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Hypoxia and Hypoxia-Inducible Factors in Kaposi Sarcoma-Associated Herpesvirus Infection and Disease Pathogenesis

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Abstract

Kaposi sarcoma-associated herpesvirus (KSHV) is the causative agent of Kaposi sarcoma and several other tumors and hyperproliferative diseases seen predominantly in HIV-infected and other immunocompromised persons. There is an increasing body of evidence showing that hypoxia and hypoxia inducible factors (HIFs) play important roles in the biology of KSHV and in the pathogenesis of KSHV-induced diseases. Hypoxia and HIFs can induce lytic activation of KSHV, and KSHV can in turn lead to a hypoxic-like state in infected cells. In this review, we describe the complex interactions between KSHV biology, the cellular responses to hypoxia, and the pathogenesis of KSHV-induced diseases. We also describe how interference with HIFs can lead to decreased tumor growth and/or death of infected cells and KSHV-induced tumors. Finally, we show how these observations may lead to novel strategies for the treatment of KSHV-induced diseases.

Keywords

Gammaherpesvirus; Kaposi sarcoma; Hypoxia; HIF-1; HIF-2; Viral malignancy; hypoxia response element

Introduction

Within weeks after acquired immunodeficiency syndrome (AIDS) was recognized as a new disease in 1981, it was observed that persons with other AIDS manifestations often had Kaposi sarcoma (KS), a hitherto very rare skin cancer [1,2]. KS was originally reported in 1872 by Moritz Kaposi as a multicentric cutaneous tumor in elderly men [3]. Before the AIDS epidemic, several epidemiologic forms were recognized: (1) classic KS, occurring in elderly men in Mediterranean regions; (2) endemic KS, occurring in younger individuals in sub-Saharan Africa, and (3) iatrogenic KS, occurring in transplant recipients and other medically immunosuppressed individuals [4]. The cause of KS and its association with human immunodeficiency virus (HIV) infection was a mystery until Yuan

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Chang and Patrick Moore reported in 1994 on the discovery of a new gammaherpesvirus called Kaposi sarcoma-associated herpesvirus (KSHV) or human herpesvirus-8 (HHV-8) in Kaposi sarcoma (KS) lesions [5]. Further studies showed that areas with a high incidence of KS, such as sub-Saharan Africa, and persons at risk for HIV/AIDS in the United States, especially men who had sex with men, had a high prevalence of infection with KSHV [6,7]. By contrast, the prevalence of KSHV infection was found to be low in the general population in the United States. KSHV is now known to be the cause of all forms of KS. Soon after the discovery of KSHV, it was found that KSHV is also the cause of a form of multicentric Castleman disease (MCD) and an unusual lymphoma that has been named primary effusion lymphoma (PEL) [8,9]. It was recently recognized that some patients with KSHV infection develop severe inflammatory symptoms without MCD or PEL, and this disease is known as KSHV-inflammatory cytokine disorder (KICS) [10–12].

KSHV has now been studied in extensive detail to understand how the virus enters, replicates in, and survives in cells; avoids immune recognition; and leads to specific diseases [13,14]. Cell lines from KSHV-infected patients with PEL were developed and have been widely used to study the KSHV life cycle; these have been indispensable in the understanding of KSHV pathogenesis[15–17]. In addition, endothelial cell models have been created to gain a better understanding of the KSHV life cycle in different cells and the pathogenesis of KS, PEL, and MCD [16,18–20]. Interestingly, research by several different groups, including our own, has revealed a pivotal role for hypoxia and hypoxia inducible factors (HIFs) in viral maintenance, replication, and pathogenesis.

Studies of the potential role of hypoxia in the KSHV life cycle were initiated in our laboratory when we sought to understand the clinical observation that KS preferentially involves the skin of the feet and lower extremities, which often has poor oxygenation, especially in the elderly men in whom classical KS is seen. Interestingly, this observation had been made by Moritz Kaposi in his original description [3] Exploring this phenomenon, we found that hypoxia could activate virus replication in KSHV-infected PEL cells [21]. This provided one of the first potential mechanisms by which virus lytic replication might be activated in people infected with KSHV and promote disease progression and virus dissemination. Since then, a vast amount of research by many laboratories investigating KSHV around the world have made it clear that hypoxia and hypoxia inducible factors (HIFs) play a central role in the pathogenesis of KSHV-associated diseases. This review describes the latest understanding of the central role in which hypoxia and HIFs participate in KSHV survival, lytic replication, and pathogenesis.

Hypoxia Response Elements in the KSHV Genome and their Contribution to Lytic Replication

When cells or tissues experience low oxygen tension (<5%−1%), they respond by rapidly increasing the cellular levels of hypoxia inducible factor alpha subunits (HIF-1α and HIF-2α) [22,23]. HIF-1α and HIF-2α mRNAs are constitutively expressed and translated, but under normoxic conditions are regularly ubiquitinated and undergo proteasomal degradation. Upon exposure to hypoxia, this process is blocked, and cells rapidly accumulate

HIF-1α and for certain cells HIF-2α within just a few hours. HIF-1α and HIF-2α then dimerize with HIF-1β, a constitutively produced protein already present in the cell, and enter the nucleus. In the nucleus, HIFs activate a variety of genes by binding to promoters through hypoxia response elements (HREs) (see Figure 1). Also, certain stimuli can also increase HIF production by affecting its transcription and translation through the MEK/ERK/PI3K/mTOR pathway.

The core HRE sequence is (RCGTG), although there is considerable variation among various HREs [24,25] Genes upregulated by HIFs enable the cell and organism to sustain hypoxia and restore homeostasis by promoting angiogenesis, iron uptake, vascularization, and hematopoiesis (for review see [26]). Although there is a significant overlap in the genes activated by HIF-1α and HIF-2α, there are certain genes that respond strongly primarily to HIF-1α or HIF-2α [27–29] (and references therein). Also, studies of cells exposed to hypoxia have demonstrated that the expression levels of the individual HIF-1α and HIF-2α proteins vary depending on the cell type studied. In some cell types, only HIF-1α is primarily expressed, while in others HIF-2α is more prominent and in some cases both proteins are made at comparable levels. For example, HIF-2α, also known as endothelial PAS domain protein-1 (EPAS-1), is preferentially expressed in endothelial cells while other cell types express lower levels [30]. Due to these cellular variations, the responses to hypoxia can vary greatly among cell types and this may possibly impact the different diseases caused by viral infection.

The discovery in 2001 that KSHV lytic replication could be activated by hypoxia [21] raised the possibility that promoters within the KSHV genome might contain HREs like those found in the host cell genome. Analyzing the KSHV genome for the core HRE consensus sequence (RCGTG), we noted several potential HREs, including a cluster in the promoter region of open reading frame 34 (ORF34), a lytic gene. Exploring this further, we found that ORF34, and also the replication and transcription activator gene (RTA, encoded by ORF50), the master switch for lytic replication, responded to HIFs. Interestingly, the RTA promoter was preferentially upregulated by HIF-2α [31]. Further studies showed that the entire ORF34-ORF37 lytic gene cluster could be activated by hypoxia and HIFs [32]. This observation also provided evidence that certain lytic genes of KSHV could be directly activated by a cellular signal without necessarily involving the entire lytic pathway. Around this same time, Glaunsinger et al. reported ORF37 of KSHV was a shutoff exonuclease (SOX) that degraded mRNA and suppressed expression of most cellular genes [33]. Interestingly, this gene spared HIF-1α mRNA [33]. This report was particularly revealing in that it demonstrated that KSHV had developed another mechanism to facilitate the activation of virus by HIF. The sparing of HIF mRNA by SOX was further described in several subsequent papers [34,35]. These reports came on top of an earlier report by Sodhi et al. demonstrating that vGPCR (a viral G protein-coupled receptor encoded by ORF74) plays a role in increasing HIF levels by stabilizing HIF-1α [36]. Taken together, these reports provided evidence that KSHV could facilitate increases in HIF activity by several mechanisms, and HIF in turn could stimulate KSHV replication or the activity of specific viral genes.

A detailed analysis of the promoter for the lytic switch protein RTA was subsequently reported by Cai et al. [37]. They noted that the RTA promoter contains several potential HRE elements. They further showed that the KSHV latency-associated nuclear antigen (LANA, encoded by ORF73), one of several proteins expressed during latency, associates with HIF-1α and cooperates in upregulating the RTA promoter in hypoxia to induce lytic replication.

Since these initial studies, functional HREs have been identified in the promoters of other latent and lytic genes of KSHV including LANA [38], vBCL-2 [39] and vGPCR (ORF 74) [40]. Indeed, a recent study showed that a number of areas of the KSHV genome show HIF-1 binding in infected cells [41]. Also, it should be noted that KSHV is not the only virus known to be regulated or affected by HIF and hypoxia. It has been demonstrated that hypoxia and/or HIF activate lytic replication of other viruses including MHV68 [42], EBV [43], and Marek's Disease virus [44] and may play key roles in HBV/HCV and HTLV-1 pathogenesis [45].

Effects of KSHV on HIF and HIF-mediated genes

KSHV infection can in turn affect the expression of HIF and HIF-modulated cellular genes through a variety of mechanisms (Figure 2). The net effect of this is to reprogram the cell to a hypoxic-like state. The first report of this was a study by Sodhi et al. showing that vGPCR could increase HIF levels in KSHV infected cells through p38/MAPK-mediated phosphorylation and suppression of the inhibitory domain of HIF-1α [36]. An additional mechanism for the vGPCR-induced increase in HIF-1 activity was subsequently discovered involving the activation of the mTOR pathway via paracrine signals originating from a small percentage of vGPCR-expressing cells [49]. Additionally, it has been shown that certain KSHV proteins (K1, vIL6, and ORF45) [50–54] as well as KSHV miRNAs (miR-K1 and miR-K4) [55] can enhance mTOR signaling, thus potentially upregulating HIF levels and/or activity indirectly.

Interestingly, several of the viral proteins that directly or indirectly affect HIF-1α and/or HIF-2α levels are upregulated directly by HIF-1α and/or HIF-2α via hypoxia response elements. These include SOX, which spares HIF-1α mRNA; vGPCR which stabilizes HIF-1α through its phosphorylation; vIRF3 by associating with and blocking HIF degradation in normoxia [47]; and LANA which binds to and accumulates HIF-1α in the nucleus of cells [46]. These can all act as positive feedback loops.

While virus-induced regulation of HIF levels may be central for controlling gene expression in KSHV-infected cells (see Figure 2), as well as disease outcomes, it should be noted that the mechanisms can differ in different cells. One reason is the variation in the expression of HIF-1α and HIF-2α in different cell types. In particular, under conditions of hypoxia, HIF-2α is prominently expressed in endothelial cells (believed to be a precursor for KS spindle cells), while HIF-1α is more highly expressed in a wide variety of other cells including PEL cells. This is an area that has been studied only to a limited extent, and further research may help understand variations in the outcome of KSHV infection in different cells.

In this regard, it has been recently discovered that the ORF34 gene product, which can be upregulated by hypoxia or HIF, can stabilize HIF-2α while at the same time cause the degradation of HIF-1α [56,57]. Deletion of ORF34 from the KSHV genome resulted in decreased early gene expression and blocked late gene expression. Together these studies suggest that the ORF34–37 gene products play a key role in the regulation of KSHV lytic replication induced by hypoxia. As will be discussed in more detail below, KSHV manipulates the hypoxic state in a way that favors survival and replication while bypassing hypoxic pathways that would be detrimental to viral survival. For example, while hypoxia usually leads to inhibition of DNA replication by restricting transcriptional activity, vCyclin of KSHV (encoded by ORF72) appears to play a role in balancing HIF levels in hypoxia by promoting its degradation and bypassing the usual inhibitory effect of HIF on DNA replication [41]. Also, some typical HIF-responsive cellular genes such as such as PDK1, LDHA and P4HA appear to be suppressed by certain KSHV-associated viral factors in hypoxia allowing the infected cell to bypass cell cycle arrest.

KSHV Infection Reprograms the Cell to A Hypoxic-Like State

The net result of the effects of KSHV on HIFs is to reprogram KSHV-infected cells to a hypoxic-like state, even in the absence of hypoxia. Many of these changes can aid in cell survival. In 2017, Viollet et al. reported that 34% of the gene expression changes induced by hypoxia or induced by latent KSHV infection of SLK cells were similarly dysregulated [58]. The mRNAs for integrin alpha 5 (ITGAV), nuclear enriched abundant transcript 1 (NEAT1), and baculoviral IAP repeat-containing 3 (BIRC3) were particularly elevated. ITGAV has been shown to bind to glycoprotein B of KSHV and have a role in viral entry while NEAT1, through its upregulation by HIF-2α, promotes cancer cell survival. BIRC3 is a known anti-apoptotic gene, and its expression can be further upregulated by the KSHV-lytic gene K15 [59].

Interestingly, although KSHV infection clearly changes the mRNA landscape of the infected cell by upregulating or downregulating certain host genes, there was very little in common among the microRNA (miRNA) profiles seen during the hypoxic response and KSHV infection. The vast majority of dysregulated miRNAs in hypoxia (88%) and KSHV infection (73%) were found to be downregulated, yet these dysregulated populations showed little overlap. Also, there were only two miRNAs that were upregulated in common among hypoxia and KSHV-infected cells. These two notable exceptions were miR-210 and miR-3074–3p, and there is evidence that they likely play key roles in KSHV pathogenesis[58]. It is important to note that hypoxia itself also alters the miRNA landscape and certain miRNAs termed hypoxamirs (such as miR210) are induced or suppressed by hypoxia [60].

miR-210 is a known HIF-responsive microRNA with a functional HRE in the promoter that plays a role in downregulating hypoxia-responsive genes not needed for cell survival and prevents cells from undergoing cell cycle arrest [61,62]. Also, miR-210 has as one of its targets the mitochondrial iron sulfur scaffold protein (ISCU). The downregulation of ISCU by miR210 can ultimately lead to an increase in the Warburg effect (increased

glycolysis under aerobic conditions) thus promoting cell survival [63,64]. The Warburg effect contributes to the maintenance of KSHV latently-infected endothelial cells [65].

At the time of publication, Viollet et al. had not identified a potential role for miR-3074–3p. However, a potential role for the upregulation of miR-3074–3p in KSHV pathogenesis has since been identified through its effect on Caveolin-1. Caveolin-1 (CAV1) is known to be responsive to both HIF-1α and HIF-2α [66]. In 2020, CAV1 was identified as a primary target for miR3074–3p, leading to its downregulation in myoblasts [67]. In addition, CAV1 gene expression has also been reported to be suppressed by KSHV vIL-6; this results in the activation of AKT signaling, promotion of cell invasion, and growth transformation induced by KSHV [68]. Therefore, it appears that KSHV infection can downregulate CAV1 mRNA by two mechanisms, one of which involves miR3074–3p. This is an example of KSHV evolving more than one way to inhibit the expression of a hypoxia-responsive cellular gene that would otherwise interfere with survival of KSHV-infected cells. Although many hypoxic pathways can favor KSHV pathogenesis, certain other hypoxic responses can be detrimental to the survival and growth of KSHV-infected cells (cell cycle arrest and inhibition of DNA replication). In the cases described above for mir210 and miR3074–3p, the microRNAs would act to promote Akt signaling and the Warburg effect. (Figure 3).

Additional Metabolic Reprogramming Induced by KSHV

Although KSHV-infected cells in latency demonstrate at least a partial hypoxic-like signature, Singh et al. have further demonstrated that KSHV- infected cells in hypoxia (or treated with $CoCl_2$, a hypoxic mimic) have additional metabolic reprogramming during lytic activation [40]. The changes include glucose dependency, high glucose uptake, and high lactate release. Interestingly, they demonstrated that vGPCR encoded by ORF74 could alone impart many of the changes they were seeing in hypoxic infected cells and provided evidence that ORF74 was itself upregulated by hypoxia and HIFs through several HREs within the promoter of vGPCR. They also demonstrated a synergy between certain KSHV proteins and hypoxia. In other studies, Cai et al. demonstrated that HIF and LANA cooperate to activate lytic replication via the HREs present in the RTA promoter [37,48]. According to their findings, although KSHV infection can promote the accumulation of HIFs in normoxia, the exposure to hypoxia appears to tip the virus further toward lytic replication This may in part be explained by the “redistricting” of HIF binding to different sites on the KSHV genome upon hypoxic exposure [41]. This may be further supported by the recent work by Lee et al. demonstrating that hypoxia can promote lytic gene activation during initial infection prevent the establishment of latency [69]

Although there is no animal model that can be used to directly study KSHV and the diseases it causes, there is a mouse herpesvirus counterpart, murine herpesvirus 68 (MHV68) that manifests many of the properties of KSHV. Lopez-Rodriquez et al. demonstrated that MHV68-herpesvirus-encoded vGPCR and hypoxia had a role in lytic activation of MHV68 [42]. They also found that MHV68 infection elevated HIF-1α levels as well as cellular genes activated by HIF-1α. Inactivation of HIF-1α significantly decreased viral lytic replication and silencing of HIF-1α also reduced the expression of viral genes found to contain HREs. Deletion of HIF1α impaired viral expansion during acute infection *in vivo* and affected

reactivation from latency. These data suggest that while KSHV or MHV68 infection may reprogram the cell to a hypoxic-like state, exposure of the infected cell to hypoxia will enhance this effect and may tip the scale towards lytic replication and viral spread.

Further supporting a role for hypoxia in viral reactivation, Singh et al. demonstrated that hypoxia mediates a number of effects on both transcriptional activators and repressors on the KSHV genome [40]. This phenomenon is not simply due to a direct effect of hypoxic exposure but also involves interactions with KSHV-encoded proteins. For example, in hypoxia, DNA polymerase 1α is usually degraded; however, KSHV LANA protects it from being degraded in hypoxia [46]. Proteins that are usually degraded in hypoxia such as those in the origin recognition, pre-initiation, and replication initiation complexes, all were protected by KSHV infected cells under conditions of hypoxia, thus allowing for continued DNA replication. LANA was found to be the primary means by which KSHV protects the cells in hypoxia in a way that allows this continued DNA replication, amplification, and cell division.

While much of this review has touched on the role of HIF-1α, it is important to keep in mind that HIF-2α can also play a role, especially in endothelial cells. Indeed, latent KSHV infection of endothelial cells induces both HIF-1α and HIF-2α expression and these factors are increased in KS tissue [18,49,70]. A study published by Dr. Mesri's group in 2021 revealed a novel role for HIF-2α in initiation of translation of viral lytic mRNAs [71]. In this study they found that lytic replication in iSLK cells initially increased HIF-2α levels within 24h but decreased its expression level in later stages (48–72h). They also found that suppressing HIF-2α led to a 50% decrease in viral reactivation that did not occur with HIF-1α suppression. The unique role of HIF-2α in this model was the translocation of HIF-2α to the ER and subsequent increased lytic replication through the HIF-2α -regulated eIF4E2 translation-initiation complex. It is important to realize that these changes were all observed in normoxia and again supports a role for the viral induced expression of HIFs absent hypoxia.

HIF as a Potential Target for KSHV-Associated Diseases

The hypoxic state of KSHV-associated malignancies and their reliance on HIF proteins suggests that HIFs could be a potential target for therapy of these diseases. In one approach, Davis et al., showed that two of the KSHV lytic genes, ORF21 (encoding a thymidine kinase) and ORF36 (encoding a phosphotransferase), can phosphorylate two FDA-approved antiviral agents, zidovudine (azidothymidine, AZT) and ganciclovir, respectively, into their higher phosphorylated moieties that are toxic to cells in addition to having anti-viral activity [72]. The production of these phosphorylated forms in PEL cell lines was enhanced during hypoxia, and was associated with not only higher cytotoxicity, but also suppression of KSHV lytic reactivation [72]. Interestingly, these KSHV genes are also activated in KSHV-associated MCD. KSHV-MCD is a serious B-cell disorder caused by KSHV that is characterized by elevated levels of inflammatory cytokines hIL-6, hIL-10, and KSHV vIL-6. It is usually fatal within two years if not treated. The pathology of KSHV-MCD is characterized by KSHV-infected plasmablasts in lymph nodes, many of which express the lytic genes of KSHV including ORF21 and ORF36 [73]. The expression of ORF21 in these

cells was later found to be stimulated in part by the high levels of spliced X-box binding protein (XBP-1) that are found in developing B cells and KSHV-MCD plasmablasts [74]. Interestingly, XBP-1 is upregulated by hypoxia and required for cell survival in hypoxia [75] Therefore, hypoxia either directly or indirectly may play a role in activating ORF21 and ORF36. These observations led to a pilot clinical study assessing a combination of high dose valganciclovir (a prodrug of ganciclovir) and high dose zidovudine in KSHV-MCD [76]. Eighty-six percent of the patients in this study showed major clinical responses with significant decreases in the levels of hIL-6, hIL-10, and KSHV viral load. High dose AZT/GCV is now included as one of the treatment choices for KSHV-MCD following failure of other regimens. However, for unclear reasons, relapses from this regimen are common, and other regimens such as rituximab-based therapies are now more often utilized [11,77,78].

Over the past several years, there has been a substantial interest in the use of inhibitors of HIF to treat various cancers. One broad concern is that given the importance of HIFs overall, their inhibition can potentially have toxic effects. However, there has already been some clinical success with this approach and given the key role of HIFs in KSHV biology and the pathogenesis of KSHV diseases, this is a potentially promising area for therapeutic research. KS tissues express both HIF-1α and HIF-2α, and evidence from *de novo* infection of endothelial cells suggests that KSHV directly leads to the stabilization and activation of these proteins; this leads to increased glycolysis, which can contribute to the survival of KSHV-infected endothelial cells [18,65]). Additional key evidence supporting the use of HIF inhibitors in KS comes from a study conducted by Zham et al., which showed that paracrine signaling from KSHV vGPCR leads to an mTOR-pathway-dependent upregulation in HIF-1α, HIF-2α, and VEGF in endothelial cells [49]. More importantly, they observed that endothelial cells expressing the KSHV oncogenes vCyclin and vFLIP can form KS-like tumors in nude mice in the presence of vGPCR, and the growth of these tumors as well as HIF expression in the tumor lesions could be blocked by treating the mice with two FDA-approved drugs, rapamycin and digoxin, both of which have been shown previously to inhibit HIF-1α and HIF-2α levels and activity [49,79–81]. Additional evidence for the dual targeting of HIF-1α and cMyc in KS and PEL xenograft models with echinomycin was recently reported [82]. Echinomcyin dramatically regressed cell growth in KS and PEL mice models suggesting this strategy of dual targeting may be more effective than HIF inhibitors alone. These observations provide evidence that suppressing HIF-related pathways could be beneficial for KS. In fact, it has been reported that replacing cyclosporin with rapamycin (sirolimus) is effective against Kaposi sarcoma in renal transplant recipients [83]. Rapamycin inhibits mTOR, and among other effects, it inhibits the transcription of HIF-1A mRNA. Rapamycin has been found to be particularly effective when it is substituted for other immunosuppressive agents in solid organ transplant recipients, and in fact is recommended as first line therapy for such patients in the National Comprehensive Cancer Network (NCCN®) guidelines [[84].

There is also evidence that PEL cells are dependent on HIF-1α for their growth. One key piece of evidence comes from a preclinical study conducted by Shrestha et al. [85], which showed that knocking down HIF-1α expression in PEL cell lines causes a dramatic decrease in the expression of both latent and lytic KSHV genes, inhibition of basal as well

as hypoxia-induced lytic reactivation of KSHV, and suppression of glycolysis and lipid metabolism [85]. They also showed that HIF-1α inhibition using a HIF-1 inhibitor, PX-478, led to selective growth inhibition of PEL cell lines but not uninfected non-PEL cell lines, providing direct evidence that HIF-1α inhibition is a rational strategy for treating PEL. PX-478 was found to be safe in a phase I trial but no further studies have been reported [86]. However, these results suggest that HIF inhibitors may be worth exploring further. In this vein, several FDA-approved drugs that are known to indirectly inhibit HIF-1α signaling have shown activity against PEL in preclinical models. The AKT/PI3K/mTOR pathway, a known activator of HIF-signaling pathway, is constitutively active in PEL cells [79,87]. HIF-1 activation mediated by this pathway can be inhibited by rapamycin and everolimus, two mTORC1 inhibitors approved by FDA for use in various diseases [79,80]. Rapamycin, which as noted above is effective in KS, has been shown to prevent the growth of PEL cell lines as well as murine xenograft models of PEL [87]. Everolimus has been shown to induce apoptosis of PEL cell lines, decrease KSHV latent gene expression and virus production, as well as increase their recognition by and activation of dendritic cells, thus not only leading to direct cytotoxicity of PEL cells but also potentially facilitating immune-mediated clearance of PEL cells [88]. These observations provide a potential rationale for testing or repurposing these drugs for use in PEL.

Given that many advanced tumors are hypoxic and are dependent on HIF proteins for their continuous growth/metastasis, there is a continued interest in developing novel HIF inhibitors. Although several of the FDA-approved anti-cancer agents have been found to inhibit HIF-1 and/or HIF-2 signaling (see reviews [22,89], development of direct and specific inhibitors of the HIF proteins has proven difficult. To this end, belzutifan, a specific HIF-2α inhibitor that binds to HIF-2α and prevents its dimerization with HIF-1β, was recently approved by the FDA for the treatment of various tumors in patients with VHL (von Hippel-Lindau) disease [90]. Since HIF-2α is expressed in KS tissues from patients where it likely contributes to KS pathology based on several *in vitro* and *in vivo* studies [18,49,65,71] belzutifan provides a potential drug candidate for testing in KS. As for a direct inhibitor of HIF-1α, acriflavine, an FDA-approved compound, was identified as the most potent inhibitor of HIF-1α in a study screening 3,120 drugs that are either FDA-approved or in phase II clinical trials [91]. Acriflavine was shown to bind to the PAS-B subdomain of HIF-1α or HIF 2α, blocking their heterodimerization with HIF-1β, thus inhibiting their DNA-binding and downstream transcriptional activity. Various groups have now provided preclinical evidence of anti-cancer activity of acriflavine in a variety of solid cancers as well as in lymphomas [92]. It is important to note that, while both belzutifan and acriflavine have been shown to inhibit HIFs, they have not yet been tested in the context of KSHV-associated diseases. Given the highly complex signaling pathways involving HIF-1α and HIF-2α, their disparate effects on KSHV biology in different KSHV-diseases, and potential activation of compensatory signaling pathways, preclinical analyses should first be conducted.

Another possible approach involves galectin-1 (gal-1), which is upregulated by both hypoxia and KSHV, and has been shown to be necessary for hypoxia-driven angiogenesis in mice [93]. Angiogenesis and growth of the KS-derived cells *in vivo* could be suppressed by administration of therapeutic anti gal-1 monoclonal antibody. Several classes of galectin-modulating agents are currently under development for use in various diseases [94].

Belapectin, a polysaccharide inhibitor of galectin-3 (gal-3) that was recently approved by the FDA for use in head and neck squamous cell carcinoma, can also bind to extracellular gal-1, albeit with lower affinity compared to gal-3 [95,96], and thus presents a potential agent to test in KS.

Considerations for Future Research

Although we now have a significant body of knowledge to demonstrate that hypoxia and HIF favor KSHV replication and pathogenesis and that inhibiting HIF expression is a reasonable therapeutic option for KSHV diseases, there are still several areas with unanswered questions that have yet to be explored or have only been touched upon. The answers to these questions may directly impact on the success or failure of therapeutics used to target HIFs in KSHV diseases. For example, several viral proteins both bind to and stabilize HIF-1α. How do these proteins affect HIF-2α? At least one KSHV protein, the ORF34 gene product, is known to increase degradation of HIF-1α while stabilizing HIF-2α [56,57]. Another KSHV protein, vCyclin, also leads to degradation of HIF-1α at certain stages of infection to allow for cell cycle progression but what is its effect on HIF-2α? In some cases, the cells being examined may not express HIF-2α so the point may be moot. Yet, for endothelial cells that express both HIFs, the answer to this question becomes more relevant. What is the role for HIF-1 versus HIF-2 in endothelial cells infected with KSHV? A related question is: what is the relative effect of each of these HIFs on the individual viral genes? It will be important to understand the role of HIF-1 vs HIF-2 in various cells infected with KSHV to develop a fuller picture of the effects of HIFs on this virus. This is a largely underexplored area. Another consideration is that long term KSHV infection could lead to a prolonged hypoxia-like state in infected cells. In this case, do these cells undergo switches to HIF-2 and/or HIF-3, a third HIF whose effects are at present poorly understood [97]? Studies suggest that miRNAs can mediate this hypoxic switch [98] and so their potential role in this area should not be ignored.

In summary, there is a complex panoply of interactions between KSHV and hypoxia or HIFs. Hypoxia and HIFs can substantially affect expression of various KSHV genes and KSHV replication, and KSHV infection can at the same time influence on the levels of HIF. The effects of hypoxia can affect the manifestations of KSHV-induced diseases (e.g., the predilection of KS to develop on the feet), and as noted, it may provide avenues for targeted therapy. While much has already been learned, there is much we still do not understand, and this will be a fruitful area for future research.

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related to internalization of target receptors, epigenetic analysis, and ephrin tyrosine kinase inhibitors. All rights, title, and interest to these patents have been assigned to the U.S. Department of Health and Human Services; the government conveys a portion of the royalties it receives to its employee inventors under the Federal Technology Transfer Act of 1986 (P.L. 99–502).

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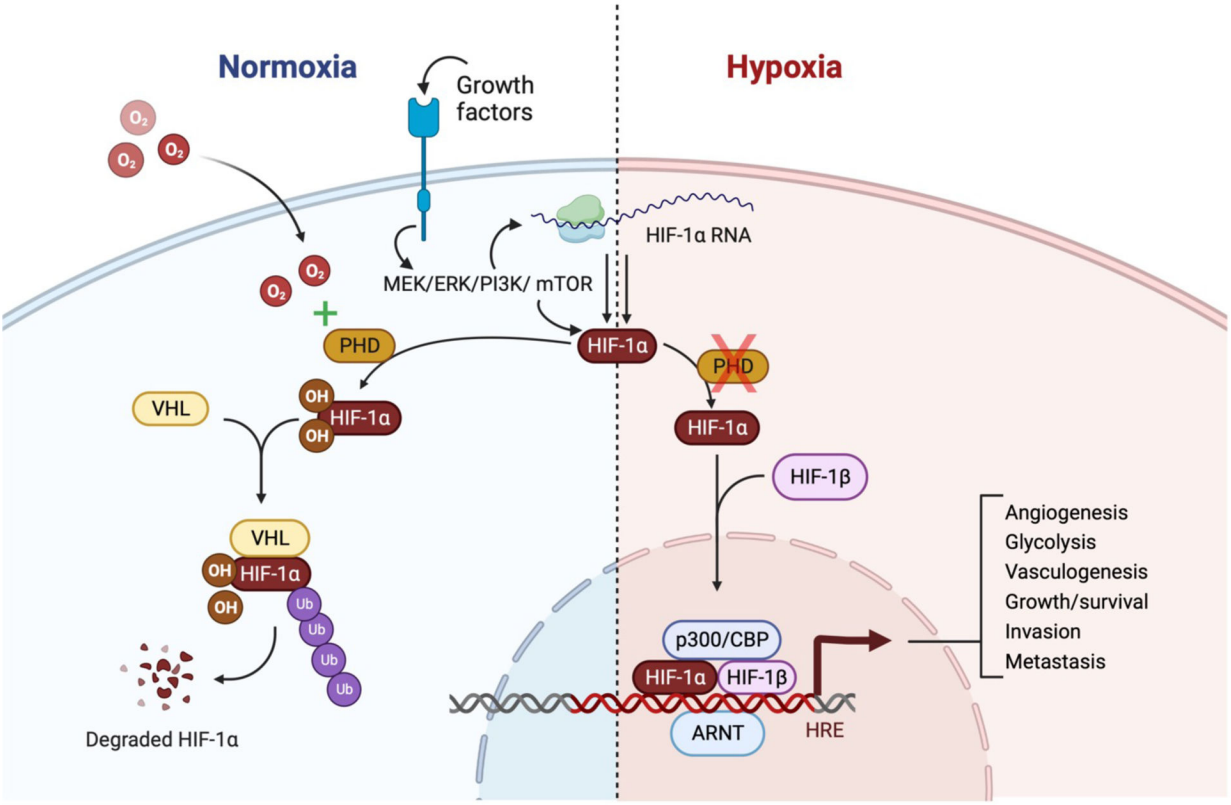

Figure 1. Regulation of HIF-1α.

HIF-1α mRNA is constitutively expressed, and the regulation of HIF-1α protein levels in the cells occurs primarily via oxygen-dependent degradation of HIF-1α protein. In the presence of normal oxygen concentrations, HIF-1α is hydroxylated by prolyl hydroxylase domain-containing enzymes (PHDs). The hydroxylated HIF-1α is then recognized and polyubiquitinated by an E3 ubiquitin ligase, pVHL (protein von hippel-Lindau), leading to HIF-1α degradation. During hypoxia, PHDs are unable to hydroxylate HIF-1α, thereby sparing it from being degraded. HIF-1α then forms a heterodimer with HIF-1β and is translocated to the nucleus where the HIF-1α/HIF-1β complex binds to HREs and recruits transcription factors p300/CBP, resulting in the transcription of HIF-1α-responsive genes involved in a variety of cellular processes. In addition to oxygen levels, certain growth factors and metabolites can also regulate the production of HIF-1α. MEK/ERK/PI3K/mTOR pathways activated by growth factors can increase both transcription and translation of HIF-1α, thereby enhancing HIF-1α level and activity. HIF-2α is similarly regulated.

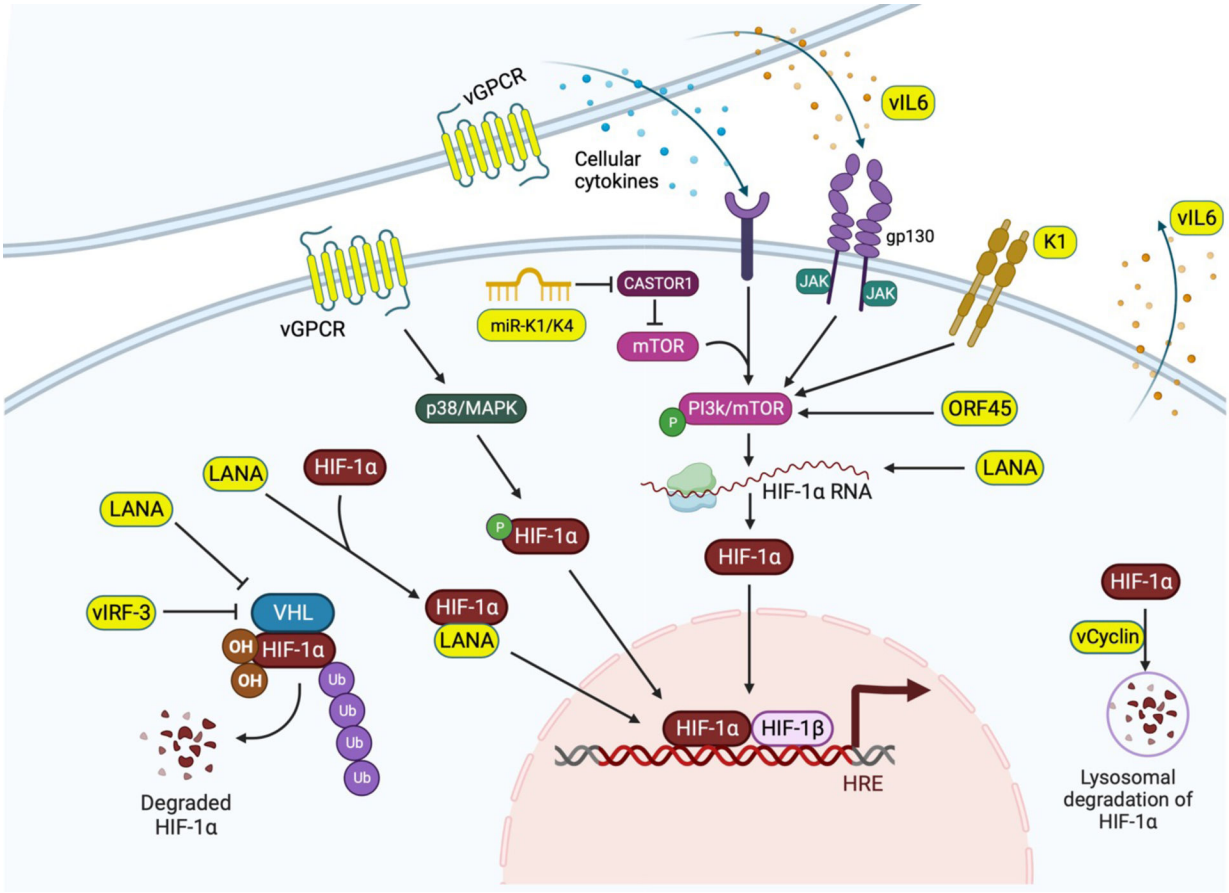

Figure 2. Regulation of HIF-1α by KSHV.

KSHV infection can modify HIF levels and activity in a variety of ways. LANA directly activates the HIF-1α promoter to increase HIF-1α transcription [37]. In addition, LANA binds to HIF-1α protein, which leads to its stabilization and translocation to the nucleus, thus increasing its activity during normoxia in activating RTA [46]. KSHV LANA and vIRF-3 can also block VHL-induced degradation of HIF-1α during normoxia [47,48]. KSHV vGPCR enhances the p38/MAPK pathway, which causes phosphorylation of the inhibitory domain of HIF-1α, leading to increased HIF-1α-mediated transcriptional activity [36]. Paracrine signaling by cytokines upregulated by vGPCR can also lead to the phosphorylation of mTOR in neighboring cells, thus inducing an mTOR-dependent increase in the transcription as well protein synthesis of HIF-1α [49]. Other KSHV proteins, including K1, viral interleukin-6 (vIL6), and ORF45, have also been shown to activate the PI3K/mTOR pathway and can also potentially regulate HIF-1 levels indirectly [50–54]. Two KSHV-encoded microRNAs (miRNAS), miR-K1 and miR-K4, can activate the mTOR pathway by downregulating CASTOR1 (cytosolic arginine sensor for mTORC1), an mTORC1 inhibitor, thus potentially increasing HIF-1 levels via mTOR activation [55]. Not shown in this model is the KSHV SOX protein encoded by ORF37, which degrades most cellular mRNAs but spares HIF-1α mRNA[33]. By contrast to the above, which enhance HIF activity, vCyclin negatively regulates HIF-1α levels during hypoxia by facilitating

its degradation via lysosomal pathway to prevent HIF-1α-mediated inhibition of DNA replication [41].

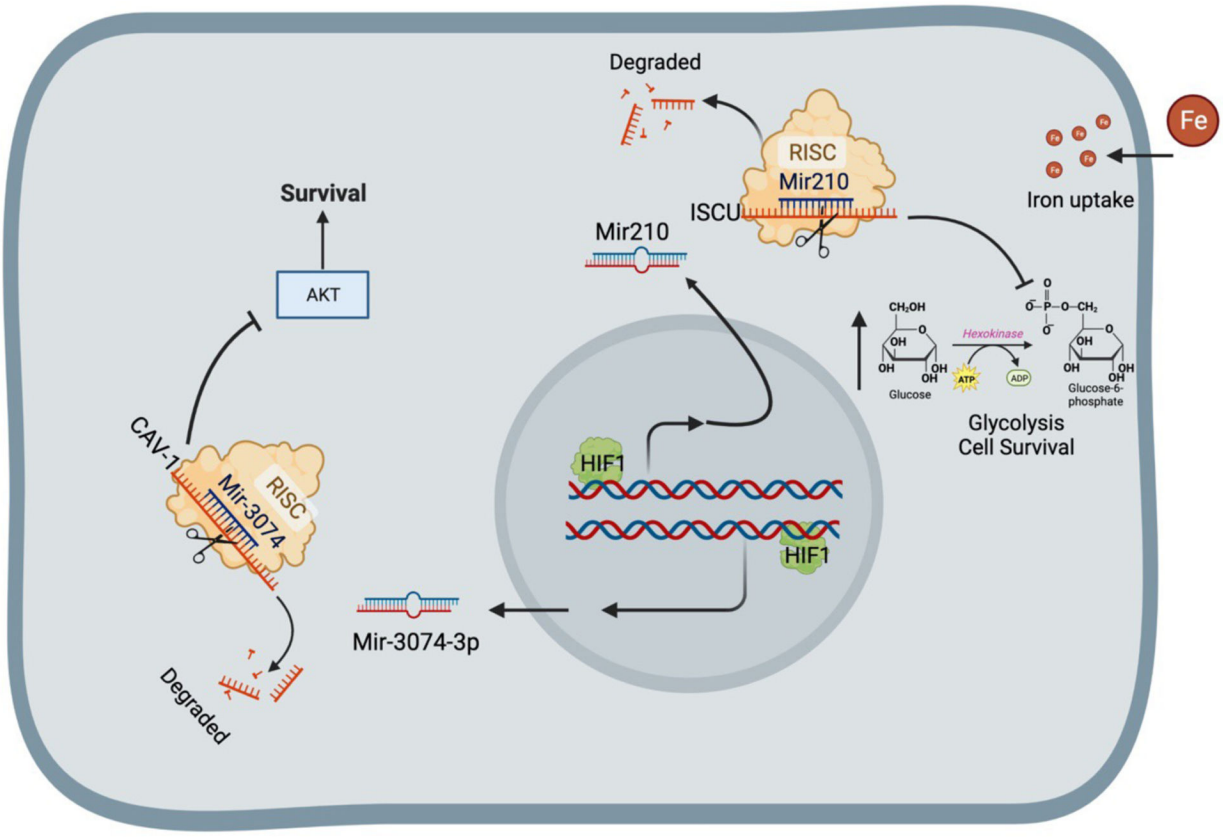

Figure 3. KSHV infection and hypoxia upregulate Mir210 and Mir3074–3p resulting in increased cell survival.

Both KSHV infection and hypoxia significantly upregulate two cellular micro RNAs. The first, Mir210, can downregulate ISCU and this can contribute to a shift to glycolysis, enhance cell survival [61,62], and mediate an increase in iron uptake required for cell growth [63,64]. The second, Mir307403p, targets Caveolin 1 (Cav1), a HIF-responsive gene [66]. Mir307403p is elevated in hypoxia and infection and can target Cav1 expression by degradation of its mRNA resulting in the activation of AKT signaling, promotion of cell invasion, and growth transformation induced by KSHV [67,68].