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Review

# THE SIGNALING PATHWAYS OF EPSTEIN-BARR VIRUS-ENCODED LATENT MEMBRANE PROTEIN 2A (LMP2A) IN LATENCY AND CANCER

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**Abstract:** Epstein-Barr virus (EBV) is a ubiquitous virus with infections commonly resulting in a latency carrier state. Although the exact role of EBV in cancer pathogenesis remains not entirely clear, it is highly probable that it causes several lymphoid and epithelial malignancies, such as Hodgkin's lymphoma, NK-T cell lymphoma, Burkitt's lymphoma, and nasopharyngeal carcinoma. EBV-associated malignancies are associated with a latent form of infection, and several of these EBV-encoded latent proteins are known to mediate cellular transformation. These include six nuclear antigens and three latent membrane proteins. Studies have shown that EBV displays distinct patterns of viral latent gene expression in these lymphoid and epithelial tumors. The constant expression of latent membrane protein 2A (LMP2A) at the RNA level in both primary and metastatic tumors suggests that this protein might be a driving

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Abbreviations used: BCR – B-cell receptor; BL – Burkitt's lymphoma; EBER – *EBV*-encoded small non-polyadenylated RNA; EBNA – *EBV* nuclear antigen; EBV – Epstein-Barr virus; ERK – extracellular signal-regulated kinase; GSK-3β – glycogen synthase kinase-3 beta; HL – Hodgkin's lymphomas; IkBα – inhibitor of NF-κB alpha; IL – interleukin; ITAM – immunoreceptor tyrosine-based activation motif; JNK – c-Jun-N-terminal kinase; LMP – latent membrane protein; MAPK – mitogen-activated protein kinase; NHL – non-Hodgkin's lymphomas; NK-κB – nuclear factor-kappa B; NPC – nasopharyngeal carcinoma; PI3-K – phosphotidylinositol 3-kinase; PTK – protein tyrosine kinase; STAT – signal transducers and activators of transcription; TGF-β – transforming growth factor-beta

factor in the tumorigenesis of EBV-associated malignancies. LMP2A may cooperate with the aberrant host genome, and thereby contribute to malignant transformation by intervening in signaling pathways at multiple points, especially in the cell cycle and apoptotic pathway. This review summarizes the role of EBV-encoded LMP2A in EBV-associated viral latency and cancers. We will focus our discussions on the molecular interactions of each of the conserved motifs in LMP2A, and their involvement in various signaling pathways, namely the B-cell receptor blockade mechanism, the ubiquitin-mediated (Notch and Wnt) pathways, and the MAPK, PI3-K/Akt, NK-κB and STAT pathways, which can provide us with important insights into the roles of LMP2A in the EBV-associated latency state and various malignancies.

**Key words**: Epstein-Barr virus, Latent membrane protein, Cancer, Latency

## INTRODUCTION

Epstein-Barr virus (EBV) is a ubiquitous virus that infects humans worldwide [1]. More than 90% of the world's population contract the virus at an age when they are most vulnerable to becoming carriers [2-4]. In most cases, the virus has a harmonious co-existence with the host. Early reports speculate that EBV possibly plays a causative role in the pathogenesis of several human lymphoid and epithelial malignancies such as Burkitt's lymphoma (BL) [5, 6], Hodgkin's lymphomas (HL) [7], non-Hodgkin's lymphomas (NHL) [8, 9], nasopharyngeal carcinoma (NPC) [10], breast cancer [11-14] and gastric carcinoma [15, 16].

Most EBV-positive malignancies are associated with a latent form of infection [17], and several of these EBV-encoded latent proteins are known to mediate cellular transformation. The latently expressed viral proteins consist of six nuclear antigens (EBNAs 1, 2, 3A, 3B, 3C, and -LP), three latent membrane proteins (LMPs 1, 2A and 2B), and two EBV-encoded small non-polyadenylated (non-coding) RNAs (EBER 1 and 2) and BamH1-A rightward transcripts (BARTs) [17, 18]. Studies have shown that EBV displays distinct patterns of viral latent gene expression in the development of both lymphoid and epithelial tumors [19-23]. Specific EBV latent gene expression patterns have been reported from different latency stages. Four major forms of EBV latent gene expression have been described [24]. Type 0 latency means quiescent, memory B cells where no viral genes are expressed [25]. Type I latency, in which EBNA1 and EBER RNAs are expressed, occurs in BL [26]. EBV infection of NPC, HD and peripheral T-cell lymphomas is the type II latency pattern, in which EBV-encoded EBNA1, LMP1, LMP2 and EBER RNAs are expressed [27]. Type III latency infection means the expressions of EBNA1, EBNA2, EBNA3, LMP1, LMP2, EBER and EBNA-LP RNAs [28], which are detected in patients with infectious mononucleosis (IM) [29], post-transplantation lymphoproliferative disorders and acquired immunodeficiency syndrome-related lymphomas [30]. Therefore, EBER RNAs are consistently present in all 3 latency infection types [31]. It has been reported that LMP2A and LMP2B proteins are constantly

detected in HD and NPC tumor tissues [19, 21-23, 32]. LMP2 transcripts are frequently expressed not only in primary NPC but also in metastatic tumors [19, 33]. The persistence of LMP2 gene expression suggests that LMP2 is important in maintaining tumour growth in EBV-associated NPC, possibly by contributing to epithelial cell transformation and providing growth advantage [33].
This review summarizes the role of EBV-encoded LMP2A membrane proteins in EBV-associated malignancies. Focus will be given to the involvement of LMP2A in protein-protein interactions that establish cellular signaling pathways, the elucidation of which may reveal the role of LMP2A in oncogenesis and viral latency.

## STRUCTURE OF LMP2A

The EBV LMP2 gene encodes 2 isoforms of the hydrophobic integral membrane protein, namely LMP2A and LMP2B [34]. LMP2A and LMP2B are transcribed from two different promoters separated by 3 kb through the fused terminal repeat of the EBV episome [34]. The LMP2A promoter is upstream of the LMP2 gene, while LMP2B shares a bidirectional promoter with LMP1 [35]. The LMP2A and LMP2B transcripts are respectively 2.0 kb and 1.7 kb in length. These transcripts share eight common 3' exons, but have unique 5' exons. The eight common exons shared by both LMP2A and LMP2B encode a hydrophobic stretch encompassing 12 membrane-spanning domains and a 27-amino acid cytoplasmic C-terminal domain. The 5' exon of LMP2A encodes a hydrophilic N-terminal 119-amino acid cytoplasmic-signaling domain, whereas the 5' exon of LMP2B is non-coding [34]. Since LMP2A and LMP2B are coded by the same gene, they are identical except for the first exon. Therefore, it is speculated that both proteins might possess some common functions and might localize to the same cellular compartments [36, 37]. A recent study suggested that LMP2B modulates LMP2A activity. When LMP2B was expressed in conjunction with LMP2A, there was a restoration of normal B-cell receptor (BCR) signal transduction upon BCR cross-linking [37]. The expression of LMP2B did not alter the cellular localization of LMP2A, but did bind to and prevent the phosphorylation of LMP2A [37]. However, further in-depth studies are required to scrutinize the effect of LMP2B expression on LMP2A, because the loss of function of LMP2A does not appear to be dependant solely on the heterodimerazation of LMP2A and LMP2B [37].

## MOLECULAR INTERACTION OF LMP2A MOTIFS

LMP2A is composed of a variety of highly conserved motifs with functional significance (Fig. 1). The sequence homology of these conserved motifs led to speculation of their involvement in signal transductions [38, 39]. The N-terminal domain of LMP2A contains tyrosine, serine and proline motifs, while the transmembrane and C-terminal domains contain multiple cysteine motifs.

A number of studies have been carried out to reveal their functional significance in signal transduction. In this section, we will focus on the molecular interactions of each conserved motif in LMP2A, while their roles in viral latency and signaling pathways in cancer will be discussed in the next section.

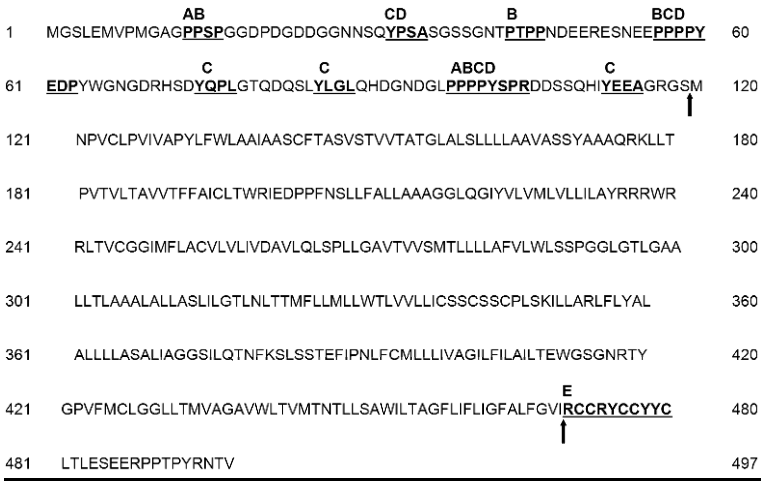


Fig. 1. The sequences and conserved motifs of LMP2A. LMP2A is composed of 497 amino acids (UniProtKB/TrEMBL accession: Q1HVJ2; NCBI accession: ABB89217). The N-terminal domain (NTD) of the LMP2A contains 119 amino acids. NTD contains serine (A) and proline (B), tyrosine (C) and PY (D) motifs. The serine-containing motifs (A) represent the consensus sequences of MAPK-binding sites. The proline-rich regions (B) contain a consensus sequence which is required for the interaction with the protein which carries the SH3 domains and WW domains. The tyrosine-containing motifs (C) play an important role in the BCR-signaling blockade upon phosphorylation. Two PY (D) motifs, Y101 and Y112, play an important role in the interaction with the WW domains of the Nedd4 family ubiquitin-protein ligases, which leads to the BCR signaling blockade. The C-terminal domain (CTD) of LMP2A has multiple cysteine (E) motifs, which are required for LMP2A palmitoylation. The arrow indicates the boundary between NTD, the transmembrane domain and CTD.

## THE N-TERMINAL DOMAIN

Point-mutation and deletion studies of the N-terminal domain of LMP2A revealed that three tyrosine-containing motifs play an important role in the BCR signaling blockade upon phosphorylation [39, 40]. LMP2A tyrosine residues Y74 and Y85 (Y74/85) with conserved sequences of paired tyrosine and leucine (YXXL) constitute the immunoreceptor tyrosine-based activation motif (ITAM) [41, 42]. A mutation study showed that an increase in cellular protein phosphorylation is dependent on the LMP2A ITAM motif, thereby proving that the LMP2A ITAM participates in signal transduction events [43]. Tyrosine 112 (Y112) within the YEEA motif of LMP2A demonstrates sequence homology to the PTK SH2 binding motif (YEEI) of the Src family. This binding motif

belongs to a consensus Src binding site, especially for Lyn [40, 44]. It has been established that Lyn can bind to Y112. The binding of Lyn to Y112 leads to phosphorylation of ITAM at Y74 and Y85. When phosphorylated, Y74, Y85 and Y112 become the interacting site for the tandem SH2 domains of Syk [40, 41]. It has been shown that tyrosine residues Y74, Y85 and Y112 of LMP2A are essential for LMP2A in latency activation, and that the deletion or mutation of these motifs does not cause a BCR blockade upon phosphorylation [40, 41].

The sequence homology of five other tyrosine residues (Y23, Y31, Y60, Y64 and Y101) of the LMP2A N-terminal to ITAM might imply their molecular interaction and functional significance. However, a point mutation study showed that Y60, Y64 and Y101 do not affect tyrosine phosphorylation, calcium mobilization and the induction of BZLF1 expression [45], and *in vitro* studies have shown that Y23 and Y31 are non-essential for LMP2A function. Therefore, these tyrosine motifs are not essential for blocking BCR signal transduction in EBV-immortalized cell lines [45]. That study showed that the regulation of LMP2A phosphorylation is different in epithelial cells and B cells. It was suggested that there might be different specific regulatory factors that mediate the phosphorylation of LMP2A in epithelial cells. To date, we have no concrete information on the functional significances of these tyrosine motifs, and further experimental designs are needed to address this issue.

The LMP2A N-terminal proline-rich motifs are conserved among different EBV clinical isolates and EBV-related herpes virus papio [46, 47]. These proline-rich regions contain a consensus sequence that is required to interact with SH3 and WW domains, interactions which in turn yield various biological effects [48-52]. The SH3 domain consists of 50 to 70 amino acid sequences often present in eukaryotic signal transduction and cytoskeletal proteins. It was assumed to play a role in mediating protein-protein interactions or directing cell compartmentalization. The WW domain interacts with a number of proline-containing ligands yielding a great deal of functional diversity. It has been shown that WW domain interactions can also be differentially regulated by tyrosine phosphorylation.

Experiments showed that two of the N-terminal PY motifs, with a consensus sequence of PPXY, interact strongly with class I WW domains, but not with class II WW domains. However, there are no interactions detected between LMP2A and any of the five different SH3 domains [53]. Several studies involving microsequence and mutation analysis and structural approaches showed that the HECT-domain containing the Nedd4 ubiquitin protein ligase family, which contains WW domains, participates in WW-PY interaction [54, 55]. The ubiquitin protein ligase family includes AIP4/Itchy, WWP2, Nedd4 and KIAA0439/Nedd4-2 [49, 56, 57]. Microarray analysis has identified a number of LMP2A-mediated transcriptional changes in the genes encoding ubiquitination [58]. It was shown that proteasome subunits (Psm a4, c3, d3, d8, d9, d11), ubiquitin-conjugating enzyme E2C (Ube2c, 21), WW domain-containing protein 4 (Wwp2), Ubiquitin fusion degradation 1-like 11 (Ufd11),

ubiquitin-specific protease (Usp15), and F-box and leucine-rich repeat protein 7 (Fb17) are induced by LMP2A, while autophagy 12-like (Apg121), ubiquilin 1 (Ubqln1), ubiquitin-specific protease 15 (Usp15) and WW domain-containing protein 4 (Wwp2) are repressed by LMP2A in different cell types [58]. The binding of these ubiquitin ligases to LMP2A is important for targeting LMP2A and LMP2A-associated proteins such as Lyn for ubiquitination, followed by internalization and degradation [54]. This is supported by the fact that Lyn PTK exhibits a dramatically reduced half-life in BJAB cells expressing LMP2A when compared to other cellular proteins [54]. The degradation of LMP2A and its associating proteins may play an important regulatory role in EBV latency, B-cell signal transduction and malignancies. It is shown that Itchy down-regulates LMP2A activity in B-cell signaling [54]. Various experimental studies have yielded two proposed models for the ubiquitin-mediated pathway induced by LMP2A, namely the Wnt and Notch pathways [59].

Two serine motifs lie within the proline motifs at the N-terminal domain of LMP2A. A preliminary sequence analysis of 13 serine residues in LMP2 exon 1 revealed 2 consensus sequences of MAPK binding sites. These binding motifs are located in serine 15 (S15), with the sequence of PPSP, and in serine 102 (S102), with the sequence of PYSP [60]. It was demonstrated that S102 lies within a motif that is evolutionarily highly conserved between the human and simian LMP2 genes. Besides, this residue and its flanking sequences are conserved among 28 isolates, and 2 of these isolates had mutations converting S15 into asparagine [46]. The S102 lies between the ITAM motif (Y74/Y85) and Y112, and within the proline-rich motifs, which suggests that the phosphorylation of S102 might alter LMP2 docking interactions with signal transduction kinases and/or adapter molecules. We might consider a possible role of this configuration in a switching mechanism for the latent to lytic status of latently infected B cells [60].

## THE C-TERMINAL AND TRANSMEMBRANE DOMAINS

The cysteine-containing motifs in the C-terminal and transmembrane domains of LMP2A are thought to be the protein interaction motifs involved in molecular clustering, which is required for LMP2A function [61, 62]. These cysteine-containing motifs localized in the C-terminal and inner leaflets of the transmembrane domains are the major sites of LMP2A palmitoylation, a process of post-translational modification [61, 62]. The transient expression of LMP2A in B cells showed that LMP2A is palmitoylated, suggesting palmitoylation plays a functional role in B-cell signaling [61-63]. Several studies have been carried out to investigate the functional significances of this modification and their biological effects.

*In vitro* deletion studies show that the transmembrane and C-terminal domains are not required for primary B-cell infection, in which the growth transformation does not show any difference from wild-type viral infection. These findings

suggest that the transmembrane domain and C-terminal domain do not take part in latent infection and lytic virus replication [64, 65]. A study by Matskova *et al.* demonstrated that the palmitoylation of the two cysteine residues in the C-terminal is not required for the clustering of LMP2A and localization of LMP2A to lipid rafts [62]. A more recent study reported that palmitoylation in different cysteine residues in the C-terminal and transmembrane domains is not required to localize it to buoyant complexes or for lipid raft association, suggesting that palmitoylation of LMP2A in different residues is cell-type specific [61]. All these studies conclude that the palmitoylation of LMP2A is not required for lipid raft association.

Besides this, palmitoylation is known to be associated with protein trafficking and protein turnover [66]. However, the mutations of these cysteine residues do not interfere with the binding of LMP2A with AIP-2, or with ubiquitination [61]. These studies propose that there must be some unknown mechanisms for lipid raft association other than palmitoylation [61].

To date, no known function has been found for LMP2A palmitoylation. Some authors suggested that palmitoylation is required for LMP2A-mediated survival signal and function, and to regulate the protein interaction or localization required for LMP2A-mediated cell survival [61].

## THE SIGNALING PATHWAY OF LMP2A IN VIRAL LATENCY AND CANCER

LMP2A is a crucial mediator in the viral latency of B-cells, with involvement in various signaling mechanisms (Fig. 2). LMP2A mimics BCR signaling and contributes to the long-term survival of B-cells [67]. In latent infected B-cells, BCR is blocked, and a series of events renders the necessary survival signals to prevent viral replication and promote B-cell differentiation into resting memory cells [68-70]. The role of LMP2 in malignancy remains an enigma. LMP2A are consistently expressed at both the RNA and protein levels in NPC [19, 71], and LMP2-specific antibodies are detected in the sera of these patients [72]. In addition, the expression of LMP2A is consistently detected in Hodgkin's lymphoma and nasopharyngeal carcinoma tissue [23, 73]. These findings suggest that LMP2A probably plays some specific roles in malignancy [74]. However, initial genetic studies proposed that neither LMP2A nor LMP2B were essential for B-cell transformation *in vitro* [64, 65]. A recent study showed that the transforming function of LMP2A is present in the immortalized epithelial cell line, but not in normal epidermal cells [75]. It induces both the differentiation blockade and malignant transformation in the HaCat epithelial cell line [75]. It is also suggested that the transformation properties associated with LMP2A expression are more subtle than those of LMP1, and that it perhaps only manifests in certain cellular contexts [76]. Another study showed that LMP2A is essential for the growth transformation of germinal center B cells, which do not express genuine BCR because of deleterious somatic

hypermutations in their immunoglobulin genes. The study suggested that LMP2A has potent, distinct antiapoptotic and/or transforming characteristics, indicating its role as an indispensable BCR mimic in certain B cells from which human B-cell tumors such as Hodgkin's lymphoma originate [77].

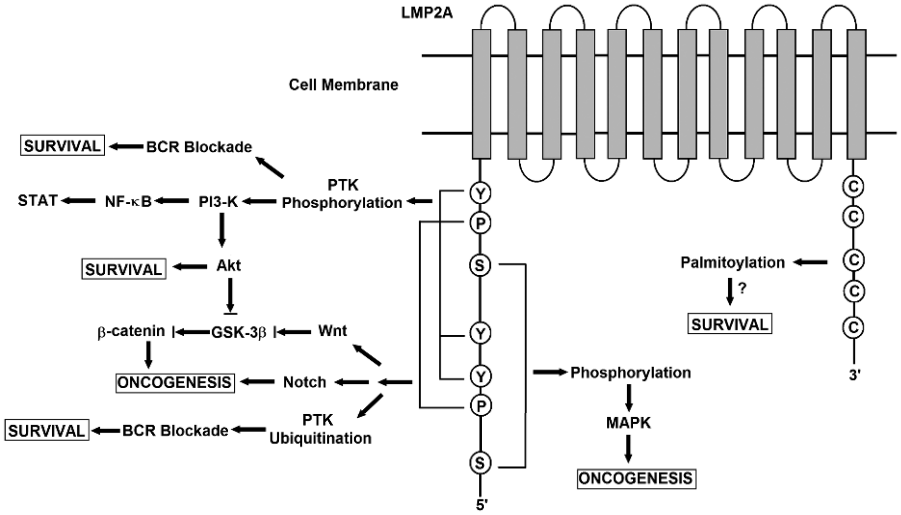


Fig. 2. A schematic diagram showing the signaling pathways engaged by the LMP2A gene. The N-terminal domain of LMP2A prevents BCR signaling by recruiting Nedd4-like ubiquitin-protein ligases and B-cell signaling molecules, leading to the degradation of LMP2A and its associated molecules in a ubiquitin-dependent manner. LMP2A also provides a survival signal to BCR-negative B-cells through the activation of the Ras/PI3K/Akt pathway. Activating this pathway induces the transcription of anti-apototic genes, the expression of which is controlled by NF-κB. Notch signaling regulates various cellular processes including cell survival and proliferation. In LMP2A-expressing splenic B cells, Notch activation is reported. Notch signaling is closely related to the pathogenesis of HL. LMP2A perturbs the turnover of β-catenin and other proteins that are involved in Wnt signaling. β-catenin is stabilized and activated by LMP2A through PI3K/Akt activation, which inhibits glycogen synthase kinase-3β (GSK-3β). The association of LMP2A with MAPK was implicated in the development of B-cell malignancy while the activation of MAPK was not observed in LMP2A-expressing epithelial cells. The multiple cysteine motifs within the C-terminal of LMP2A are required for LMP2A palmitoylation. Studies of the proposed palmitoylation are required for LMP2A-mediated survival signal and function as they regulate the protein interaction or localization required for LMP2A-mediated cell survival.

The latency and immortalization functions of LMP2 involve various signaling pathways. It is proposed that viral latency is either a separate (parallel pathway model) or downstream consequence (serial pathway model) of immortalization events [78]. Studies on LMP2 and its involvement in various signaling pathways provide important clues on viral latency and malignant transformation. LMP2 may cooperate with genetic aberrations of the host genome and thereby

contribute to malignant transformation by its intervention in multiple points in signaling pathways, especially of the cell cycle and apoptotic pathway. However, the exact pathogenetic mechanism of LMP2 in malignant transformation has not been elucidated. In the following sections, we will focus on the various signaling pathways induced by LMP2, and their involvement in viral latency and malignant transformation.

## THE BCR BLOCKADE AND UBIQUITIN-MEDIATED PATHWAY

The development of normal B-cells is mediated through the signal transduction of BCR [79, 80]. ITAM is a tyrosine-containing motif present in Fc receptors and B- and T-cell antigen receptor-associated molecules that form a consensus binding site for SH2-containing protein, i.e. Src family PTK and Syk PTK [41, 79, 81, 82]. Upon tyrosine phosphorylation, BCR recruits and activates the Src family of PTK and Syk PTK, which are subsequently recruited to lipid rafts. They act as sorting sites for the proteins and lipids required for signaling transduction [83, 84]. The association with lipid rafts leads to Ig receptor stimulation and the signaling protein complex called BCR signalosome is organized in the plasma membrane [85]. As a consequence, the downstream signal transduction events take place.

The expression of LMP2A interferes with BCR signaling and function. It has been demonstrated that low LMP2A expression does not inhibit Ig rearrangement, BCR expression or normal B-cell differentiation into follicular and marginal zone B cells, while high LMP2A expression inhibits BCR expression and results in exclusive B-1 differentiation in the bone marrow and peripheral lymphoid organs [49, 86, 87]. Most of the phosphotyrosine activity in latently infected B-cells is associated with LMP2A patches, and these clustered membrane patches mimic the activated BCR [70, 82, 88, 89]. LMP2A has been shown to localize in the lipid rafts and prevent recruitment to them of activated BCR [63, 85, 90, 91]. It was shown that LMP2A negatively regulates BCR signaling by excluding BCR from lipid rafts and by targeting the Src family members of the Lyn and Syk protein tyrosine kinases for ubiquitin-mediated degradation [54, 85]. This event is achieved by sequestering PTK away from BCR and/or ubiquitination degradation of PTK, leading to BCR signal transduction blockade [39, 41, 57]. The blocking of BCR-induced $Ca^{2+}$ mobilization, tyrosine phosphorylation, and BCR gene transcription of subsequently leads to the prevention of EBV lytic cycle activation and promotes the survival of B-cells and viral latency [40, 44, 79]. Studies have shown LMP2A drives B-cell development and survival in the absence of normal B-cell receptor signals [86, 92].

The binding of ubiquitin ligases to LMP2A is important for the targeting of LMP2A and its associated proteins, such as Lyn, for ubiquitination, and thus for internalization and degradation [54]. This ubiquitination is an important mechanism for the regulation of LMP2A and associated proteins. Itchy, a Nedd4

ubiquitin ligase, down-regulates LMP2A activity in B-cell signaling [54]. One study suggested that the WW domain of ubiquitin ligases binds to the prolines of the PY motif of LMP2A only when the tyrosine residue is not phosphorylated [54]. We might propose that these ubiquitin ligases remove excess LMP2A in tightly regulated signaling pathways. Recent studies proposed a model in which two ubiquitin-mediated signaling pathways are involved in these processes, namely the Wnt and Notch pathways [59]. LMP2A activates and stabilizes β-catenin in epithelial cells through PI3-K and Akt activation, which negatively regulates glycogen synthase kinase-3β (GSK-3β) [93]. GSK-3β, in turn, is tightly regulated by Wnt signaling [94]. Notch signaling pathways influence a variety of cellular processes, including lineage specification, cell survival and proliferation [95, 96]. Upon binding to a ligand, Notch translocates to the nucleus and interacts with RBP-Jκ to promote gene transcription. The suppressors of deltec [Su(dx)] and AIP4/Itchy associate with Notch and promote ubiquitination and proteasomal degradation, which is enhanced by Numb and Cbl [97, 98]. Notch1 inhibits E2A activity by degrading E47. LMP2A might utilize the Wnt and Notch pathways to regulate the strength of its own signal and various B-cell processes, such as differentiation, activation or survival [59]. These are important to maintain B-cell survival during viral latency and malignant transformation. To date, we have no direct evidence to show that these pathways are mediated or altered by LMP2A. Further studies are needed to determine the precise mechanisms by which LMP2A alters these signaling pathways during viral latency and malignant transformation.

## MAPK PATHWAY

MAPK is a group of serine/threonine kinases that is activated in response to the extracellular environment and delivers a signal into the nucleus. This is followed by a series of biological events. The MAPK family includes 3 parallel pathways, namely ERK/MAPK, JNK/MAPK and p38/MAPK. MAPK has been shown to directly and indirectly participate in growth factor receptor regulation by phosphorylating receptors and their associated molecules [99-102]. Under normal physiological conditions, the MAPK signaling pathways are involved in a variety of key events, such as proliferation, differentiation, apoptosis and migration [103, 104]. When deregulated, these pathways contribute to the development of malignancy. In this section, we will discuss the involvement of the MAPK signaling pathways triggered by LMP2A and their role in the development of malignancy.

Several direct and indirect pieces of evidence suggest that LMP2A is involved in the activation of MAPK signaling in various EBV-infected cell lines *in vitro* [20, 58, 60]. A study on lymphoblastoid B-cell lines and Burkitt lymphoma cell lines suggests that LMP2A activates ERK/MAPK [60]. In that study, GST1-119, a glutathione S-transferase (GST) fusion protein containing 119 amino acids of the LMP2A cytoplasmic domain, coprecipitates with the ERK1 form of MAPK,

suggesting that phosphorylation on the serine residues of LMP2A is induced by ERK1, followed by activation of MAPK signaling in B-cell lines [60]. A recent study on transgenic LMP2A mice showed that constitutive activation of the ERK/MAPK and PI3K/Akt pathways is involved in proliferation and survival [105].

A recent report claims that LMP2A activates the kinase activity of ERK/MAPK and JNK/MAPK in NPC cell lines, but not that of p38/MAPK [20]. The above study shows that LMP2A phosphorylation can be inhibited by the ERK pathway inhibitor PD98059, thus implying that ERK/MAPK are activated in LMP2A-expressing cells. C-Jun is a crucial downstream effector of the JNK/MAPK pathway. Under mitogen stimulation, c-Jun is induced as an immediate early factor, playing an important role in the promotion of cell growth [106-108]. C-Jun is a strong inhibitor of differentiation, and LMP2A has been shown to transform and inhibit the differentiation of keratinocytes in organic raft cultures [91, 109, 110]. These observations suggest a link between JNK/MAPK and LMP2A. By using the protein synthesis inhibition assay, it has been shown that MAPK activation confers c-Jun phosphorylation, and thus increases c-Jun protein stability [20]. These findings imply that the LMP2A pathway begins with the activation of MAPK kinase and changing the phosphorylation status of c-Jun would stabilize it [20]. It had been reported that stable phosphorylation of c-Jun confers oncogenicity [111, 112].

Regulation of the upstream effectors of the MAPK signaling pathway by LMP2A provides important clues as to its involvement in this signaling circuit. Microarray studies reveal that the gene transcription alteration of many MAPK-related molecules is induced by LMP2A. These include the upregulation of Ras, the Ras homolog, Ras GTPase-activating protein-binding protein 1, MAPK2K2, and MAPK2K1; and the suppression of Ras suppressor protein [58]. It has been shown that c-Jun can cooperate with activated Ras to effectively carry out the transformation [113, 114].

The proposed role of MAPK signaling in the upregulation of LMP2A expression and an increased metastasis rate remains controversial [20]. It has been shown that the ERK/MAPK pathway is involved in cell migration and tumor progression [115-119]. In the three-dimensional collagen gel assay, LMP2A expression renders migratory ability, and promotes the mobility of EBV-infected NPC cell lines [20]. However, another study shows that the MEK inhibitor, UO126, is ineffective at blocking the spread and motility of LMP2A- and LMP2B-expressing epithelial cell lines on the extracellular matrix, suggesting that the MAPK pathway is not involved in the LMP2A- and LMP2B-mediated spread and motility of epithelial cells [120]. It is possible that different signaling pathways are employed by LMP2A in the metastasis of different cancer types, and further investigations are necessary to unravel the exact mechanisms of LMP2A influence on cell motility.

Although the involvement of LMP2A in MAPK signaling has been established, the exact mechanism and molecular interactions remain unclear. Our current

understanding of LMP2A and MAPK signaling is mostly derived from *in vitro* studies of different cell lines. There has been no *in vivo* study supporting the connection between LMP2A and MAPK signaling.

## THE PI3-K/AKT PATHWAY

The phosphotidylinositol 3-kinase (PI3-K)/Akt signaling pathway plays an important role in mediating transformation, anti-apoptotic effects, invasion and adhesion [121-125]. Akt is a serine/threonine kinase, which phosphorylates and regulates the activities of cell cycle regulatory proteins, such as glycogen synthase kinase 3 and cyclin D [126, 127], and apoptotic proteins, such as Bad, pro-caspase 9 and Forkhead transcription factors [128, 129].

Several lines of evidence indicate that LMP2A may activate the PI3-K/Akt pathway through protein kinase Syk and Lyn, which play a crucial role in cell survival [130]. It was observed that LMP2A-associated PI3-K/Akt activation leads to an enhanced cell growth and anti-apoptotic effect in B-cells, lymphoma, gastric carcinoma, and epithelial cells [56, 76, 131]. The PI3-K inhibitor, LY294002, can result in the inhibition of LMP2A-induced colony formation in soft agar. This phenomenon suggests that PI3-K activation is critical for the anchorage-independent growth of epithelial cells [59, 132]. B-cells from LMP2A transgenic mice are sensitive to apoptosis induction after treatment with specific inhibitors of Ras, PI3-K and Akt, which suggests that LMP2A activates Ras and subsequently the PI3-K/Akt pathway, leading to B-cell survival [59]. In the human Burkitt's lymphoma cell line and gastric carcinoma cell line, the PI3-K inhibitor blocks the anti-apoptotic effect of LMP2A, suggesting that LMP2A utilizes the PI3-K signaling pathway in mediating anti-apoptotic effects [132]. In addition, one study suggested that the LMP2A-induced Syk activity in 293 and HaCat human epithelial cell lines is involved in cell migration, and this activation requires the tyrosine residues in the LMP2A ITAM motif [133].

LMP2A regulates cell survival and apoptotic inhibition through various routes. The inhibition of the TGF-β pathway is one example. TGF-β1 induces apoptosis by activating caspase [134-137]. LMP2A inhibits TGF-β1-induced caspase activity and apoptosis through the activation of the PI3-K/Akt pathway via phosphorylation of Akt at its serine residue [132, 138]. This is further supported by suppression of B-cell apoptosis being associated with an increased level of activated Ras and a high level of Bcl-XL expression [59]. The other pathway regulated by LMP2-associated PI3-K/Akt activation is integrin. Recent studies suggested that PI3-K activation by integrin leads to invasive and adhesive phenotypes, and subsequently protection from apoptosis [124, 125].

The activation of the PI3-K/Akt pathway is important in the development of EBV-associated malignancies, by maintaining EBV persistence and latency, rather than cell transformation [76, 77]. It is likely that the activation of Ras/PI3-K/Akt by LMP2, in combination with other genetic changes unique to the epithelial cells, contributes to its aggressive tumorigenicity [76]. Ectopic

expression of LMP2A in HaCaT cells was subsequently analyzed in an organotypic raft culture, in soft agar, and by subcutaneous injection into nude mice, which showed that LMP2A affects the cell growth and differentiation pathways, in part through activation of the PI3-K/Akt pathway [76]. However, the involvement of the LMP2-associated PIK-3/Akt pathway and other apoptotic regulators such as caspase remains not entirely clear. The regulation activation of this pathway and differential expression in various tumor types have also yet to be discovered. Further studies will provide detailed signaling pathway profiling of LMP2A, which is important in our understanding of EBV-associated cancer pathogenesis.

## THE NF-κB AND STAT PATHWAYS

STATs are transcription factors that mediate interferon-regulated gene expression and the cytokine signaling pathway [139]. The members of the NF-κB family are transcription factors involved in cellular responses to stimuli such as stress, cytokines, free radicals, radiation and bacterial or viral antigens [140]. Constitutive activation of the STAT and NK-κB pathways commonly occurs in malignancies as a consequence of either genetic or autocrine/paracrine alteration [141, 142]. The activation of NF-κB in epithelial cells induces IL-6 production, and leads to the activation of STAT [141, 143]. This subsequently results in cell growth and survival. It also mediates inflammatory responses by inducing cytokines and chemokines, which result in the recruitment and activation of immune cells with the consequent stimulation of anti-tumor activity [141]. The balance between tumorigenesis and the anti-tumor immune response exists in the NK-κB pathway, while altering this balance results in tumor development [141].

The involvement of LMP2A in the down-regulation of the STAT and NF-κB pathways in human carcinoma cell lines infected by EBV is demonstrated *in vitro* by using wild-type recombinant EBV (rEBV) and mutant rEBV, in which the LMP2A gene is deleted (rEBV-2A) [144]. The results show that transient expression of LMP2A in LMP2A-deficient carcinoma cells suppressed LMP1 expression, IL-6 secretion, and STAT3 and NF-κB activity, while down-regulation of LMP2A resulted in LMP1 induction [144]. It was also shown that LMP1 expressed in rEBV-2A cells is driven from the STAT-responsive L1-TR promoter. The production of IL-6 is regulated by the NF-κB pathway [145]. Transfection of a recombinant adenovirus expressing the dominantly active mutant IkBα, and the luciferase assay in rEBV HONE-1 cells showed that the activity of the IL-6 promoter is remarkably decreased [144]. These results imply the role of LMP2A in modulating the STAT pathways and indirectly in the modulation of LMP1 expression through NF-κB activity in epithelial cells [144]. This experiment thus explains that consistent LMP2A expression [19, 71] is associated with undetectable LMP1 expression at the protein level in most EBV-infected carcinoma cell lines. However, the presence of IL-6 can release the

suppression of LMP1 expression by LMP2A [146-149]. This suggests that the expression of LMP1 does not depend solely on the LMP2A, but can also be induced by the cellular environment or by local secretion of IL-6 [144].

The NF-κB and STAT pathways are likely to contribute to various cancer phenotypes in EBV-associated malignancies. For example, the suppression of NF-κB induces epidermal hyperplasia, which then leads to the formation of NPC, an undifferentiated tumor [150]. NF-κB is also shown to be positively regulated by Akt, which leads to an increased level of Bcl-xL in B-cells, confering an anti-apoptotic effect and cell survival [59, 151].

There are no available proven details on the mechanism and the consequence of LMP2A-induced suppression of the NF-κb and STAT pathways. We propose that further studies of the molecular crosstalk between tumor cells and the tumor microenvironment are essential to define the precise mechanisms in mediating oncogenesis, and to enable further insights into EBV-associated malignancies.

## CONCLUSION

In summary, research shows that LMP2A probably plays significant roles in EBV-associated malignancies and viral latency. These processes are modulated through a variety of signaling pathways. Identifying the LMP2A signaling molecules that are involved in the pathogenesis of EBV-associated malignancies is important for the development of potential therapeutic targets. Potent small molecule inhibitors that impair the LMP2A oncogenic signaling pathways could possibly become novel therapeutic modalities. However, the precise mechanisms by which LMP2A alters these pathways remains elusive. Differential manifestation of these signaling pathways is likely due to the different cell types examined, suggesting that some processes may be cell-type specific. Instead of *in vitro* studies on certain signaling pathways, such as MAPK pathway, *in vivo* studies are needed for further clarification. The molecular interaction of LMP2A and other effectors, such as components of the PIK-3/Akt pathway and caspase, remain elusive. The NF-kB- and STAT-mediated effect of LMP2A on gene transcription and the cellular phenotype is not clear. Therefore, the development of potential therapeutics targeting LMP2A is hindered by the lack of understanding of the role of LMP2A in oncogenesis. Additional experiments to dissect the role of LMP2A in carcinogenesis are crucial in our understanding of EBV-associated tumorigenesis. The activation of signaling pathways by LMP2A is not an independent process, and the combination with other genetic changes and cellular properties might be crucial in tumorigenesis. In addition to this, LMP2A might work in concert with other EBV proteins to alter the signal transductions. High-throughput studies with genomic and proteomic approaches will provide a broader picture of the signaling pathways associated with LMP2A. The power of the high-throughput approach has been demonstrated in DNA microarray studies [58]. We must note that the interaction between the virus and host factors is essential for the tumorigenesis of EBV-associated

malignancies. We propose that proteomics approaches and the generation of animal models for *in vivo* studies might provide further insights into the understanding of the important regulatory elements in the LMP2A signaling pathways. The targeting of LMP2A and LMP2B in cancer treatment has been highlighted in many studies [152-155]. Complete pictures of the signaling pathways where LMP2A is involved would facilitate the design of treatments for EBV-associated malignancies.

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