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Review

Epstein-Barr virus lytic reactivation regulation and its pathogenic role in carcinogenesis

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Abstract

Epstein-Barr virus (EBV) has been associated with several types of human cancers. In the host, EBV can establish two alternative modes of life cycle, known as latent or lytic and the switch from latency to the lytic cycle is known as EBV reactivation. Although EBV in cancer cells is found mostly in latency, a small number of lytically-infected cells promote carcinogenesis through the release of growth factors and oncogenic cytokines. This review focuses on the mechanisms by which EBV reactivation is controlled by cellular and viral factors, and discusses how EBV lytic infection contributes to human malignancies.

Key words: Zta, Rta, Epstein-Barr virus, latency, reactivation, carcinogenesis

1. Introduction

EBV is an oncogenic virus that is linked with several malignancies, including nasopharyngeal carcinoma (NPC), Hodgkin's lymphoma (HL), Burkitt's lymphoma (BL), and gastric carcinoma [1]. In the host, EBV can establish two types of infection known as latent and lytic. During latency, only a limited number of viral genes are expressed and the viral genome exists in the nucleus as an episome. Upon reactivation, EBV briefly passes through three consecutive lytic phases, including immediate early (IE), early (E), and late (L) [2]. The viral IE genes *BZLF1* and *BRLF1* are first transcribed to encode the transactivators, Zta and Rta, respectively, followed by expression of the early genes required for EBV genome replication. After EBV DNA replication, late genes are expressed that encode mainly viral structural proteins, including capsid antigens and membrane proteins, followed by viral genome encapsidation and the production of mature virions. Although all EBV-associated cancers involve the latent cycle of EBV, the viral lytic cycle also contributes to the development and maintenance of malignancies through the induction of growth factors and oncogenic cytokine production [3-5].

In this review, we describe recent advances regarding the mechanisms underlying EBV reactivation, focusing on the control of the host and the virus itself, and discuss the contribution of viral lytic infection to EBV-associated malignancies.

2. Zta and Rta synergistically trigger EBV reactivation

Following various stimuli, such as 12-*O*-tetradecanoylphorbol-13-acetate (TPA), sodium butyrate, anti-Ig, and transforming growth factor-beta (TGF-β), EBV reactivation can be triggered by two immediate early (IE) transactivators, Zta and Rta. Together, both IE proteins turn on the entire lytic viral cascade of gene expression and EBV replication. Zta, a member of the basic-region leucine zipper (bZIP) family of transcription factors, activates the expression of lytic EBV genes by binding to the

activator protein (AP)-1-like motif known as Zta response elements (ZREs) [6,7]. In addition, it also functions as a replication factor for EBV genomic DNA by binding the lytic origin of replication, oriLyt [8]. Similar to Zta, the Rta protein can transactivate lytic target promoters by direct binding to Rta response elements (RREs) [9]. Although Rta is unable to recognize oriLyt, it plays an indispensable role in the process of lytic DNA replication by activating the expression of the *BHLF1* gene which encodes replication proteins [10]. This synergy is achieved because Zta and Rta activate both their own and one another's promoters, which greatly amplifies their lytic-inducing effects [11]. Zta can directly activate transcription from its own *BZLF1* promoter (Zp), by binding to the ZIIIA and ZIIIB elements of Zp [12] and the *BRLF1* promoter (Rp) by binding to three known ZREs (ZRE1, ZRE2 and ZRE3) within Rp [13]. However, Rta activates its own promoter through an indirect mechanism involving a direct interaction with specificity protein (Sp1) through an intermediary protein, MCAF1, to form a complex on Sp1-binding sites [14]. Rta also activates Zp indirectly through activation of the mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3-kinase (PI3-K) pathways, resulting in phosphorylation of transcription factors that bind to a ZII cyclic AMP response element, such as activating transcription factor-2 (ATF-2) or c-Jun [15,16].

3. Host factors contributing to the regulation of EBV reactivation

3.1. The role of post-translational modifications in the functional activities of Zta and Rta

The balance between EBV latent and lytic infection in host cells is initially implicated in transcriptional control of the *BZLF1* and *BRLF1* genes. Cellular transcription factors and their binding motifs within Zp and Rp have been well-studied [17,18]. However, activation of both IE promoters is not sufficient for induction of EBV reactivation. The ability of Zta and Rta to trigger EBV reactivation is also regulated through post-translational mechanisms. Among them, phosphorylation is the most common post-translational modification and modulates the transcriptional potential of transcription factors regardless of whether they are encoded by the host cell or the virus. Phosphorylation of serine residue 173 (Ser173), located in the DNA binding domain of Zta, promotes viral replication by enhancing Zta's affinity for DNA, but is not required for activation of early lytic genes [19]. Ser186 of Zta is phosphorylated by protein kinase C after stimulation with TPA. The phosphorylation of Ser186 is essential for the full functional activity of Zta during the lytic cycle [20]. In addition to Ser173 and Ser186, Zta was shown to be constitutively phosphorylated at multiple sites [21]. Nonetheless, the role of phosphorylation in the functional activity of Zta remains largely unknown. Unlike phosphorylation, sumoylation modification often negatively affects Zta transcriptional activity [22,23]. Recent evidence revealed that sumoylation of lysine 12 results in Zta repression of viral gene expression, promoting EBV latency and, also, that the EBV-encoded protein kinase (EBV-PK) reverses the sumoylation of Zta during EBV reactivation [22]. Subsequently, Murata *et al.* demonstrated that the inhibitory effect of sumoylation on Zta activity is mainly mediated by recruiting histone deacetylase (HDAC) complexes [23].

In addition, post-translational modifications have been shown to affect Zta and Rta activities through protein-protein interactions. In EBV-infected cells, the transcription factors Ikaros, Oct-1, and TAF4 and the retinoblastoma (Rb) protein directly interact with Rta, and the interactions are thought to be important for Rta-mediated disruption of viral latency [14,24-26]. Mutation analysis revealed that the interactions require the DNA-binding/dimerization domain of Rta. Transducer of regulated CREB protein 2 (TORC2) and C/EBP have been identified as co-activators for Zta to activate its own promoter, Zp [27,28]. Interestingly, both EBV IE transactivators, Zta and Rta, have been shown to interact with cAMP response element binding (CREB)-binding protein (CBP), which exhibits histone acetylase activity. The interactions enhance Zta and Rta transactivator activity and increase their ability to induce the lytic form of EBV infection in latently-infected cells [29]. Alternatively, protein-protein interactions are also related to the reduced transcriptional activity and weak affinity for DNA of IE proteins. For example, the cellular transcription factors, Oct-2, Pax-5, NF-κB, and c-Myc, inhibit the induction of EBV lytic reactivation by interacting with Zta [30-33].

3.2. Cellular signaling pathways involved in EBV reactivation

In the human host, B-cell receptor (BCR) antigen stimulation is known to reactivate EBV latency. An accumulation of data demonstrated that the PKC, MAPK, and PI3-K signaling pathways are involved in BCR induction of the EBV lytic cycle [34]. Eventually, a network with crosstalk of these signaling pathways leads to activation of several positive transcription factors on Zp or Rp, thereby stimulating the latent-lytic switch (Figure 1).

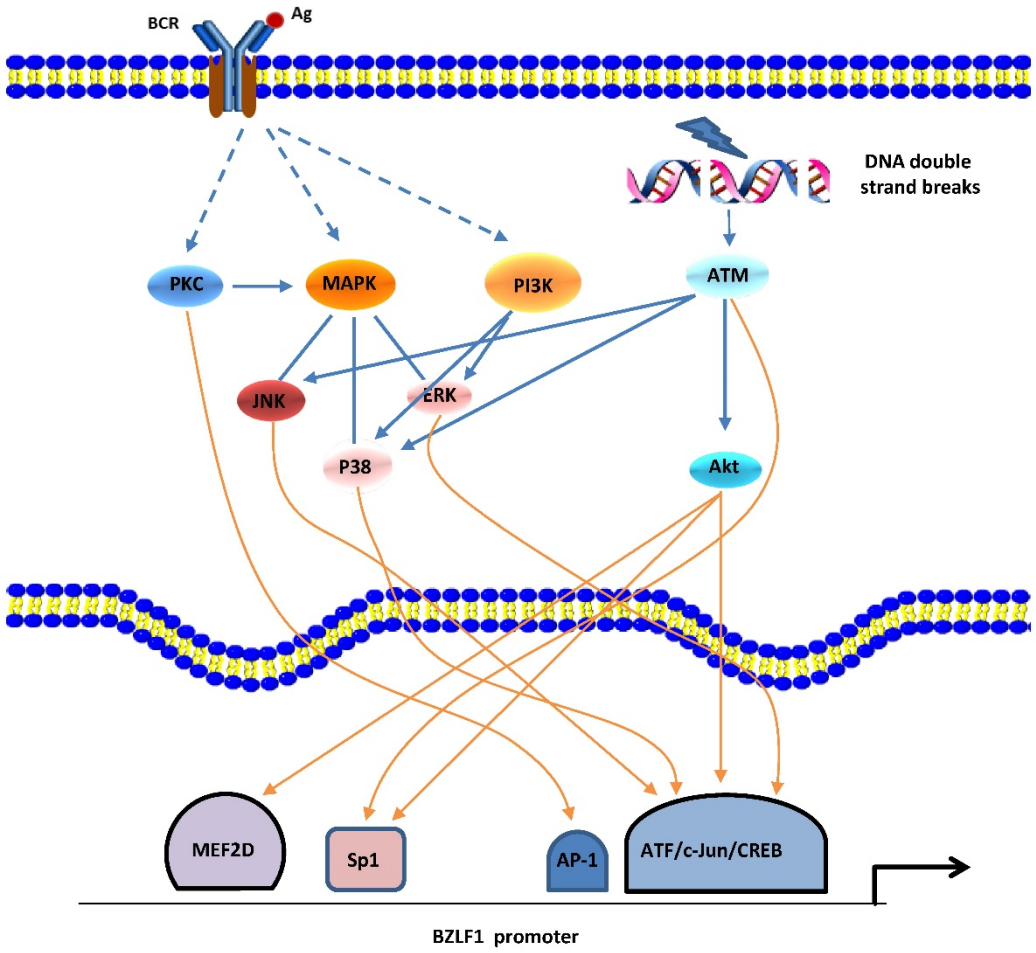

Figure 1. Signaling pathways of *BZLF1* promoter activation. EBV reactivation can be induced by BCR-mediated signaling pathways or cellular stress (e.g., DNA damage). PKC, MAPK (ERKs, JNKs, and p38), and PI3K pathways as well as ATM-dependent mechanisms appear to be involved. A network with crosstalk of four major signaling pathways leads to activation of several positive transcription factors, followed by transcription from Zp.

Gao *et al*. first reported the involvement of the PKC pathway in the latent-lytic switch [35]. The switch, marked by Zta expression, can be induced by active NF-κB and AP-1 through PKC. This report was followed by similar reports from other groups [36,37]. The MAPK family consists of 3 member cascades, extracellular signal regulated kinases (ERKs), c-Jun NH_2-terminal kinases (JNKs), and p38 and is usually activated as a partner of the PKC signaling pathway. JNK signaling leads to the phosphorylation of c-Jun and c-Jun/c-Fos cooperate with Smads proteins to bind the AP-1 motif and the Smad4-binding element within Zp, followed by expression of the *BZLF1* gene [38]. By inducing the phosphorylation of c-Jun/ATF2, the MAPK signaling pathways are also required for Rta-mediated activation of Zp [25]. Iwakiri and colleagues demonstrated that PI3-K/Akt signal transduction contributes to transcription from the promoters of the *BZLF1* gene [39], which is consistent with our recent study showing that the PI3-K/Akt and ERKs pathways are involved in the EBV spontaneous lytic cycle cascade [40]. PI3-K/Akt signaling has been shown to activate cellular transcription factors c-Jun, ATF2, CREB, Sp1, and myocyte-specific enhancer factor 2D (MEF2D) that activate Zp and/or Rp [18]. Moreover, in cells that are not responsive to BCR-mediated EBV reactivation, active PI3-K activates signaling cascades for the ERKs and p38 pathways, resulting in initiation of the EBV lytic cycle [39].

Recently, the ataxia telangiectasia mutated (ATM) activation that occurs in response to DNA damage or oxidative stress has been shown to induce EBV reactivation through a p53-dependent mechanism [41,42]. ATM and downstream signaling pathways p38 and JNKs are responsible for phosphorylation of p53 at multiple sites, and the activated p53 protein mediates expression of the *BRLF1* gene by directly binding Rp.

3.3 Epigenetic regulation of EBV reactivation

Epigenetic factors, including viral genome methylation and histone modifications, also play an important role in regulating the state of EBV infection in host cells.

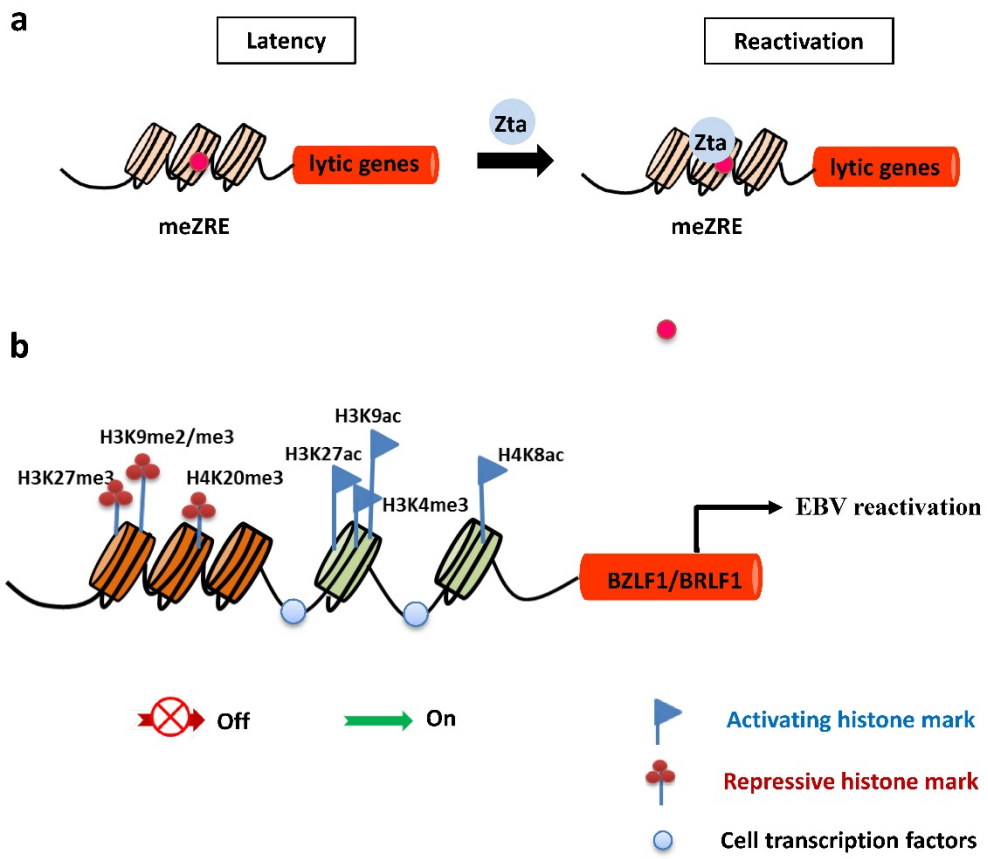

Figure 2. Epigenetic control of EBV lytic reactivation. a. During latency, EBV lytic promoters are silenced by host-driven methylation. The IE protein Zta preferentially binds to methylated ZREs (meZREs) in lytic promoters to initiate replication from this epigenetic repressed state. **b.** The IE promoters Zp and Rp are controlled by several repressive histone modifications during EBV latent infection. After nucleosomes are removed locally, activating histone marks are established and allow the access of transcription factors to induce expression of both IE genes.

DNA methylation

In latently-infected cells, the promoters of EBV lytic genes are intensively repressed and silenced by host-driven DNA methylation [43]. The repressive epigenetic player, CpG DNA methylation of the viral genome decreases the ability of Rta to activate most early lytic promoters [44]. Importantly, the virus has evolved a strategy to overcome the silencing of DNA methylation and withdraw into a latent state. The unique ability of Zta to bind preferentially and activate methylated CpG-containing ZREs is essential for initiating EBV reactivation in cells latently infected with a methylated viral genome (Figure 2). Of note, Ser186 is required for Zta activation of the methylated form of EBV lytic promoters [45].

Histone modifications

Histone modifications have a central epigenetic role in regulating activation of *BZLF1* and *BRLF1* genes. The local acetylation state of histones H3 and H4 around Zp and Rp, such as histone H3 Lys27 acetylation (H3K27ac), H3K9ac, and H4K8ac, help to establish the open chromatin configuration so as to allow access of transcription factors, followed by transcription of the *BZLF1* and *BRLF1*genes [46-48]. In contrast, the methylation modifications of histones were suggested to be marks of heterochromatin formation and transcriptional repression. Repressive histone modifications, such as H3K27me3, H3K9me2/me3, and H4K20me3 have been identified at Zp or Rp, and correlate with inactivation of both IE promoters, maintaining viral latency. Nevertheless, H3K4me3 allows the virus to express Zta [49-51] (Figure 2).

3.4 Cellular stresses contributing to EBV reactivation

Increasing evidence suggests that severe host cell stress in response to many different toxic stimuli, including chemotherapy and γ irradiation, can induce lytic EBV infection, which ensures that the virus spreads from host to host.

Oxidative stress

Oxidative stress, generally induced by chemotherapy and irradiation (IR), leads to EBV reactivation through the induction of *BZLF1* gene expression [52]. Increasing evidence suggests a role

for reactive oxygen species (ROS), resulting from oxidative stress, as intermediates of intracellular signal transduction pathways [53]. Huang, *et al.* demonstrated a novel signaling mechanism by ROS for induction of EBV reactivation [42]. They found that various signaling pathways including ATM, p38 and JNKs are activated by ROS and involved in the induction of EBV reactivation in a p53-dependent manner. Also, phosphorylation of the ATF2 transcription factor by p38 and JNKs has been reported to activate Zp [16], implying deregulated ROS signaling might similarly induce EBV reactivation from latency through modification of other redox-sensitive transcription factors that activate Zp and/or Rp, such as early growth response 1 (EGR1) [54,55], Sp1 [56], Stat3 [57], and c-Jun [58].

Hypoxia

Hypoxia-inducible factor 1 (HIF-1) is a transcription factor that consists of α and β subunits, and is responsible for hypoxia induction of EBV reactivation [59]. In hypoxic conditions, HIF-1 accumulates to a high level, consequently the α subunit up-regulates expression of the *BZLF1* gene by binding to HIF-1-responsive elements (HREs) in Zp (Kraus RJ, Yu X, Sathiamoorthi S, Ruegsegger N, Nawandar DM, Kenney SC, et al. Unpublished data. n.d.).

Autophagy

Although autophagy normally serves as a defense mechanism against viral infection, recent research findings showed that EBV manipulates this mechanism to promote viral replication [60-63]. Results from various studies reveal that autophagic activation through the Rta-mediated ERKs pathway [60] and the PKCθ-p38 signaling axis [61] promotes viral lytic development in the early phase of EBV reactivation, but is soon inhibited by the early lytic products so as to prevent viral degradation in the degradative phases of autophagy [62,63].

Inflammation

Inflammatory responses against viral infection is one of the predisposing factors associated with virus-mediated tumorigenesis [64]. In the case of EBV, lytic reactivation induces expression of inflammatory cytokines, including interleukin-6 (IL-6), IL-8, IL-10, and IL-13, contributing to pathogenesis of NPC or lymphomas [65-68]. In a recent study, Gandhi, *et al.* elucidated the role of inflammation in EBV lytic reactivation [69]. They found that COX-2, a key mediator of inflammatory processes, induces EBV lytic reactivation through prostaglandin E2 (PGE2) by modulating the prostaglandin EP receptor-signaling pathway.

4. The viral self-regulation of EBV reactivation

4.1. Viral encoded proteins tend to maintain the temporal modes of EBV infection

In latent infection, the EBV latent membrane protein 1 (LMP1), a viral mimic of constitutively active CD40, intensifies latency in part through NF-κB activity [70]. LMP2A blocks BCR-induced EBV reactivation by inhibiting activation of tyrosine kinases by BCR [71,72]. And yet, some EBV early lytic proteins affect the activities of Zta or Rta through interactions between EBV proteins [73-75]. For example, the BRRF1 (Na)-Rta interaction enhances induction of viral lytic replication [73]. Unlike Na, the interaction of LF2 with Rta is critical for altering Rta subcellular localization and consequent functional repression [74,75]. In an earlier study, the BMRF1 protein was found to directly interact with Zta *in vitro* as well as *in vivo*, enhancing transcription from their common early *BHLF1* promoter [76]. Similarly, EBV tegument protein BGLF2, encoded in the late phase of the lytic cycle, was suggested to enhance Zta expression through activation of the p38 signaling pathway [77].

EBV nuclear antigen1 (EBNA1) is expressed in both latent and lytic modes of EBV infection. Notably, two roles for EBNA1 in the EBV latent-lytic switch have been identified. First, EBNA1 is known to be required for maintenance of latency; and second, when the lytic cycle is induced, it also has a role in viral reactivation and lytic infection [78,79]. For example, EBNA1 was shown to organize the *oriP* regions into replication domains for lytic replication and transcription [78], and induce EBV reactivation by overcoming the PML protein- and nuclear body (NB)-suppression of lytic infection [79].

These results indicate that the viral proteins preferentially maintain the modes of EBV infection, which is most likely to facilitate viral optimal replication in host cells.

4.2. The inhibitory effects of EBV-encoded microRNAs on reactivation from latency

EBV is the first virus found to encode microRNAs (miRNAs) [80]. The miRNAs encoded by EBV can be divided into two clusters, including 29 miRNAs located in the introns of the viral BART gene and 3 located adjacent to the *BHRF1* gene [81]. By identifying target genes, the roles of miRNAs in EBV latent-lytic switch have been established (Figure 3). For instance, the cellular miR-200b and miR-429, members of the miR200 family, are able to induce lytic replication by targeting ZEB1/2 and blocking their repressive effect on Zp [82,83]. On the other hand,

miR-155 inhibits BMP-mediated lytic reactivation by targeting multiple members of the BMP signaling pathway, including SMAD1, SMAD5, and CEBPB [84]. As for EBV-encoded miRNAs, the fact that a subset of viral miRNAs is present at high copy numbers in latently-infected cells implicates them in establishing and maintaining latency [81,85]. This occurs by inhibiting expression of viral lytic genes that play essential roles in the latent-lytic switch [86,87]. Barth, *et al.* demonstrated that EBV-encoded miR-BART2 directly targets the transcript of the viral DNA polymerase BALF5 to inhibit the transition from latent to lytic viral replication [86]. A recent study demonstrated that the EBV-encoded miR-BART20-5p directly targets the transcripts of the lytic switch proteins, Zta and Rta [87]. The EBV-encoded miRNAs also enhance latency by targeting host transcripts [88,89]. For example, EBV-encoded miR-BART5 counteracts the pro-apoptotic function of the p53/PUMA pathway by targeting transcripts of PUMA, optimizing cellular conditions for EBV latency [88]. Additionally, EBV-encoded miR-BART18-5p directly targets the transcript of MAPKKK2 (MAP3K2), which modulates the MAPK signaling pathways that are known to be important in EBV reactivation [89].

5. The pathogenic role of lytic infection in EBV-associated malignancies

Previous studies have focused on the contributions of EBV latent infection in the pathogenesis of EBV-induced malignancies and revealed that LMP1 is an essential oncoprotein [90]. In recent years, the viral lytic cycle has been shown to play an important role in carcinogenesis through several potential mechanisms. By enhancing transmission of the virus from cell to cell, EBV lytic infection may increase the total number of latently-infected cells and thus is an essential aspect of viral pathogenesis. A small subset of lytically-infected cells is commonly detected in biopsies of EBV-associated malignancies [91-93], suggesting a potential role for viral lytic infection in promoting tumor growth *in vivo*. Furthermore, some studies indicated that the viral lytic cycle in a fraction of B cells promotes the transformation of B-lymphocytes *in vitro* [94] and growth of B cell lymphoma *in vivo* [7,95] through the release of paracrine growth factors and angiogenic factors. Focusing on the study of NPC, Wu, *et al.* found that recurrent EBV reactivation promotes genome instability, invasiveness and tumorigenesis of NPC cells, and that the contribution of the lytic cycle is more profound than latent infection [5,96,97]. Additionally, lytic replication enhances secretion of the angiogenic factor, vascular endothelial growth factor (VEGF), in NPC cells, contributing to angiogenesis and consequent metastasis or relapse of NPC after remission [98]. Clinical and epidemiological studies have revealed that individuals with elevated plasma EBV DNA load and antibody titers against the lytic viral capsid antigen (VCA) and early antigen (EA) have a high risk of NPC [99,100]. These studies also show that fluctuation of EBV antibody titers occurs prior to the onset of NPC [101,102]. These results suggest the importance of lytic infection for the initiation, progression, and metastasis or relapse of NPC (Figure 4).

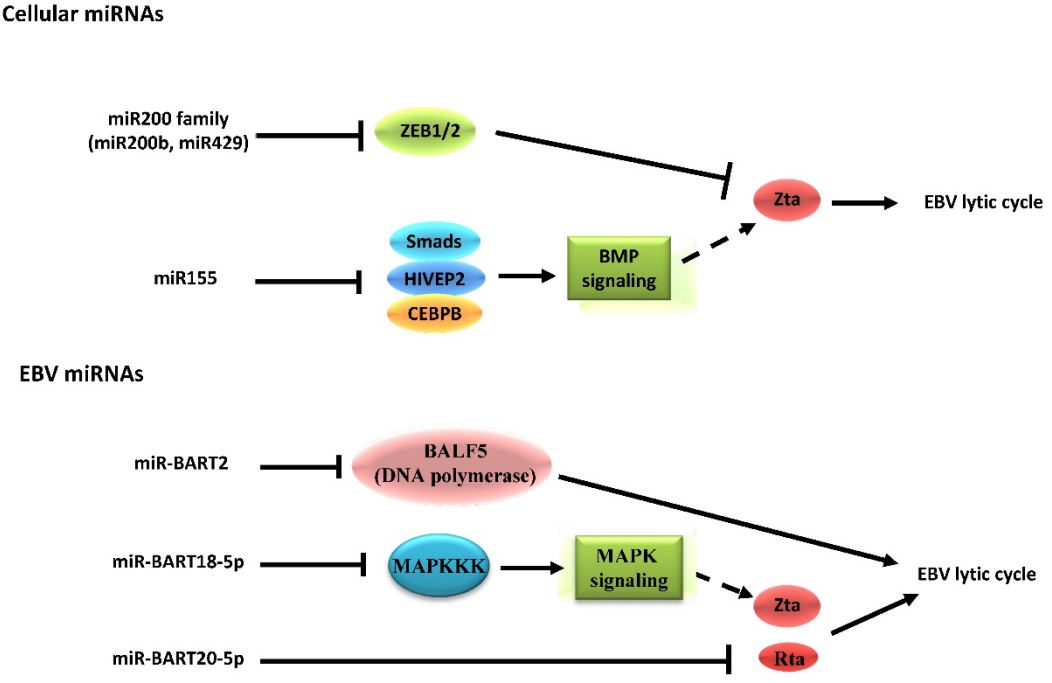

Figure 3. Schematic indicating the role of cellular and EBV miRNAs in EBV latency and reactivation. Cellular miRNAs have distinct functions in EBV latency and lytic reactivation by directly targeting transcription factors on Zp or regulating the signaling pathway related to expression of Zta. Nevertheless, the EBV-encoded miRNAs inhibit the transition from latent to lytic viral replication, which occurs both through modulation of specific signaling pathways as well as through the restriction of its own gene expression. Notably, the EBV-encoded mi-BART20-5p can directly target the transcripts of the *BZLF1* and *BRLF1* genes.

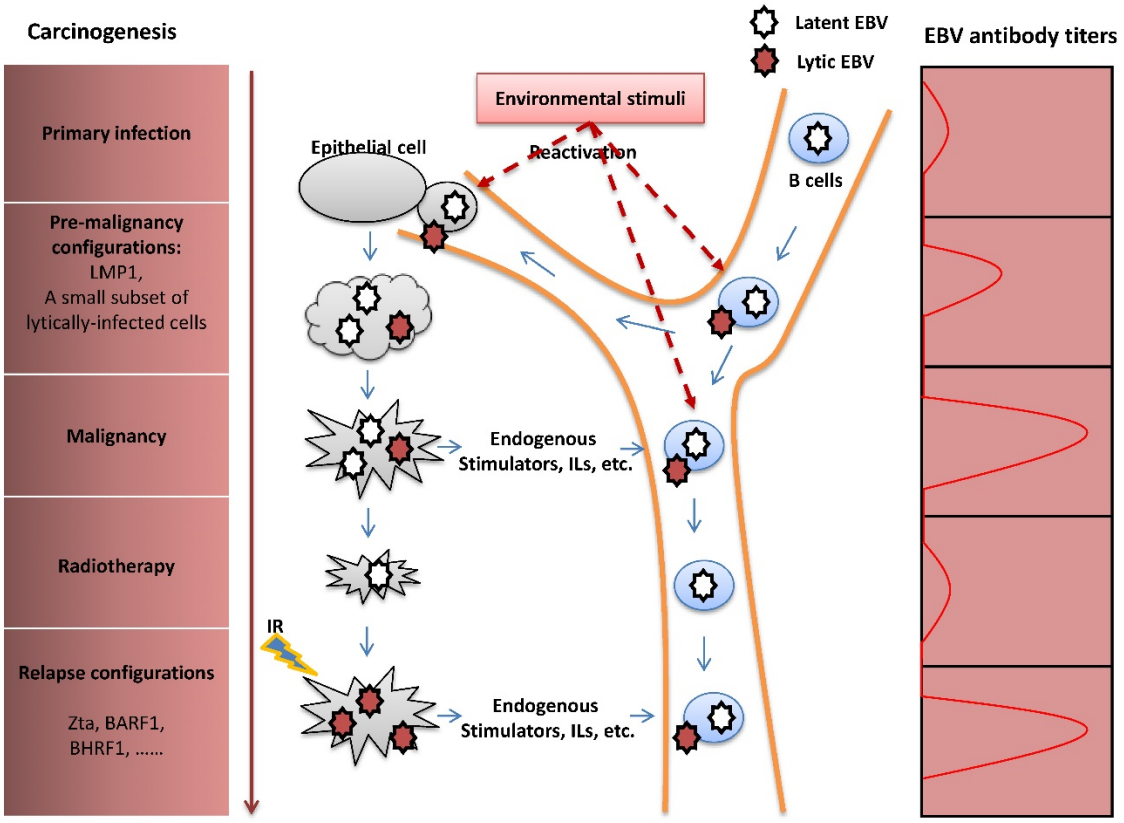

Figure 4. The pathogenic role of EBV lytic infection in NPC carcinogenesis. After primary infection, EBV establishes latent infection in B cells. Upon reactivation, lytically-infected B cells travel through the nasopharynx tissue, followed by infection of the nasopharyngeal epithelial cells by cell-to-cell contact. Similar to the LMP1 oncoprotein, the presence of a limited number of lytically-infected epithelial cells induces genome instability and release of oncogenic cytokines, consequently promoting NPC carcinogenesis. Although NPC is sensitive to radiotherapy, extensive resistance to radiation often causes tumor metastasis or relapse after remission. IR –induced recurrent expression of lytic proteins is a potential factor that mediates the impact of EBV on NPC relapse. The fluctuation of EBV antibody titers reflects tumor progression of NPC.

In addition, understanding the function of the EBV lytic proteins in malignancies is clearly essential in determining the role of lytic infection in the carcinogenic process (Table 1). The IE protein Zta triggers paracrine secretion of the angiogenic factor VEGF [98] and several oncogenic and inflammatory cytokines, including IL-6, IL-8, IL-10, and IL-13 [3,94,95,103], and thereby, participates in the tumorigenesis of EBV-associated malignancies. Some lytic proteins, such as BGLF4 protein kinase (EBV-PK) and BGLF5 nuclease (EBV DNase), have been reported to promote genomic instability and enhance tumor progression of NPC cells [96,104]. Intriguingly, EBV encodes a series of important proteins that show homology to diverse human anti-apoptotic molecules and oncogenic cytokines. For example, early gene *BCRF1*, also known as *viral interleukin-10 (vIL-10)*, encodes a homolog of IL-10 that functions as a paracrine growth factor in EBV-associated lymphomas [105]. BHRF1 exhibits homology to the human oncoprotein Bcl-2 and delays cell death during EBV lytic replication [106,107]. Chiu, *et al.* demonstrated that BALF3, a homologue to terminase, is not only involved in the induction of host genomic instability, but also mediates the impact of EBV on NPC relapse [108]. Another early gene *BARF1* encodes a homologue to the colony-stimulating factor-1 (CSF-1) receptor, which is a product of the human oncogene, *c-fms*. BARF1 inhibits apoptosis by activating Bcl-2 [109], hence contributing to the tumorigenicity of NPC cells [110,111]. Thus, effective strategies that inhibit EBV lytic reactivation might be valuable in the prevention or treatment of EBV-associated malignancies and improve the clinical outcome.

Table 1. EBV lytic proteins and tumorigenic functions [a]

Lytic protein	Lytic phase	Human homologue	Tumorigenic function
Zta	Immediate Early	–	Induction of IL-6, IL-8, IL-10, IL-13, and VEGF secretion
BARF1	Early	C-fms receptor	Anti-apoptosis
BHRF1	Early	Bcl-2	Anti-apoptosis
BALF3	Early	Terminase	Induction of genomic instability
BCRF1	Early	IL-10	Anti-apoptosis
BGLF4	Early	–	Induction of chromosomal abnormality and DNA damage
DNase	Early	–	Induction of genomic instability

[a] Summarized from references 3, 94-96, 98, 103-111.

6. Conclusions

Focusing on host and viral factors, this review has covered recent advances with respect to the mechanisms underlying EBV reactivation. In host cells, in addition to transcription control, post-transcriptional modifications, signal transduction, and epigenetic regulation, can together determine whether EBV infection remains latent or becomes lytic. Simultaneously, the virus has evolved strategies to exploit cell epigenetic machinery to establish a lifelong latent infection and use a strategy to escape from the latent state. To propagate, EBV spontaneously enters into lytic replication under severe cellular stress, and this adds to our understanding of how these host-viral interactions modulate the microenvironment contributing to EBV reactivation. In addition, regulation of the latent-lytic switch in the EBV life cycle is dependent partly on gene products encoded by the virus itself. Importantly, identification of lytic proteins that result in tumorigenesis determines the pathogenic role for lytic infection in human malignancies. In summary, a better understanding of the mechanisms beneath EBV lytic reactivation and the pathogenic role of viral lytic infection in carcinogenesis helps in the design of new virus-targeted therapies aiming at lytic cycle for EBV-associated malignancies.

Acknowledgements

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Competing Interests

The authors have declared that no competing interest exists.

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