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PERSPECTIVE

Consideration of Epstein-Barr Virus-Encoded Noncoding RNAs EBER1 and EBER2 as a Functional Backup of Viral Oncoprotein Latent Membrane Protein 1

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ABSTRACT **The Epstein-Barr virus (EBV)-encoded noncoding RNAs EBER1 and EBER2 are highly abundant through all four latency stages of EBV infection (III-II-I-0) and have been associated with an oncogenic phenotype when expressed in cell lines cultured *in vitro*. *In vivo*, EBV-infected B cells derived from freshly isolated lymphocytes show that EBER1/2 deletion does not impair viral latency. Based on published quantitative proteomics data from BJAB cells expressing EBER1 and EBER2, we propose that the EBERs, through their activation of AKT in a B-cell-specific manner, are a functionally redundant backup of latent membrane protein 1 (LMP1)—an essential oncoprotein in EBV-associated malignancies, with a main role in AKT activation. Our proposed model may explain the lack of effect on viral latency establishment in EBER-minus EBV infection.**

The Epstein-Barr virus (EBV) is a gammaherpesvirus that primarily infects B cells and in some cases epithelial cells. EBV infection leads to a lifelong latent phase in which the viral DNA episome remains attached to the host's genome in the nucleus of the EBV-infected cells. Like other latent viruses, EBV has evolved the capability to evade the immune system and reprogram host gene expression and intracellular signaling patterns in ways that favor the perpetuation of the EBV-induced viral latency phenotype (1, 2). Up to 95% of the human population carries this virus in memory B cells in an asymptomatic manner (1, 2). The association of EBV latency with lymphomas (the most common EBV-associated malignancy) is typically observed only in immunocompromised individuals after transplants or in people suffering from debilitating chronic inflammation (e.g., HIV infection or malaria) (1, 2). Uniquely, the relatively rare cases of EBV-associated epithelial tumorigenesis, such as nasopharyngeal and gastric carcinoma, occur in healthy individuals, albeit in many cases with an apparent genetic predisposition (1–3).

EBV latency gene expression stages. The EBV life cycle follows four sequential latency stages (III-II-I-0) in which the virus modulates its gene expression program. Each EBV latency stage is unique in its gene expression repertoire from up to 9 viral proteins (EBNA1 [EBV nuclear antigen 1], EBNA2, EBNA3A, EBNA3B, EBNA3C, EBNALP, latent membrane protein 1 [LMP1], LMP2A, and LMP2B), two noncoding RNAs (ncRNAs) (EBER1 and EBER2), and 44 mature microRNAs (miRNAs), primarily derived from two loci, the BART and BHRF clusters (1, 2, 4). While the EBV-encoded proteins show regulated expression across the four latency stages, EBER1 and EBER2 are expressed at all times (1, 2). The expression of miRNAs derived from the BHRF loci is restricted to the latency III stage. In contrast, the miRNAs derived from the BART loci show varied expression levels in different latency stages, depending on cell and tumor type (4).

Upon infection of naive B cells, EBV initially activates its latency III gene expression program (Fig. 1), characterized by the production of all 9 viral proteins, the EBER RNAs, and potentially the full complement of 44 mature viral miRNAs (1, 2, 4). The latency III gene expression program favors host cell growth and establishment of viral latency, referred to as viral transformation (1, 2). Latency stage III is highly immunogenic and activates the host's immune surveillance pathways (1, 2).

After successful viral transformation, EBV progresses to the latency II gene expression program, where it limits the production of immunogenic viral proteins to a variable number (~4)—EBNA1, LMP1, and LMP2A/B (1, 2) (Fig. 1). The latency II gene expression program contributes to the establishment of a memory B-cell phenotype (1, 2). This change in cell fate is commonly triggered by the viral proteins LMP1 and LMP2A, which are constitutively active transmembrane receptors that mimic the signaling requirements for memory B-cell commitment (5–7).

Once the memory B-cell phenotype has been established, EBV switches its gene expression program to latency I, also known as the EBNA1-only stage because EBNA1 is the single viral protein produced (1, 2) (Fig. 1). EBNA1 (EBV nuclear antigen 1) is a multifunctional viral protein associated with tumor growth in *in vivo* mouse assays (8–10). One of EBNA's main functions in latency maintenance is chromosome anchorage and stabilization of the viral episome (8) (Fig. 1). EBNA1 has particularly low immunogenicity, due to its unusual amino acid sequence, which helps it to evade major histocompatibility complex class II (MHCII) (11).

Through a mechanism not yet well understood, EBV-infected B cells in latency I enter a lifelong dormant stage named latency 0 (EBNA-minus), characterized by the absence of EBV-produced proteins (1, 2) (Fig. 1). EBV-infected memory B cells in latency 0 are thought to be quiescent (12) (Fig. 1). Every time an EBV-infected cell exits this quiescent stage and divides, EBV reenters latency I to produce EBNA1, which then promotes the faithful duplication of the EBV episome (1, 2). Latency 0 is regarded as true latency and thought to be prevalent lifelong in healthy indi-

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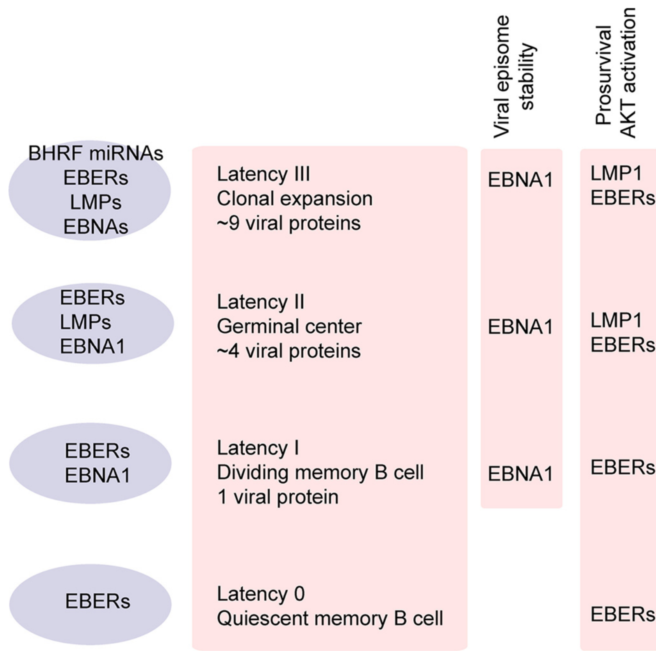

FIG 1 EBER1/2 functional redundancy in EBV latency. The cartoon depicts the most relevant features of EBV latency stages and aims at guiding the reader through the text. The purple circles depict in a condensed manner the genes expressed in each stage. "BHRF" is the locus that contains miRNAs known to be expressed in latency III. "EBERs" refers to EBER1 and EBER2; "LMPs" refers to LMP1, LMP2A, and LMP2B; and "EBNAs" refers to the broad repertoire of EBV nuclear antigens expressed during latency—EBNA1, EBNA2, EBNA3A, EBNA3B, EBNA3C, and EBNALP. The first (from left to right) pink inset depicts the phenotype induced by each latency stage in EBV-infected B cells and the number of viral proteins produced. The middle pink inset illustrates the role of EBNA1 in EBV episome stability in the first three latency stages (the so-called transient ones). The last pink inset illustrates the stages in which the activation of AKT by LMP1 and EBER1/2 (EBERs) overlap.

viduals (12). The other 3 latency stages are transitory and are thought to pave the way for long-term EBV persistence by mimicking the B-cell signaling requirements that lead to the establishment of a memory B-cell phenotype (13) (Fig. 1).

EBER1 and EBER2. While viral protein expression varies across latency stages, it is well established that the EBV-encoded ncRNAs EBER1 and EBER2 are expressed in all four viral gene expression programs (14) (Fig. 1). The EBERs are ~180 nucleotides each, transcribed by the host RNA polymerase III (15), and their expression is tumorigenic in *in vitro* cell line experiments and *in vivo* mouse assays (16–19). More than 30 years after their discovery (15), however, the EBERs continue to pose a challenge to the study of EBV latency. Their functional role is still a riddle, primarily because gene deletion studies show that EBER1/2-minus EBV bacmids show no apparent loss of viral latency establishment or tumorigenic potential in freshly isolated lymphocytes (20, 21).

Evidence from fluorescence *in situ* immunohistochemistry (FISH) studies indicates that the EBERs accumulate to ~10^6 copies per EBV-infected cell in the nucleus, where they assemble into ribonucleoprotein complexes (RNPs) (22). Heterokaryon assays suggest that while known binding partners shuttle from the nucleus to the cytoplasm (i.e., La), the EBERs themselves are nuclear (23). However, it is possible that the EBERs are not strictly nuclear at all times, since a high-resolution microscopy study of B cells in interphase shows their presence in the perinuclear region of the cytoplasm (24). Most remarkably, recent evidence shows that EBER1 may be a component of secreted exosomes (endosome-derived vesicles) that bud off EBV-infected cells—EBER2 is not found consistently secreted (25, 26).

The composition of the functional EBER1 and EBER2 RNPs is currently not well defined. We know so far that both EBER1 and EBER2 interact with the protein La, which is a nuclear RNA chaperone known to bind RNA polymerase III transcripts, such as the EBERs in EBV-infected lymphomas cultured *in vitro* (15, 23). Other known EBER1-specific interactors reported so far are the ribosomal small protein L22 (27) and the mRNA decay factor hnRNP D–AU-rich element binding factor 1 (AUF1) (28). EBER1 may also be a specific interactor with the latent EBV-encoded protein EBNA1 (29), and while further experimental evidence is necessary to corroborate this interaction, an EBER1-EBNA1 RNP is not surprising from a functional point of view. EBNA1 is produced in all latency stages along with the EBERs (1, 2), and it is known to upregulate the transcription of EBER1 and EBER2 (30). Given the similarity in the secondary structures between the EBERs and the adenoviral ncRNAs VAI and VAII, two known La interactors (31), it was originally proposed that like VAI and VAII, the EBERs could interact with the double-stranded RNA (dsRNA)-binding kinase PKR, an innate immunity regulator. While direct *in vivo* evidence for the EBER-PKR interaction is still missing, it has been confirmed *in vitro* (32). *In vitro* studies have also shown that PKR dimerization (a requirement for activation) is inhibited in the presence of either EBER1/2 or VAI/II (33). Despite the reported *in vitro* EBER-PKR interaction and the consequential disruption of active PKR dimers, a study has shown that the EBERs do not inhibit PKR activity *in vivo*, challenging the hypothesis that EBER function is mediated by its inhibitory interaction with PKR (34).

Besides PKR, the EBERs have also been reported to interact with and inhibit the innate immunity regulator RIG-I (35). The EBER interactions with PKR and RIG-I are supported by the observation that EBER expression in cell lines leads to inhibition of apoptosis and interferon-mediated innate immunity (36, 37). However, these interactions are highly contested, as the EBERs are considered to be strictly nuclear (23) whereas PKR and RIG-I are cytoplasmic (36, 37). Therefore, another mechanism for EBER inhibition of apoptosis and interferon-mediated innate immunity is sought. The latest report of an EBER interaction is that of EBER2 in complex with the host transcription factor Pax5 (38). In accordance, the proteomics and transcriptomics study of EBER-expressing BJAB cells reported recently shows an approximate 1.5-fold increase in the mRNA and protein levels of Pax5 in response to EBER1 and EBER2 expression (39).

EBER-specific activation of the oncogenic PI3K-AKT signaling pathway. The absence of an effect on viral latency establishment and a tumorigenic phenotype in the EBER-deletion studies that use freshly isolated lymphocytes reported so far (20, 21) is puzzling given the effects of EBER1 and EBER2 when expressed in isolation in cultured cell lines (16–19). In an attempt to rationalize this discordance between experimental data sets, we recently hypothesized that this apparent lack of phenotype could be due to a yet-undisclosed form of functional redundancy (39). To test this hypothesis, we performed a proteomics and transcriptomics study of BJAB cells (a Burkitt's lymphoma cell line) stably transfected

with the EBER1 and EBER2 genes. The proteome profile in this study revealed, in EBER-expressing cells relative to non-EBER-expressing cells, an increase of the protein PIK3AP1 (39), a B-cell-specific protein adapter involved in the activation of the phosphatidylinositol 3-kinase (PI3K)–AKT signaling cascade (40). As predicted from the known PIK3AP1 function, we found that an increase in PIK3AP1 in EBER-expressing BJAB cells correlated with higher levels of active/phosphorylated AKT (pAKT) (39). These data prompted us to formulate a working model that proposes functional redundancy between the EBERs and the main EBV-encoded oncoprotein LMP1 (39), which has a well-established role in the activation of the PI3K-AKT signaling cascade in asymptomatic and oncogenic EBV latency (41–44) (Fig. 1).

Memory B-cell commitment upon EBV latency establishment. Tonic (ligand-independent) AKT signaling downstream of the B-cell receptor (44) is known to ameliorate the apoptotic collateral downturn, typically observed in prolonged activation of B cells, a requisite for memory B-cell commitment (6). Prolonged PI3K-AKT signaling is therefore a survival cue required for memory B-cell differentiation in uninfected, otherwise healthy lymphocytes (6). Similarly, the main effect of EBV latent infection in B cells is tonic signaling activation, followed by the acquisition of a memory B-cell phenotype (13). Experimental evidence indicates that one of the functions of LMP1 in latency II is to promote B-cell survival by activating AKT (13). Not surprisingly, the PI3K-AKT pathway is the main oncogenic signaling cascade activated during latency, mainly due to the specific signaling functions of LMP1 (3, 42, 44) (Fig. 1). Our recently published data (39) support the hypothesis that the EBER1/2-mediated activation of the AKT signaling pathway helps provide a robust signaling cue that ensures latency stage progression, especially in latency I and 0, when LMP1 is no longer expressed (Fig. 1). An interesting way of testing this hypothesis would be to knock out the EBERs and LMP1 simultaneously in the latency stage I cell lines AKATA and/or MUTU. The knockout of the EBERs in EBV-infected AKATA cells has been reported elsewhere (45). To our knowledge, the simultaneous knockout of EBERs and LMP1 in these cell lines has not been reported so far. We are also not aware of experiments reported with a recombinant EBV in which the EBERs and LMP1 have been simultaneously deleted. The lack of this experiment is less important to test our model because EBV infection of freshly isolated B cells establishes a strict latency III infection, with no progress toward the subsequent stages (II, I, and 0).

Still puzzling is how the quiescent phenotype in EBER1/2-expressing latency 0, prevalent in long-term EBV asymptomatic infection, correlates with the reported tumorigenic effects of EBER1 and EBER2 when expressed in *in vitro* cell lines (16–19). We propose that in healthy individuals, the EBER prosurvival effects are kept from triggering tumorigenesis by the immunological surveillance system. In chronically debilitating conditions, the otherwise harmless EBER prosurvival signals may contribute to lymphoma outbursts in the pool of the typically infected memory B cells.

Conclusions. Based on the upregulation of the B-cell-specific protein adapter PIK3AP1 (a mediator of AKT signal activity) upon EBER1/2 expression published recently (39), we postulate that EBER1/2 expression may be used during EBV latency as a redundant source of prosurvival signaling. A redundant AKT activation, through its survival cue necessary for memory B-cell commitment, may ensure transient latency stage progression and true latency maintenance, as the number of immunogenic viral proteins decreases (Fig. 1). In particular, the EBER-mediated activation of AKT may act as a "backup" antiapoptotic signal, required by activated B cells to persist during memory B-cell development. Once in latency I, with LMP1 not expressed, the EBERs may become the main AKT-activating source. While enticing, the hypothesis proposed here is based on data gathered from experiments performed with BJAB cells and should therefore be tested with a larger collection of cell lines and optimally in an *in vivo* model system.

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