interact with membrane receptors including integrins, VEGFRs, FGFRs, and PDGFRs, thus altering post-transcriptionally the receptor functions and cell phenotype (Eisenach et al., 2010; Lehti et al., 2005; Sugiyama et al., 2010). Therefore, as a potent regulator of mesenchymal cell invasion, proliferation, and differentiation, PDGFR-β could act as a logical partner with MT1-MMP in mediating EndMT. To examine the involvement of PDGFR-β in EndMT program induced by Notch/MT1-MMP axis, we silenced PDGFR-β by using siRNA oligos in K-LECs and analyzed the silencing efficiency, spheroid sprouting, and expression of mesenchymal markers by qRT-PCR. Unexpectedly, silencing of PDGFR-β expression led to only a moderate reduction in spheroid sprouting and the induction of some of the assayed mesenchymal markers (Figure 6D-E). This suggests that in collaborating with Notch/MT1-MMP signaling pathway, PDGFR-β plays a mild role to activate the transcriptional EndMT program in 3D environment.

Taken together, the evidence indicates that in the context of K-LECs, KSHV (mainly mediated by vFLIP and vGPCR) induces Notch activation up-regulates MT1-MMP expression already in 2D KLECs. Consistently, exogenous vGPCR expression has previously been linked to MT1-MMP upregulation in pulmonary arterial endothelial cells

(Shan et al., 2007). The activated Notch and MT1-MMP are not sufficient for the virus-induced EndMT in 2D, which requires the cross-linked 3D fibrin matrix and a functional microtubule network. The cross-talk between the PDGFR-β and Notch pathways and a positive feedback mechanism through PDGFR and MT1-MMP signaling upon K-LEC sprouting further promote EndMT in 3D culture (Fig. 6F in III).

3.5. Mesenchymal cells in KS lesions are infected with KSHV

The above results reveal a novel form of cell plasticity through the virus-induced Notch-MT1-MMP axis activation, providing a possible explanation for the origin of the KS tumor cells expressing heterogenic mesenchymal markers (Kaaya et al., 1995; Sturzl et al., 1995; Weich et al., 1991; Werner et al., 1990). Importantly, by double-label fluorescent immunohistochemistry, we found cells co-expressing LANA and the mesenchymal markers (αSMA and PDGFR-α) in the majority of the primary KS tumors. Although vimentin signal was observed also in uninfected cells, a quantitative image analysis revealed that the LANA-positive cells in the tumor display a stronger, fibrous vimentin signal when compared to the LANA-negative, non-infected cells (Fig. 1 in III). These results have profound implications for our understanding of KS-lesion histogenesis.

3.6. The 3D KSHV-specific transcriptome has implications for KS invasiveness

To further assess the biological relevance of our 3D system to KS, we compared the K-LEC 3D transcriptome to previously identified gene expression profiles of KS biopsies and 2D K-LECs (Wang et al., 2004a). This analysis reveals a substantial number of genes co-regulated in all three-sample groups. Intriguingly, there are a number of genes with similar co-regulation between 3D and KS biopsies, including genes involved both in EMT/EndMT and invasive processes (Fig. S5A and S5B in III). In addition, KSHV-infected mesenchymal cells expressing MT1-MMP were found both at the invasive edge of KS tumors and within the nontumorigenic surrounding stromal tissue (Fig. 5E and S5E in III). Therefore, it is possible that the Notch-MT1-MMP axis and downstream effectors are important mediators of KS cell invasion and dissemination from the primary site. In light of these findings, we propose that the KSHV-induced invasive mesenchymal cell phenotype may contribute to the development of KS, and help to explain the progression of the disease through spread of the tumor foci into surrounding connective tissue.

3.7. The 3D system offers a permissive microenvironment for virus replication

To investigate whether the microenvironment milieu of the 3D culture and/or KSHV-induced EndMT would result in changes in the viral gene expression pattern, a timecourse mRNA analysis was performed for the expression of latent, early lytic and late lytic transcripts of the 3D and the parental 2D K-LECs from the matching same-day

cultures. Interestingly, the microenvironment during spheroid formation (16-24 hours) in the non-adherent conditions before embedding into fibrin already induced substantial viral lytic gene expression (Fig. S7A in III). The subsequent time-course qRT-PCR analysis showed a time-dependent increase in the mRNA levels of the latent and lytic viral genes in the 3D cultured K-LECs over 2D. This was supported by the concomitant immunofluorescence analysis that revealed enhanced expression of LANA and ORF59 in the K-LEC spheroids over 2D K-LECs (Fig. 7A-C in III). The higher number of LANA positive cells as well as LANA dots per cell in 3D was further confirmed by PCR for viral DNA that showed an increase of KSHV DNA in the 3D cells over 2D ones with time. This increase may result from elevated latent replication, re-infection of the cells by new progeny virions derived from an early activated lytic program upon the transfer from 2D to 3D, or improved maintenance of the viral episomes.

To address whether the observed gene expression changes involve alterations in the epigenetic landscape of the viral genome, we analyzed histone modification patterns of viral episomes in cells grown under 2D or 3D conditions. However, an epigenetic landscape scanning of viral episomes in cells grown under 2D or 3D conditions showed that, at least on the side of viral chromatin, there is no fundamental differences between 3D or 2D conditions in the overall distribution profile of tri-methylated histone H3 at lysine 4 (H3K4-me3), a modification that is commonly associated with open or active chromatin (Fig. 7D in III). One possible reason is that a significantly amount of naïve virion DNA in 3D is devoid of histones and thus refractory to immune precipitation, yet will register in the input channel of our arrays, resulting in artificially lower ChIP vs. input ratios. Another possible explanation for viral gene alteration in 3D would be the loss of another common histone modification, H3K27-me3 that has previously been found to be present on latent KSHV episomes and represses lytic gene expression via recruitment of polycomb repressor complexes (Gunther and Grundhoff, 2010; Toth et al., 2010). A more detailed investigation of multiple histone modifications at various time points of 2D and/or 3D culturing would help to address this question.

The K-LEC spheroids showed a time-dependent increase in the expression of latent genes LANA and vFLIP, as well as of a panel of lytic genes including vGPCR, over the parental 2D cultures, suggesting the importance of cell-cell interactions in 3D culture for virus replication and maintenance. Our previous results suggest that Notch signaling is activated in K-LECs by the KSHV proteins vGPCR and vFLIP, which in turn leads to Notch-dependent induction of MT1-MMP already in 2D KLECs. The Notch/MT1-MMP axis is further enhanced in 3D microenvironment and activates the EndMT program. Very interestingly, we found that culturing K-LECs in 3D microenvironment led to changes in the viral gene expression involving increases in the vFLIP and vGPCR mRNA levels (Fig. 7A in III), indicating that an active virus replication and reactivation program in 3D condition could be the key mechanism to induce LEC cell reprogramming (Summarized in Figure 9).

In KS lesions, all the tumor cells are latently infected by KSHV and express latent genes such as vFLIP and LANA. Several lines of evidence support the role of lytic viral

replication in KS tumorigenesis, and in addition to latent KSHV genes, genes expressed during the lytic replication cycle may contribute to pathogenesis either via paracrine effects or by promoting the dissemination of the virus. Our findings suggest that the 3D model may also provide the virus a permissive cellular microenvironment for efficient maintenance of infection, lytic replication and spread of the viral episomes. Hence, cellular and viral factors operative in the KSHV-induced EndMT could offer attractive targets for intervention and treatment of KS.

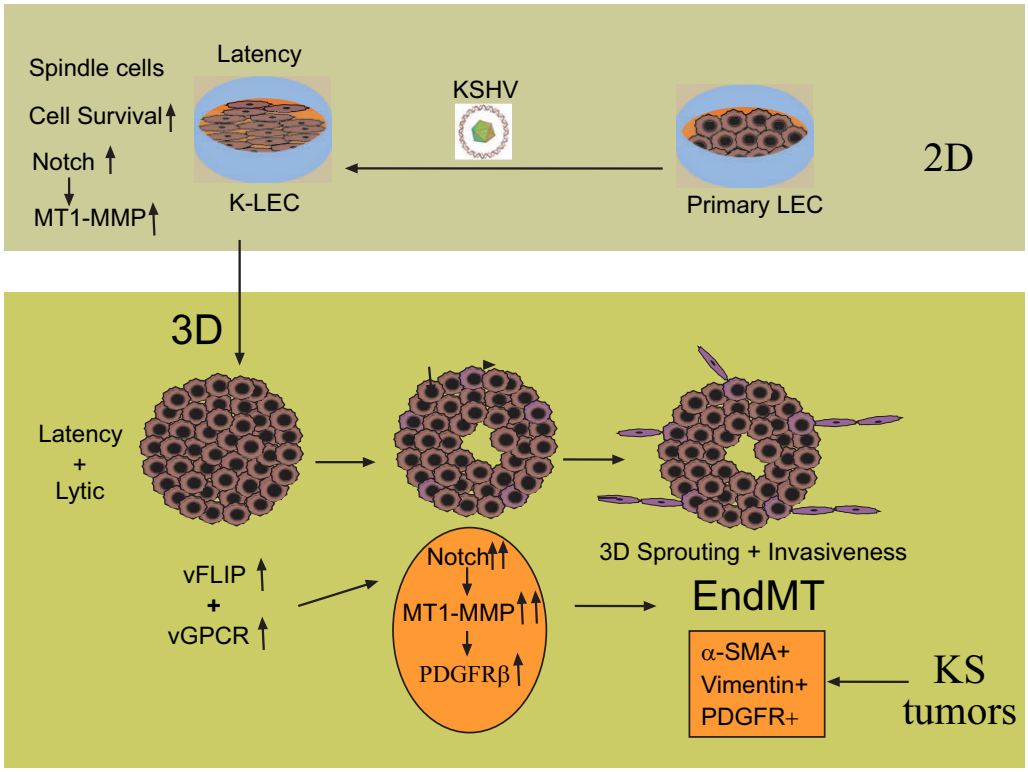

Figure 9. K-LECs cultures in 3D recapitulate pathogenetic aspects of KS. In 2D culture (top-left), KSHV infection of Primary LECs has a tendency to establish latency and acquires partial KS-like phenotypic changes. However, cells tend to lose the viral episomal genomes upon culturing, suggesting that the tumorigenic potential of KSHV on the endothelium may be dependent on the microenvironment. 3D spheroids (bottom) provide the virus a permissive cellular microenvironment for active KSHV latent and lytic replication. Increased expression of lytic viral protein vGPCR and latent viral protein vFLIP activate Notch-MT1-MMP-PDGFR-β signaling axis, giving rise to EndMT, sprouting and invasiveness. EndMT is characterized by downregulation of endothelial markers and the expression of mesenchymal markers, which were found co-distributed in the same cells with KSHV in primary KS tumor samples, suggesting that the 3D culture in this work may recapitulate the known heterogeneity of the cell types in KS tumors.

CONCLUSIONS AND FUTURE PROSPECTS

To date, accumulating evidence suggests that tumor viruses contribute to 15-20% of human cancers worldwide. KSHV is consistently associated with KS, the most widespread cancer in AIDS patients and a primary cause of cancer deaths in parts of Africa. In addition, KSHV causes devastating lymphoproliferative disorders such as PEL and MCD, but the therapies against KSHV associated malignancies are limited.

A feature common to all oncogenic infectious agents is their long-term persistence in infected individuals, allowing time for the multi-step tumorigenesis. A sustained, although low level of reactivation can contribute to progression of KS by not only producing new virions but also by contributing to other aspects of KS pathogenesis, such as the production of inflammatory and angiogenic cytokines. This thesis work demonstrates that Pim-1 and -3 are critical regulators of the KSHV lytic replication, and that silencing of these kinases by RNAi leads to inhibition of the lytic replication cascade at an early stage. Thus, Pim kinases could represent potential novel cellular targets for therapeutic intervention. As animals lacking all three Pim genes are viable with a mild phenotype, one could anticipate that small-molecule inhibitors targeting Pim-1 and -3 might be tolerated without significant side effects. Moreover, inhibition of Pim kinases does not impose mutagenic pressure to viral genes, which will minimize the acquisition of drug resistance, and provide a long-lasting block to virus replication and the progression of the disease.

A specific Pim inhibitor has been successfully optimized to inhibit reactivation in KSHV infected cell models (Sarek et al., manuscript in revision). The MDM2 antagonist, Nutlin-3, is capable of effectively restoring p53 function and eradicating PEL tumor cells both *in vitro* and *in vivo*. However, an active viral reactivation program in some tumor cells, as a side-effect of Nutlin-3 treatment, hampers the apoptotic response of p53 restoration *in vivo*. A combination treatment of Nutlin-3 and the specific Pim inhibitor restores the cell death of those Nutlin-3 resistant PEL tumor cells. Thus, the future application of Pim inhibition as a treatment calls for further evaluation of its applicability as combinatorial therapies in relevant preclinical models for KSHV-malignancies in the future.

Another important finding from the thesis work is the novel Notch-MT1-MMP signaling axis responsible for the functional properties of the emerging mesenchymal cells (motile, invasive), and its potential impact on KSHV pathogenesis. The Notch pathway has been implicated in both KSHV latency (Lan et al., 2007), lytic replication (Chang et al., 2005a; Lan et al., 2005a; Liang et al., 2002), in the survival of KSHV-infected lymphomas (Lan et al., 2009) as well as in KS tumors (Curry et al., 2005; Liu et al., 2009). The efficacy of Notch targeting can be evaluated as a pharmacological control and intervention in KS as well as similar pathological conditions in other cancers. In the future, the role of EndMT in KS pathogenesis and progression would be a new research topic to study. Besides

the Notch-MT1-MMP axis, the 3D *in vitro* viral oncogenesis system and appropriate preclinical models may allow the identification and validation of a plethora of potential new therapeutic targets or be used to test various types of drugs to alleviate this currently incurable cancer.

KS spindle cells and their ontogeny are a matter of intense scrutiny in KS research. The effects of KSHV infection of endothelial cells *in vitro* (2D results in this thesis) poorly reproduce many markers found in KS spindle cells *in vivo*, and infected cells tend to lose viral episomes upon culturing. The 3D culture in the thesis work succeeds in inducing spindle cells that express mesenchymal markers characteristic of KS tumor cells. Therefore, testing the capability of transformed foci formation in the 3D culture system using cells from fresh KS tissue biopsies would strengthen the finding, and provide relevance beyond artificial manipulation *in vitro*. Moreover, the 3D culture is able to provide a permissive microenvironment for continuous viral replication and persistence. Since cultures from primary KS lesions lose KSHV after several passages *in vitro*, 3D methods can be used to test the possibility of generating actual KS cell lines retaining the viral genome, and retention of the expression of KSHV pathogenic genes.

Oncogenic viruses have served as important experimental models to identify and investigate molecular events and regulatory networks that are critical for cancer development and progression. The finding of a novel mechanism of KSHV pathogenesis in an *in vitro* 3D context shows that sophisticated tissue culture techniques such as 3D spheroid assays can be critical to gain insights into *in vivo* viral oncogenesis mechanisms. In this regard, the current research would motivate other investigations of *in vivo*-specific mechanisms of tumor virus pathogenesis that may be reproduced *in vitro*.

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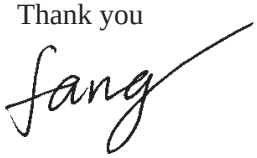
Thank you



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