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博士論文

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Doctor Dissertation

EB 病毒對 EphA4 的調控及其對 EB 病毒相關之淋巴
增殖疾病的致病性之影響

Regulation of Eph receptor A4 (EphA4) expression by
Epstein-Barr virus and its impact on the pathogenesis of
EBV-associated lymphoproliferative disorders

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中文摘要

EB 病毒 (Epstein-Barr virus, EBV)，為致癌性之疱疹病毒，與許多淋巴性細胞異

常之疾病有高度相關性，包括柏金氏淋巴瘤 (Burkitt's lymphoma)、霍金氏症

(Hodgkin disease)、瀰漫性大 B 細胞淋巴瘤 (diffuse large B-cell lymphoma，

DLBCL)、移植後淋巴增生症 (post-transplant lymphoproliferative diseases，PTLD)、

等疾病，在體外實驗中具備使人類 B 細胞 (primary B cell) 轉形成不朽化淋巴母

細胞株 (lymphoblastoid cell line, LCL) 的能力。近年來，在 EB 病毒相關之腫瘤

形成 (neoplasia) 中發現，受體酪胺酸激酶 (Receptor tyrosine kinase, RTK) 扮演

了一個很重要的角色，然而 EB 病毒是如何藉由調控 B 細胞內受體酪胺酸激酶，

進而導致 B 細胞惡性腫瘤形成的機制，目前並未十分清楚。本篇我們發現人類 B

細胞受到 EB 病毒感染會造成受體酪胺酸激酶中隸屬於最大 Eph 家族的 EphA4

在轉錄以及轉譯的表現量下降，從外送 (overexpression) 與抑制 (knockdown) 的

實驗證明 LMP1 造成 EphA4 降低。在細胞調控機制方面，LMP1 透過 ERK 路徑

去促進 Sp1 來達到抑制 EphA4 啟動子之活性 (promoter activity)；在功能上面，

外送 EphA4 會抑制 LCL 的細胞生長；在病理上，我們發現 EB 病毒陰性的扁桃

腺檢體可偵測到 EphA4 之表現 ，而 EB 病毒陽性的 PTLD 檢體則無 EphA4 表現，

除此之外，利用免疫化學染色 (Immunochemical staining) 之方法，在有無感染

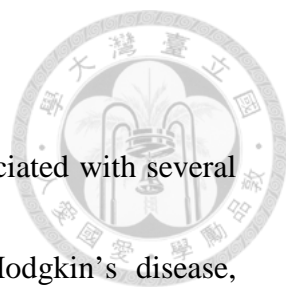EB 病毒的 DLBCL 檢體中發現，EphA4 的表現與 EB 病毒的感染呈負相關；從

資料庫分析 DLBCL 病人的存活率，發現低度表現 EphA4 的病人，其存活率較

差。我們的發現為 B 細胞中的 EphA4 如何被致癌蛋白 LMP1 調控，提供了一個

嶄新的機制，並發掘 EphA4 在 B 細胞中的功能，這些研究成果能提供 EphA4 在
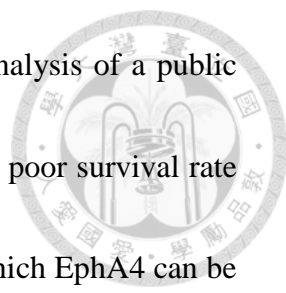
EB 病毒陽性之 PTLD 及 DLBCL 所扮演的角色一些新的觀點。

關鍵字：

EphA4 酪胺酸激酶; EB 病毒; 淋巴母細胞株; 潛伏膜蛋白 1; 移植後淋巴增生疾

病; 瀰漫性大 B 細胞淋巴瘤

Abstract

Epstein-Barr virus (EBV), an oncogenic human virus, is associated with several lymphoproliferative disorders, including Burkitt's lymphoma, Hodgkin's disease, diffuse large B-cell lymphoma (DLBCL) and post-transplant lymphoproliferative disorder (PTLD). *In vitro*, EBV transforms primary B cells into lymphoblastoid cell lines (LCLs). Recently, several studies have shown that receptor tyrosine kinases (RTKs) play important roles in EBV-associated neoplasia. However, details of the involvement of RTKs in EBV-regulated B cell neoplasia and malignancies remain largely unclear. Here, we found that EphA4, which belongs to the largest RTK Eph family, was downregulated in primary B cells post-EBV infection at the transcriptional and translational levels. Overexpression and knockdown experiments confirmed that EBV-encoded latent membrane protein 1 (LMP1) was responsible for this EphA4 suppression. Mechanistically, LMP1 triggered the ERK pathway and promoted Sp1 to suppress EphA4 promoter activity. Functionally, overexpression of EphA4 prevented LCLs from proliferation. Pathologically, the expression of EphA4 was detected in EBV-negative tonsils but not in EBV-positive PTLD. In addition, an inverse correlation of EphA4 expression and EBV presence was verified by immunochemical staining of EBV-positive and EBV-negative DLBCL, suggesting

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EBV infection was associated with reduced EphA4 expression. Analysis of a public dataset showed that lower EphA4 expression was correlated with a poor survival rate of DLBCL patients. Our findings provide a novel mechanism by which EphA4 can be regulated by an oncogenic LMP1 protein and explore its possible function in B cells. The results provide new insights into the role of EphA4 in EBV-positive PTLD and DLBCL.

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Contents

Introduction

Epstein-Barr virus (EBV) is oncogenic and its infection is associated with multiple human diseases. (Dolcetti *et al.*, 2013; Rickinson & Kieff, 2007). According to previous studies, EBV uses various strategies to manipulate cell gene expression so that it can persist in infected B cells. These include altering the expression of protein tyrosine kinases (PTKs), cytokines, adhesion molecules and anti-apoptotic genes (Chou *et al.*, 2012; Chou *et al.*, 2011; Chua *et al.*, 2008; Lai *et al.*, 2015; Lu *et al.*, 2000; Rickinson & Kieff, 2007; Tsai *et al.*, 2009). Among them, we are particularly interested in PTKs because they are important at many aspects of cell proliferation, differentiation, apoptosis, migration and tissue development. Herein, we wondered whether there are other mechanisms involved in EBV immortalization of B cells through other RTKs. In our previous study, a kinase display was used to investigate the differential expression of PTKs between primary B cells and EBV-transformed lymphoblastoid cell line (LCL). Recepteur d'Origine Nantais (RON) and Trk-related tyrosine kinase (TKT) were found to be upregulated (Chou *et al.*, 2012; Chou *et al.*, 2011; Chua *et al.*, 2008; Lu *et al.*, 2000). Using the same approach, we reveal another RTK, namely erythropoietin-producing hepatocellular receptor A4 (EphA4), which is involved in EBV lymphoproliferation. According to our kinase display data, EphA4 is clearly downregulated in LCL, compared to uninfected B cells (Appendix IA). Little

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is known about the role of EphA4 in B lymphocytes, so the expression and regulation of EphA4 in LCL and EBV-associated diseases will be explored in the thesis.
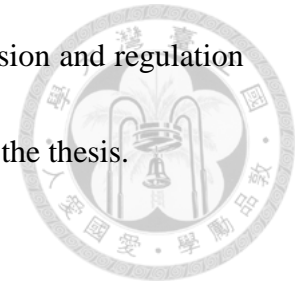
1.1 Discovery of EBV

In 1958, Dr. Denis Burkitt, a British surgeon, observed a multifocal jaw tumor prevalent in African children and later identified the tumor was lymphoma (Burkitt, 1962b). In 1964, Tony Epstein and Yvonne Barr found a herpes-like virion via electron microscopy in cultured cells from Burkitt's lymphoma and discovered a new human herpesvirus, namely Epstein-Barr virus (EBV) (Burkitt, 1962a; Epstein *et al.*, 1964; Epstein *et al.*, 1966).

1.2 EBV structure, genome and tropism

EBV, also called human herpesvirus 4 (HHV-4), belongs to the genus of lymphocryptovirus of human gamma herpesviridae. Like other herpesviruses, EBV has a linear, double stranded DNA genome with 172 kilobase pair (kbp) length in a 100 nm icosahedral nucleocapsid surrounded by tegument proteins and outside of the tegument proteins is an envelope containing both lipids and external glycoprotein spikes. (Kieff & Rickinson, 2007). The terminal repeat (TR) located at both ends of the linear EBV genome is composed of various copies of 500 base pair (bp) DNA.

EBV can infect different types of cells including primary B cells, epithelial cells, T

lymphocytes and monocytes (Guan *et al.*, 1999; Guan *et al.*, 1996; Henle *et al.*, 1967;

Nishikawa *et al.*, 1999; Pope *et al.*, 1968; Savard *et al.*, 2000). When EBV infects

host cells and establishes latent infection, the termini of the genome fuse to form an

extra-chromosomal episome in the host nucleus (Kieff & Rickinson, 2007; Yates *et*

al., 1985).
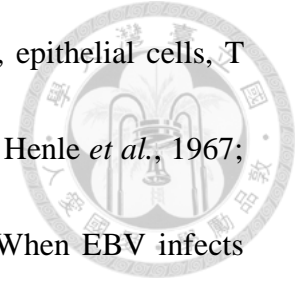
1.3 EBV infection routes

EBV persists infection in 90% human population in the world and its infection

route is through oral transmission of saliva (Macsween & Crawford, 2003; Yao *et al.*,

1985). EBV predominantly infects B cells and epithelial cells (Thompson & Kurzrock,

2004). Previous studies indicate EBV may initially infect the epithelial cells of

oropharynx , produce virion via lytic replication and the released virus from epithelial

cells subsequently infects the peripheral B lymphocytes (Allday & Crawford, 1988;

Sixbey *et al.*, 1984). The EBV envelope glycoprotein gp85 encode by BXLF2 fuses

with the cell membrane (Miller & Hutt-Fletcher, 1988) and the BKRF2-encoded gp25

which acts as a chaperon associates with gp85 for complex formation (Yaswen *et al.*,

1993). The EBV gp350/gp220 which is encoded by BLLF1 interacts with CD21, also

called complement receptor 2 (CR2), activate CD23 and triggers cell mitogenesis via

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PKCs and tyrosine kinases in B cells (Roberts *et al.*, 1996). Furthermore, the EBV

gp42 encoded by BZLF2 and gp85 forms a gp85/gp42 complex to interacts with cell

coreceptor, HLA class II to enter the B cells (Haan *et al.*, 2000; Li *et al.*, 1997; Li *et al.*, 1995; Spriggs *et al.*, 1996).
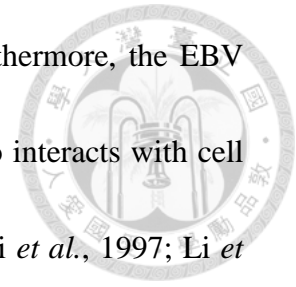

1.4 EBV-associated diseases

EBV infection is associated with several lymphoproliferative disorders including

infectious mononucleosis (ErnbergI *et al.*, 1976), Burkitt's lymphoma (Henle &

Henle, 1966), Hodgkin's disease (Johansson *et al.*, 1970), NK/T cell lymphoma (Chan

et al., 1999), diffuse large B-cell lymphoma (DLBCL) (Oyama *et al.*, 2003) and

post-transplant lymphoproliferative disorder (PTLD) (Klein *et al.*, 1969). In addition,

EBV is also associated with nasopharyngeal carcinoma (NPC), gastric carcinoma,

breast cancer, oral hairy leukoplakia (Greenspan *et al.*, 1985; Rickinson & Kieff,

2007).

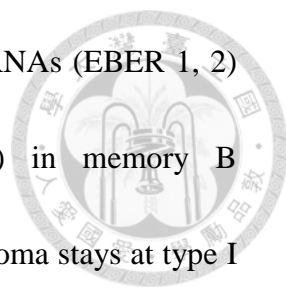## 1.5 EBV lytic replication and latency

Life cycle of EBV divides in two states: latency and lytic replication. EBV

usually stays at latent stage post infection, however, under certain conditions like

tetradecanoyl phorbol acetate (TPA) and sodium butyrate induction, EBV lytic

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protein Zta and Rta transreactivation or anti-human IgG cross-linking, EBV immediate early genes can be turned on to enter the lytic cycle.
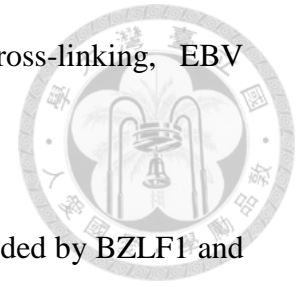
Upon lytic replication, two immediate early proteins, Zta encoded by BZLF1 and Rta encoded by BLRF1, act as transactivators and induce the following early gene and late gene expression cascades (Countryman & Miller, 1985; Feederle *et al.*, 2000; Hardwick *et al.*, 1988). Both Zta and Rta are DNA binding proteins which directly bind to the promoter regions of the lytic genes (Gruffat *et al.*, 1990; Urier *et al.*, 1989). When EBV goes to lytic cycle, Zta is the first expression gene and subsequently induces Zta and Rta expressions via binding to Zta-responsive element (ZRE) in their promoter regions (Sinclair *et al.*, 1991). Once Rta expression, it can turn on Zta and Rta promoter activities in a auto-stimulation loop (Liu & Speck, 2003; Ragoczy *et al.*, 1998). Once lytic progression starts, EBV turns on immediate early genes, Zta and Rta, sequentially activates early genes which are required for viral DNA replication, such as BALF5 (DNA polymerase) and BMRF1 (EA-D, polymerase accessory protein) (Li *et al.*, 1987; Lin *et al.*, 1991). Finally, late genes which generate viral structure proteins like viral capsid antigen (VCA) and membrane antigen (MA) are expressed for viral maturation post EBV DNA synthesis.
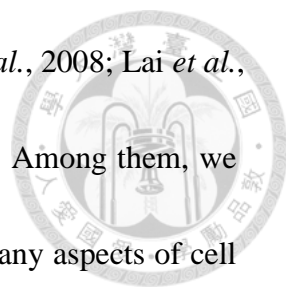
According to gene expression profile, EBV latency can be divided into four types (Klein *et al.*, 2007). Type 0 latency displays latent membrane protein 2A

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(LMP2A), non-coding RNAs including two EBV-encoded small RNAs (EBER 1, 2) and microRNAs (*Bam*H1 A rightwards transcripts, BARTs) in memory B lymphocytes (Babcock *et al.*, 1998). EBV-positive Burkitt's lymphoma stays at type I latency which exhibits EBV nuclear antigen 1 (EBNA1), EBERs and BARTs. NPC, Hodgkin's disease, and NK/T cell lymphoma are found to display type II latency which expresses EBNA1, three latent membrane proteins (LMP1, 2A, 2B), EBERs and BARTs. Type III latency displays all latent products, including six EBV nuclear antigens (EBNA1, 2, LP, 3A, 3B, 3C), three LMPs (LMP1, 2A, 2B), EBERs and BARTs in EBV-positive DLBCL of elderly, PTLD and EBV-transformed LCLs (Dolcetti *et al.*, 2013; Fox *et al.*, 2011; Rickinson & Kieff, 2007). In previous studies, EBNA1, EBNA2, EBNA3A, EBNA3C, EBNA-LP and LMP1 are essential for EBV-induced B cell immortalization through infection with recombinant EBV that has specific viral gene deficiency (Cohen *et al.*, 1989; Hammerschmidt & Sugden, 1989; Kaye *et al.*, 1993; Mannick *et al.*, 1991; Tomkinson *et al.*, 1993).
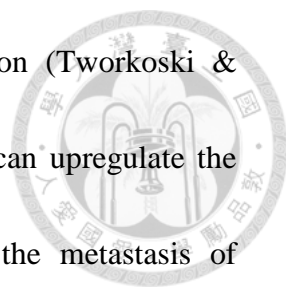
1.6 Protein tyrosine kinases (PTKs) regulated by EBV

According to previous studies, EBV uses various strategies to manipulate cell gene expression so that it can persist in infected B cells. These include altering the expression of protein tyrosine kinases (PTKs), cytokines, adhesion molecules and

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anti-apoptotic genes (Chou *et al.*, 2012; Chou *et al.*, 2011; Chua *et al.*, 2008; Lai *et al.*,

2015; Lu *et al.*, 2000; Rickinson & Kieff, 2007; Tsai *et al.*, 2009). Among them, we

are particularly interested in PTKs because they are important at many aspects of cell

proliferation, differentiation, apoptosis, migration and tissue development. In total, 90

PTKs have been defined following sequencing of the human genome (Robinson *et al.*,

2000). They can be divided into two major categories: 58 receptor tyrosine kinases

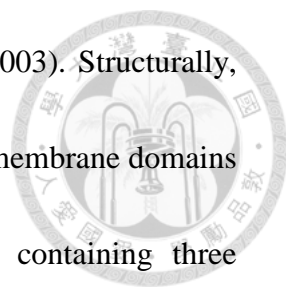(RTKs) and 32 non-receptor tyrosine kinases (Robinson *et al.*, 2000).

In the case of EBV-associated malignancies, several EBV genes are involved in

dysregulation of RTKs. EBV-encoded LMP1 elevates amounts of RON via Nuclear

factor (NF)-κB, to promote B cell proliferation and induce the migration and invasion

of epithelial cells (Chou *et al.*, 2012; Chou *et al.*, 2011). In addition, epidermal growth

factor receptor (EGFR) is induced and activated by LMP1 through PKCδ and NF-κB

(Kung *et al.*, 2011; Kung & Raab-Traub, 2010; Thornburg & Raab-Traub, 2007).

Also, c-Met is upregulated by LMP1 via Ets1 in epithelial cells (Horikawa *et al.*,

2001). Activation of discoidin domain receptor 1 (DDR1) by LMP1 contributes to the

survival of Hodgkin's lymphoma (Cader *et al.*, 2013). Recently, LMP1 has been

reported to increase the expression of fibroblast growth factor receptor 1 (FGFR1),

enhancing aerobic glycolysis and cell invasiveness (Lo *et al.*, 2015). Furthermore,

LMP1 activates the insulin-like growth factor 1 (IGF1) receptor via upregulation of its

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ligand, IGF1, to promote cell proliferation and colony formation (Tworkoski &

Raab-Traub, 2015). In addition, Zta, a lytic EBV transactivator, can upregulate the

expression of TKT (also called DDR2), which may enhance the metastasis of

nasopharyngeal carcinoma (Chua *et al.*, 2008; Lu *et al.*, 2000). Human epidermal

growth factor receptor 2 (HER2) and HER3 are dysregulated by EBV-encoded

*Bam*H1 A rightward frame 0 (BARF0), which belongs to the BART microRNAs and

found BARF0 not only increases the level of HER2/3 but also enhances cell

anchorage independence (Lin *et al.*, 2007). In the cases mentioned above, many EBV

products are responsible for increasing the expression or enhancing the kinase activity

of RTKs to deliver signaling that favors cell proliferation, migration or invasion.

Herein, we wondered whether there are other mechanisms involved in EBV

immortalization of B cells through other RTKs, especially LMP1- mediated ones.

1.7 Latent membrane protein 1 (LMP1)

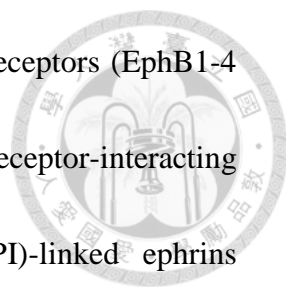LMP1 which is encoded by BNLF1 is the major oncogenic protein and plays

important roles in B cell proliferation and survival. Unlike T-cell-dependent

activation through CD40 engagement, EBV infection triggers B cell activation in

T-cell-independent manner through LMP1 by mimicking constitutively active CD40

receptor signaling to promote B cell survival and proliferation (Farrell, 1998; Gires *et*

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al., 1999; Gires *et al.*, 1997; Graham *et al.*, 2010; Li & Chang, 2003). Structurally,

LMP1 has a short N-terminal domain (amino. acid. 1-23), six transmembrane domains

(a. a. 24-186) and a long C-terminal region (a. a. 187-386) containing three

C-terminal activation regions (CTARs). The CTAR1 (a. a. 194-232) associates with

tumor necrosis factor receptor-associated factors (TRAFs) to NF-κB,

phosphatidylinositol 3-kinase (PI3K)/Akt, and mitogen-activated protein kinases

(MAPKs) including c-jun N-terminal N-kinase (JNK), extracellular signal-regulated

kinase (ERK) and p38 pathways. On the other hand, the CTAR2 (a. a. 351-386)

recruited tumor necrosis factor receptor-associated-death domain containing protein

(TRADD) to trigger the activation of NF-•B and MAPKs (Young & Rickinson, 2004).

Different from CTAR1/2, the CTAR3 (a. a. 275-330) interacted with Janus kinases

(JAKs) to stimulate signal transducers and activators of transcription (STAT)

activation (Dawson *et al.*, 2012). We identified another RTK, EphA4, was

downregulated in EBV-transformed LCLs through kinase display assay (Appendix

IA). Therefore, the detail mechanism of how EphA4 was regulated by EBV would be

explored in this thesis.

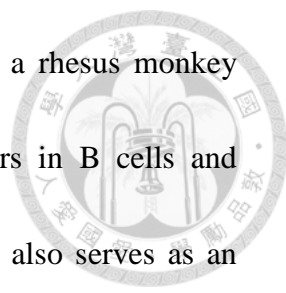**1.8 Erythropoietin-producing hepatoma (Eph) receptor family**

EphA4 belongs to Eph receptor family, the largest family of RTKs. In mammals,

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there are 9 EphA receptors (EphA1-8 and EphA10) and 5 EphB receptors (EphB1-4

and EphB6) which interact with their ligands, ephrins (Eph receptor-interacting

proteins) including 5 A-type glycosylphosphatidylinositol (GPI)-linked ephrins

(ephrin-A1-5) and 3 B-type transmembrane ephrins (ephrin-B1-3) (Kandouz, 2012;

Pasquale, 2010). The Eph-ephrin interaction is through cell-cell contact and thus

mediates bidirectional signaling: a forward signal in the Eph-expressing cells and a

reverse signal in the ephrin-expressing cells (Pasquale, 2005). The interactions

between Eph receptors and their ligands trigger both forward Eph kinase activity in

Eph receptor-expressing cells and reverse Src family kinase activity in

ephrin-expressing cells (Pasquale, 2010). These bi-directional signaling pathways

make the Eph family distinct from other RTKs.

1.9 Eph receptors and virus infection

Notably, the involvement of Eph in viral infection has been explored in some

viruses. For example, hepatitis C virus exploits EphA2 and EGFR as cellular

receptors for virus entry (Lupberger *et al.*, 2011). It has been shown that EphA2 acts

as an entry receptor of Kaposi's sarcoma-associated herpesvirus (KSHV, human

herpesvirus-8, HHV-8) via interaction with viral envelope glycoproteins H and L

(gH/gL) (Chakraborty *et al.*, 2012; Hahn *et al.*, 2012). Not only KSHV but rhesus

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monkey rhadinovirus (RRV) which belongs to the γ herpesvirus, a rhesus monkey

homolog of KSHV, uses its gH/gL binding to ten Eph receptors in B cells and

endothelial cells (Hahn & Desrosiers, 2013). In particular EphA4 also serves as an

entry receptor for RRV and KSHV infection although it is not the main target of

gH/gL (Hahn & Desrosiers, 2013). Collectively, Eph receptors not only play roles in

physiological activities but also are involved in pathogenesis (Hahn & Desrosiers,

2013).

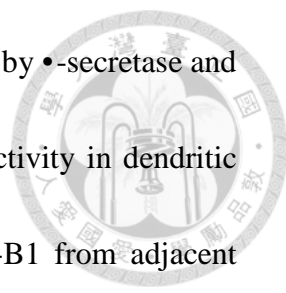## 1.10 EphA4 and its ligands-ephrins

Like most Eph receptors, EphA4 composed of 986 amino acids has an

extracellular part including N-terminal ligand binding domain, a cysteine-rich region,

2 fibronectin (FN) type III repeats and transmembrane (TM) regions followed by the

juxtamembrane (JM) region (a. a. 570-620) with tyrosine (Tyr) phosphorylation sites,

kinase domain, a sterile alpha motif (SAM) and a PSD95, DLG, ZO1 (PDZ) domain

(Pasquale, 2010). EphA4 can interact with both A-type and B-type ephrins including

ephrin-A1-5 and ephrin-B1-3 (Bowden *et al.*, 2009; Iwasato *et al.*, 2007; Kandouz,

2012; Kemp *et al.*, 2009; Murai *et al.*, 2003; Pasquale, 2005; 2010; Prevost *et al.*,

2002). Upon ligand binding, EphA4 delivers forward signaling through

autophosphorylation at Tyr596 and Tyr602 sites of JM region followed by kinase

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domain activation (Ellis *et al.*, 1996).

1.11 Function of EphA4 in neuron guidance, platelet aggregation and
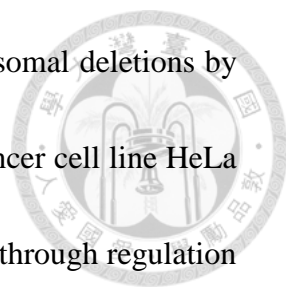
mesenchymal-to-epithelial transition (MET)

EphA4 is better-understanding in neuron guidance, platelet aggregation and MET during somite morphogenesis (Barrios *et al.*, 2003; Carmona *et al.*, 2009; Kemp *et al.*, 2009; Murai *et al.*, 2003; Pasquale, 2005; 2010; Prevost *et al.*, 2002; Prevost *et al.*, 2004). EphA4/ephrin-A3 interaction activates EphA4 forward signaling in neuron which leads to dendrite retraction and ephrin-A3 reverse signaling in astrocytes which inhibits glutamate transports (Carmona *et al.*, 2009; Murai *et al.*, 2003) (Appendix II). EphA4 activates spine-associated RapGAP (SPAR) to reduce Ras-related protein 1 (Rap1) activity, therefore suppresses integrin-mediated adhesion to the extracellular matrix and causes neuron dendrite spine retraction (Richter *et al.*, 2007) (Appendix III). In ephrin-A1 stimulated neurons, EphA4 reduces ERK phosphorylation, leads to increased activity of Tuberous sclerosis complex 2 (TSC2), further inactivates Ras homolog enriched in brain (RHEB) and downstream mTOR pathway and finally induces growth cone collapse (Nie *et al.*, 2010) (Appendix IV). Metalloprotease Meltrin β (ADAM19) interacts with EphA4 and further impedes endocytosis of EphA4/ephrin-A5 complex to avoid axon repulsion at neuromuscular junctions

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(Yumoto *et al.*, 2008). In addition, EphA4 is reported as a substrate by •-secretase and results in intracellular domain (ICD) releasing to enhance Rac1 activity in dendritic spine formation (Inoue *et al.*, 2009). Through binding of ephrin-B1 from adjacent platelets, EphA4 recruits Lyn, Fyn and the cell adhesion molecule L1 to promote integrin-mediated adhesion via Rap1B activation thus leads to platelet aggregation (Prevost *et al.*, 2002; Prevost *et al.*, 2004) (Appendix V). Interaction between EphA4 and ephrin-B2 triggers MET during somite morphogenesis in zebrafish neural tube (Barrios *et al.*, 2003; Kemp *et al.*, 2009) (Appendix VI). In summary, EphA4 induces dendrite retraction for neuron guidance, promotes platelet aggregation and triggers MET during somite morphogenesis.
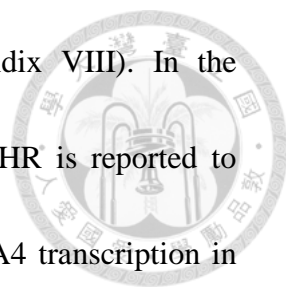
1.12 Regulation of EphA4 in cancer progression

Recently, EphA4 has been found to be involved in cancer (Easty *et al.*, 1997; Kandouz, 2012; Narayan *et al.*, 2003; Oshima *et al.*, 2008; Pasquale, 2010; Saintigny *et al.*, 2012). Of interest, various reports have indicated that Eph receptors can function in oncogenic promotion or tumor suppression in several human cancers at different stages or in different cancers (Kandouz, 2012; Narayan *et al.*, 2003; Oshima *et al.*, 2008; Pasquale, 2010). For example, EphA4 is upregulated and EphB2 is downregulated in colon cancer with liver metastasis (Oshima *et al.*, 2008). In invasive

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cervical carcinoma, EphA4 expression is decreased due to chromosomal deletions by

loss of heterozygosity (LOH) (Narayan *et al.*, 2003). In cervical cancer cell line HeLa

and U373 glioma cells, EphA4 is reduced due to mRNA instability through regulation

of 3'untranslated regions (Winter *et al.*, 2008). EphA4 mRNA is downregulated or

lost in metastatic melanoma (Easty *et al.*, 1997). Furthermore, EphA4 downregulates

ERK phosphorylation and inhibits migration and invasion in non-small cell lung

cancer (NSCLC) cells (Saintigny *et al.*, 2012). In 293T and COS-7 cells,

β2-chimaerin binds to kinase domain of EphA4 and treating with ephrin-A1

inactivates Rac1 in cells expressing EphA4 and β2-chimaerin and further causes

inhibition of cell migration (Takeuchi *et al.*, 2009) (Appendix VII). In hepatocellular

carcinoma (HCC), microRNA-10a targets EphA4 mRNA and overexpression of

EphA4 inhibits cell migration and invasion by regulation of the

epithelial-to-mesenchymal transition (EMT) process (Yan *et al.*, 2013).

Overexpression of EphA4 leads to caspase-mediated cell apoptosis in the absence of

ephrin-B3 although phosphorylation of EphA4 is not required for inducing cell death

(Furne *et al.*, 2009) .

EphA4 is also involved in tumor promoting pathway, for example, EphA4

directly interacts with FGFR1 through their cytoplasmic domains, phosphorylates

docking proteins, FGFR substrate 2-α (FRS2-α) and promotes cell migration and

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proliferation (Fukai *et al.*, 2008; Yokote *et al.*, 2005) (Appendix VIII). In the

transcriptional regulation, the oncogenic fusion protein, Pax3/FKHR is reported to

directly bind to the promoter region of EphA4 thus increase EphA4 transcription in

osteosarcoma cell line SaOS-2 (Begum *et al.*, 2005). In COS-7 cells and muscle cell

line C2C12, EphA4 is reported to interact with Jak2 and phosphorylates Stat1 and

Stat3 in the presence of ephrin-A1 to promote transformation and invasion (Lai *et al.*,

2004). Hence, EphA4 plays controversial roles in either oncogenic promotion or

tumor suppression.

1.13 EphA4 in immune cells

In hematopoietic cells, EphA4 is found to express on human CD4-positive T

cells, CD8-positive T cells and CD19-positive B cells but not on CD14-positive

monocytes (Aasheim *et al.*, 2005; Holen *et al.*, 2010). On the other hand, its ligand,

ephrin-A1 is expressed on endothelial cells in tonsils but not on T cells and B cells

(Aasheim *et al.*, 2005). EphA4 promotes human memory CD4 T cell migration in the

presence of ephrin-A1 and Lck, Fyn, Slp76 and Vav are involved in EphA4-induced

signaling in the same cells by immunoprecipitation (Holen *et al.*, 2010) (Appendix

IX). However, EphA4 has not been well studied in EBV-infected B cells.

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1.14 Aim of this study

Previous studies identified that RON and TKT were upregulated in LCLs compared to uninfected B cells through a kinase display assay (Chou *et al.*, 2012; Chou *et al.*, 2011; Chua *et al.*, 2008; Lu *et al.*, 2000). Using the same approach, we observed EphA4 was downregulated in LCLs (Appendix IA). So far, the role of EphA4 in B lymphocytes is not well-known. Herein, the expression and regulation of EphA4 in LCL and EBV-associated lymphoproliferative disorders will be investigated in this thesis.
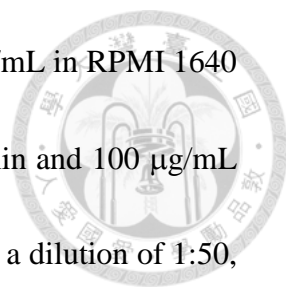
Materials and Methods

2.1 Purification of EBV virion

To induce EBV lytic replication, 240 mL of 100% confluent EBV-harboring B95.8 cells were treated with 40 ng/mL of TPA and 3 mM of sodium butyrate for 72 hours in a 75T flask. Supernatants were collected and centrifuged at 8,000 rpm for 30 min at $4^{o}C$ with JA14 rotor (BECKMAN) to remove cell debris. After ultracentrifuge at 15,000 rpm for 90 min at $4^{o}C$ with type 19 rotor (BECKMAN), supernatants were discarded and viral particles were re-dissolved in 5 mL of serum-free RPMI, filtered with 0.22 μm filter, and stored at $-80^{o}C$.

2.2 B cell purification, treatments and EBV infection

White blood cell (WBC) concentrates were obtained from anonymous donors at Taipei Blood Center of Taiwan Blood Service Foundation. Two units of human WBC concentrates were diluted with 120 mL of serum-free RPMI medium and loaded onto Ficoll-paque PLUS (GE Helthcare). Peripheral blood mononuclear cells (PBMCs) were isolated via density gradient centrifugation at 2,300 rpm for 30 min at room temperature without break. After collecting the PBMCs in the interface, cells were washed with serum-free RPMI twice and CD19-positive B cells were purified by Dynabeads (Invitrogen) according to the manufacturer's instructions. Peripheral blood

CD19-positive B lymphocytes were seeded at a density of 10^6 cells/mL in RPMI 1640

medium containing 20% FCS, 1 mM glutamine, 100 U/mL penicillin and 100 µg/mL

streptomycin in a 12 well plate.and then treated with B95.8 EBV in a dilution of 1:50,

10ng/mL recombinant human IL-4 (R&D Systems) plus 1 µg/mL anti-human CD40

agonist antibody (R&D Systems), 200 ng/mL lipopolysaccharides (LPS)

(Sigma-Aldrich) or 20 µg/mL poly I:C (GE Healthcare) for 1 to 3 days. Experiments

of human samples have been approved by Institutional Review Boards (IRB) of

National Taiwan University Hospital, Taipei, Taiwan.

2.3 Cell culture

B95.8 cell line is a marmoset B-lymphoblastoid cell line harboring EBV (Miller

et al., 1972). Human peripheral blood CD19-positive B cells and were infected by

B95.8 EBV for 28 days to establish LCLs *in vitro*. BJAB cells are derived from

EBV-negative Burkitt's lymploma from an African patient (Menezes *et al.*, 1975). All

B-cell lines were cultured in complete RPMI medium. TW01 cells are EBV-negative

cells derived from nasopharyngeal carcinoma from a Taiwanese patient with EBV

DNA loss during cultivation (Lin *et al.*, 1993). HEK293T cells are derived from

human embryonic kidney cell line and immortalized by adenovirus E1 and simian

virus 40 (SV40) large T antigen. TW01 and HEK293T cells were maintained in

complete Dulbecco modfied Eagle medium (DMEM; containing 10% FCS, 1 mM glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin).
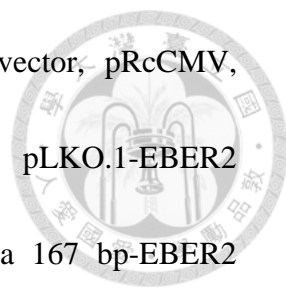
2.4 Kinase display assay

The tyrosine kinase display assays were described in a previous report (Kung *et al.*, 1998; Lu *et al.*, 2000). Briefly, total RNA was extracted and cDNAs of protein tyrosine kinases were generated and amplified by using [γ-^{33}P]-labeled degenerate primers designed for the conserved kinase domain. The resulting 170 base pairs PCR products were gel-purified and digested separately using 16 restriction enzymes, including *Aci*I, *Alu*I, *Ava*II, *BsaH*I, *Bsp*1286I, *Hae*III, *Hha*I, *Hin*fI, *Bsr*I, *Bst*NI, *Hpa*II, *Mnl*I, *Mse*I, *Mwo*I, *Nci*I, and *Rsa*I. The digestion products were resolved in a denaturing polyacrylamide gel. The patterns of restriction enzyme digestion were identified for each specific protein tyrosine kinase in the data bank.

2.5 Plasmids

pSG5-LMP1, a gift from Alan B. Rickinson (Cancer Research UK Institute for Cancer Studies, University of Birmingham, Birmingham, UK), is a B95.8 strain LMP1-expressing plasmid constructed into SV40 promoter-driven pSG5 vector. pRcCMV-EBNA2 (Lin *et al.*, 2000) and pRcCMV-Zta (Lu *et al.*, 2000) were

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constructed into the cytomegalovirus (CMV) promoter-driven vector, pRcCMV, which was purchased from Invitrogen. pLKO.1-EBER1 and pLKO.1-EBER2 plasmids were constructed by inserting a 173 bp-EBER1 and a 167 bp-EBER2 genomic DNA into the lentivirus vector, pLKO.1, respectively at the 5' *Age*I and the 3' *Eco*RI site. Both pLKO.1-EBER1 and pLKO.1-EBER2 plasmids are constructed by Dr. Shu-Chun Tsai. pcDNA3-BARF0 was constructed by inserting the genomic DNA of EBV-positive Akata cells into the HA-tagged SV40 promoter-driven pcDNA3 vector via *Bam*HI and *Eco*RI (Lin *et al.*, 2015a; Lin *et al.*, 2007). The expressing lentivirus production plasmids, pMD.G, p8.91 and pSIN, are gifts from Dr. Hsei-Wei Wang (Department and Institute of Microbiology and Immunology, National Yang-Ming University, Taipei, Taiwan). The LMP1 expressing lentivirus plasmid, pSIN-LMP1, were constructed by insertion of the full-length LMP1 cDNA fragment into pSIN vector at the 5' *Nde*I site and the 3' *Mlu*I site. Its mutant plasmids included LMP1-deleted CTAR1 (ΔCTAR1) with the deletion of LMP1 amino acids (a. a.) 194 to 232, deleted CTAR2 (ΔCTAR2) with deletion of LMP1 a. a. 351 to 386 and LMP1-deleted CTAR1/2 (ΔCTAR1/2) plasmids were reported previously (Chou *et al.*, 2011). The *EPHA4* promoter (nucleotides -1000 to +42) was inserted into the pGL3-basic vector luciferase reporter plasmid (Promega) through *Nhe*I and *Xho*I sites. For lentivirus packsge, *EPHA4* promoter (nucleotides -1000 to +42) was inserted into

doi:10.6342/NTU201603335

the pCDH-GL3-basic vector luciferase reporter plasmid through *Nhe*I and *Xho*I sites.

The pEGFP-C1 purchased from Promega is a CMV promoter-driven green

fluorescent protein (GFP)-expressing plasmid with multiple cloning sites (MCS). The

EphA4 (pCDNA3.1-EphA4) WT and mutant plasmids KD, 2M, 3M and ΔJM are

kindly provided by Dr. Tang-Long Shen (Department of Plant Pathology and

Microbiology, National Taiwan University, Taipei, Taiwan). The Flag-tagged EphA4

WT and mutant lentivirus plasmids were constructed at the 5'-*Spe*I and 3'-*Mlu*I sites

of pWPI lentiviral plasmid which is a gift from Dr. Min-Liang Kuo (Institute of

Toxicology, National Taiwan University, Taipei, Taiwan). The shLMP1 lentivirus

plasmid was constructed into the pLKO.1 lentivirus plasmid at the 5'-*Age*I and

3'-*EcoR*I sites (Chou *et al.*, 2011). For knockdown lentivirus production plasmids,

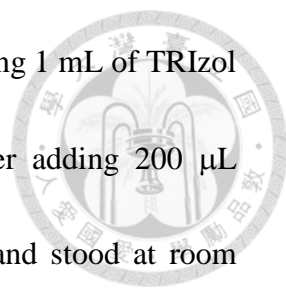pMD2.G, p8.91 and the other small hairpin RNA (shRNA)-producing plasmids

constructed into pLKO.1 vector were purchased from National RNAi Core Facility

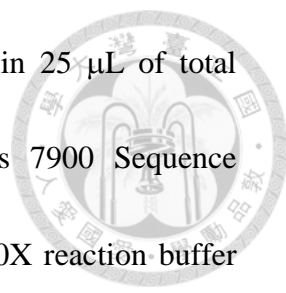(Academia Sinica, Taipei, Taiwan). All the shRNAs with targeting sequences were

listed as Appendix XI.

2.6 RNA extraction, reverse transcription and quantitative polymerase chain reaction (RT-Q-PCR)

Total RNA was extracted by using TRIzol reagent (Invitrogen) according to the
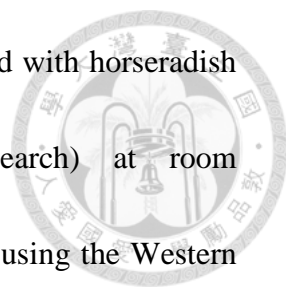
manufacturer's instructions. In brief, cell pellets were lysed by adding 1 mL of TRIzol reagent, and incubated for 5 minutes at room temperature. After adding 200 μL chloroform, the mixture was vigorously shaken for 15 seconds, and stood at room temperature for 2-3 minutes. The sample was centrifuged at 12000 x g at 4°C for 30 minutes. The colorless upper aqueous phases were collected, added equal amount of isopropanol (400 μL), gently mixed, and incubated at room temperature for 10 minutes. Next, the sample was centrifuged at 12000 x g at 4°C for 30 minutes. The RNA pellet was washed with 1 mL of 75% ethanol by centrifugation of 12000 x g at 4°C for 15 minutes and then the supernatants were removed. The total RNA pellets were dissolved in RNase-free double distilled water and the concentration was detected by NanoDrop 2000c spectrophotometer (Thermo). Reverse transcription has been described previously (Chou *et al.*, 2011). Briefly, 2 μg of RNA with 1 μg of random hexamers (Bionovas) was denatured at 70℃ for 10 minutes in a 12-μL reaction, and then adding 4 μL of M-MLV RT 5X Reaction buffer (Promega), 2 μL of 100 mM DTT (Promega), 1 μL of 2.5 mM dNTP mixture (Bionovas), and 1 μL (200 units) of M-MLV reverse transcriptase (Promega). The reaction was carried on 25℃ for 10 minutes, 42℃ for 50 minutes and 70℃ for 15 minutes to reverse transcribed RNA into cDNA. For Q-PCR analysis, the cDNA added 12.5 μL of FastStart Universal probe master (Roche), 1 μL of 10 μM forward primer, 1 μL of 10 μM

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reverse primer and 0.25 μL of Universal ProbeLibrary (Roche) in 25 μL of total volume, and performed the experiment in Applied Biosystems 7900 Sequence Detection System. For PCR analysis, the cDNA added 1 μL of 10X reaction buffer (Protech), 1.5 μL of 2.5 mM dNTP mixture (Bionovas), 0.2 μL of 10 μM forward primer, 0.2 μL of 10 μM reverse primer and 0.7 μL of ProZyme DNA polymerase (Protech) in 10 μL of total volume. The PCR condition of EBER1, EBER2 and BARF0 was 94°C for 1 minute, 60°C for 1 minute, 72°C for 1 minute, repeated for a total of 30 cycles. The PCR condition of β-actin was 94°C for 1 minute, 60°C for 1 minute, 72°C for 1 minute, repeated for a total of 23 cycles. PCR reaction was performed in T3 Thermocycler (Biometra). The specific primers used for RT-PCR and RT-Q-PCR were shown as Appendix XII.

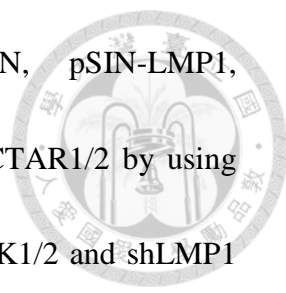## 2.7 Western blotting and antibodies (Abs)

Cells were lysed using radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris, pH7.4, 2 mM EDTA, 1.5 M NaCl, 50 mM NaF, 0.1 mM Na_3VO_4, 0.1% SDS, 0.5% Sodium deoxycholate, and protease inhibitors) and Western blotting was performed as follows. Briefly, cell lysates (20 μg) were resolved by electrophoresis in sodium dodecyl sulfate (SDS)-polyacrylamide gels and electrotransferred onto PVDF membranes (PerkinElmer Life Sciences). Milk-blocked blots were incubated with

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primary antibodies at 4°C overnight and then washed and incubated with horseradish peroxidase-conjugated secondary Abs (Jackson ImmunoResearch) at room temperature for 2 hours. The proteins of interest were detected by using the Western Lightning chemiluminescence reagent (PerkinElmer Life Sciences) and detected by exposure to X-ray films. Abs were used as follows: EphA4 (clone: S20; Santa Cruz Biotechnology and ECM Biosciences), β-actin (clone: AC-15; Sigma-Aldrich), Flag (clone: M2; Sigma-Aldrich) ,phospho-Akt S473 (Cell Signaling Technology), Akt (clone: H136; Santa Cruz Biotechnology), phospho-I•B-α-S32/36 (Cell Signaling Technology), p65(clone: C20; Santa Cruz Biotechnology), phospho-JNK T183/Y185 (Cell Signaling Technology), JNK (Millipore), phospho-ERK T202/Y204 (Cell Signaling Technology), ERK (clone: K23; Santa Cruz Biotechnology), phospho-Sp1 T739 (abcam), Sp1 (clone: PEP2; Santa Cruz Biotechnology). Abs against EBV viral products including EBNA1 (patient's serum: NPC47), EBNA2 (clone: PE2), LMP1 (clone: CS1-4; DAKO and clone: S12), Zta (clone: 1B4), BGLF4 (clone: 2616), and BMRF1 (clone: 88A9) were as reported previously (Chang *et al.*, 2004).

2.8 Lentivirus production

For lentivirus package, the 2×10^6 HEK 293T cells were seeded in 10 cm dish overnight before transfection. Cells with 90% confluency were co-transfected with 3

μg of pMD.G, 6 μg of p8.91,and 9 μg of pSIN, pSIN-LMP1, pSIN-LMP1-ΔCTAR1, pSIN-LMP1-ΔCTAR2, or pSIN-LMP1-ΔCTAR1/2 by using lipofectamine^{TM} 2000 (Invitrogen). For shLuciferase, shSp1, shERK1/2 and shLMP1 lentivirus production, HEK 293T cells were co-transfected with 1 μg of pMD.G, 9 μg of p8.91 and 10 μg of pLKO-shLuciferase, shSp1, shERK1/2 or shLMP1. For EphA4 WT and mutant expressing lentivirus package, HEK 293T cells were co-transfected with 1 μg of pMD2.G, 9 μg of p8.91 and 10 μg of pWPI-EphA4 WT, pWPI-EphA4 KD, pWPI-EphA4 2M, pWPI-EphA4 3M or pWPI-EphA4 ΔJM. After 16 hours transfection, the medium was replaced with 20 mL complete DMEM medium with 1%BSA, incubated for 48 hours, collected the supernatants which containing lentiviruses by filtering through 0.22 μm filters, and stored at -80°C.

2.9 Infection of lentivirus

The method of production and infection with lentivirus was reported previously (Tsai *et al.*, 2009). For EphA4 WT and mutant expressing lentivirus infection, 5×10^5 LCLs were infected with lentivirus at a multiplicity of infection (MOI) of 4 for 5 days. For LMP1 and Sp1 knockdown, LCLs were seeded at a density of 1×10^6 cells/mL and infected with shLuc control or shLMP1 lentivirus at MOI of 1 for 5 days and then selected with 2 μg/mL puromycin for 48 hours. BJAB cells were seeded in 1×10^6

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cells/mL and infected with pSIN-LMP1 and its deletion mutant expressing lentivirus

at a range of 0.5-4 MOI for 5 days.

2.10 Treatment with inhibitors

LCLs were seeded in 1×10^6 cells/mL and treated with 20 µM of PI3K inhibitor

LY294002, 20 µM of JNK inhibitor SP600125, 20 µM of MEK inhibitor U0126, 2.5

µM of NF-κB inhibitor BAY 11-7082 for 48 hours, respectively. TW01 cells were

treated with 500 nM of Sp1 inhibitor mithramycin for 24 hours. The inhibitors

mentioned above were purchased from Merck Millipore. Dimethylsulfoxide (DMSO)

was served as a solvent control.

2.11 Reporter assay

TW01 cells were seeded at a density of 1×10^5 cells/well in a 12-well plate and then

cotransfected with 0.5 µg EphA4 promoter luciferase reporter plasmids, 0.25 µg

pSG5-LMP1 and 0.05 µg green fluorescent protein (GFP) expressing plasmids

(pEGFP-C1, Promega) by using T-Pro Non-liposome transfection Reagent II (T-Pro NTR

II, T-Pro Biotechnology) according to the manufacturer's instructions. Cells were

harvested and the luciferase activities and GFP fluorescent intensity were detected using

the Bright-Glo Luciferase Assay System kit (Promega) 3 days post transfection. The

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relative fold induction of luciferase activity from each transfectant was first standardized

with EGFP, followed by normalization to the control vector pGL3.

2.12 Proliferation assay

LCLs were infected with EphA4 WT, mutants or vector control expressing

lentiviruses for 2 days and lentivirus-infected LCLs were seeded at 1×10^4 cells per

well in 96 well plates for 5 days. Before the indicated time point, cells were treated

with AlamarBlue (Thermo) for 4 hours. The absorbance was measured at 570 nm and

600 nm by ELISA reader (Beckman Coulter DU 730) according to the manufacturer's

instructions. The percent reduction of AlamarBlue was calculated as follow equation.

$$\text{Shuf hqvwdj h#dhgxf vwr qr #i#Dodp duEoxh}^{\text{a}} = \frac{(117216 \times A1) - (80586 \times A2)}{(155677 \times N2) - (14652 \times N1)}$$

A1= observed absorbance reading for test well at 57 0nm

A2= observed absorbance reading for test well at 600 nm

N1= observed absorbance reading for negative control well at 570 nm

N2= observed absorbance reading for negative control well at 600 nm
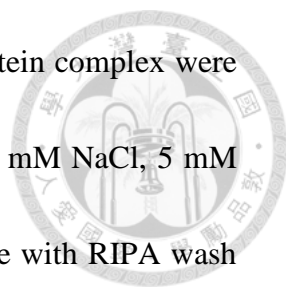
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2.13 Propidium iodide staining and flow cytometry analysis

LCLs with ectopically expressed EphA4 WT and mutants were fixed in 75% ethanol, stained with 10 μg/mL propidium iodide (Sigma-Aldrich) and analyzed through FACSCalibur flow cytometer (BD) as described previously (Tsai *et al.*, 2013).
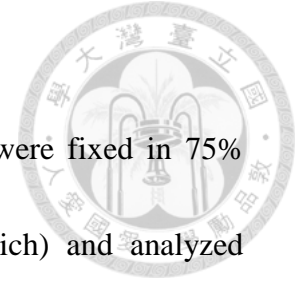
2.14 Chromatin immunoprecipitation (ChIP) assay

BJAB cells were infected with pSIN vector control and pSIN-LMP1 expressing lentiviruses for 5 days. Cells were crosslinked with 1% formaldehyde at room temperature for 10 minutes and added 0.125 M Glycine to stop crosslinking at room temperature for 10 minutes. The crosslinked cells were washed twice with PBS and harvested in 1 mL of cell lysis buffer (5 mM HEPES, pH8.0, 85 mM KCl, 0.5% NP40, 1 mM DTT, and 0.1 mM PMSF) at 4°C for 30 minutes. After centrifugation of 3000 x g for 20 minutes, the pellets were lyzed by nuclei lysis buffer (50 mM Tris, pH8.0, 10 mM EDTA, 1% SDS, and 1 mM DTT, 0.1 mM PMSF, 100 mM NaF, and 1 mM Na_3VO_4) and sonicated DNA fragments size from 500 to 1000 bp. After centrifugation of 16000 x g for 10 minutes, the supernatants were collected and quantified its protein concentration. The 500 μg of the DNA-protein complexes were immunoprecipitated using anti-Sp1 antibody or isotype control-rabbit IgG overnight

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and incubated with protein A beads for 2 hours. The DNA and protein complex were washed twice with RIPA wash A buffer (50 mM Tris, pH8.0, 150 mM NaCl, 5 mM EDTA, 1% NP40, 0.1% SDS, and 1% sodium deoxycholate), twice with RIPA wash B buffer (50 mM Tris, pH8.0, 300 mM NaCl, 5 mM EDTA, 1% NP40, 0.1% SDS, and 1% sodium deoxycholate), twice with LiCl wash buffer (50 mM Tris, pH8.0, 150 mM NaCl, 5 mM EDTA, 300 mM LiCl, 1% NP40, 0.1% SDS, and 1% sodium deoxycholate), and three times with TE buffer (10mM Tris, pH 8.0, 1 mM EDTA). For decrosslinking the DNA-protein complexes, the fresh prepared elution buffer (50 mM $NaHCO_3$ and 1% SDS) was added and incubated at $67^{o}C$ overnight. After centrifugation of 2000 x g for 2 minutes, the supernatants were collected and added 10 mg/mL of proteinase K at $45^{o}C$ for 2 hours The DNA was extracted by phenol/chloroform with equal amount and precipitated with 100% ethanol and glycogen at $-80^{o}C$ for 2 hours. The DNA was washed with 1 mL of 70% ethanol by centrifugation of 16000 x g for 15 minutes, air dried, and dissolved with 20 μL distilled water. The 2 μL DNA was analyzed by PCR with specific primers of *EPHA4* promoter spanning the Sp1-binding sites. The PCR condition of *EPHA4* promoter was $95^{o}C$ for 30 seconds, $60^{o}C$ for 30 seconds, $72^{o}C$ for 30 seconds, repeated for a total of 40 cycles. The PCR condition of *GAPDH* promoter was $95^{o}C$ for 45 seconds, $55^{o}C$ for 45 seconds, $72^{o}C$ for 30 seconds, repeated for a total of 40 cycles. The specific

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primers used for ChIP-PCR were shown as Appendix IV. The total DNA was served as input control and the results of PCR were analyzed on 2% agarose gel and visualized by staining of ethidium bromide (EtBr).
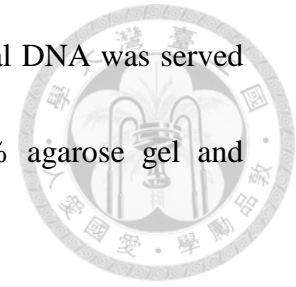
2.15 Immunohistochemisty (IHC) and in situ hybridization of EBER assays

Tonsil, PTLD, and DLBCL biopsies were obtained from the National Taiwan University Hospital. Paraffin-embedded sections were dewaxed and rehydrated and IHC assays of EphA4 and LMP1 were performed by using the Starr Trek Universal Detection system (Biocare) according to manufacturer's protocol. For EBER detection, *in situ* hybridization assays were performed by using an EBER probe (5'-CTCCTCCCTAGCAAAACCTCTAGGACGGCG-3') labeled with a DIG-labeling Kit (Roche) according to manufacturer's manual. In brief, sections were dewaxed, rehydrated, digested with proteinase K (Roche), hybridized with the EBER probe and then incubated with alkaline phosphatase-conjugated DIG antibody (Roche). The signal was detected with nitroblue tetrazolium chloride/bromochloroindolyphosphate chromogen (Roche) and counterstained with nuclear fast red. EphA4 (clone: S20; Santa Cruz Biotechnology) and LMP1 (clone: CS1-4; DAKO) Abs were used for IHC assay. The photographs were taken by a photomicroscope (Olympus Corporation).

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2.16 Kaplan-Meier plots

The microarray datasets of DLBCL were downloaded from the National Center for Biotechnology Information (NCBI) and Gene Expression Omnibus (GEO) database (accession number GSE4475). Overall survival was analyzed by generating Kaplan-Meier plots. Patients were divided into high and low EphA4 groups, according to the median expression of EphA4.

2.17 Statistical Analysis

All statistical analysis was conducted using GraphPad Prism software program. Quantitative data were reported as mean ± standard error of the mean and compared using the unpaired Student's t test. Clinical correlation was determined by Fisher's exact test. For Kaplan-Meier survival analysis, a log-rank test was employed to compare the difference between two groups. Data were considered statistically significant at $p < 0.05$ (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

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Results

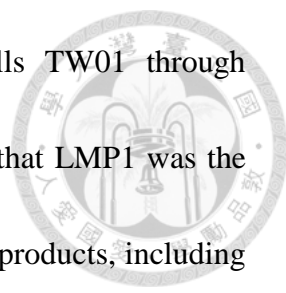## 3.1 EphA4 is downregulated post-EBV infection

Because RTKs play a critical role in EBV infected cells, the RTK expression

profile was analyzed by kinase display assay using degenerate primers (Lu *et al.*,

2000). According to the *BstN*I, *Hha*I, and *Mnl*I restriction enzyme digestion profile,

downregulation of EphA4 was found in three LCLs compared to control primary B

cells (Appendix IA). To verified the results of kinase display assay, EphA4 specific

primers are used for RT-PCR assay. Of note, expression of EphA4 is detected in

primary B cells but none or lower in tested LCLs (Appendix IB). To confirm this

result, mRNA and protein expression levels of EphA4 were detected in cells infected

with EBV for 3, 7, 14, 21 or 28 days (defined as LCL), compared to uninfected

primary B cells. Clearly, mRNA and protein expression levels of EphA4 were

reduced to approximately 50% to10% post-EBV infection, compared to primary B

cells (Figure 1A-B). Expression of EBNA1 and LMP1 indicated that the primary B

cell was successfully transformed by EBV (Figure 1B). In addition to EBV infection,

B cells can be activated by various treatments including anti-CD40 antibody plus IL-4,

lipopolysaccharide (LPS) and poly I:C to mimic T-cell dependent or independent

activation (Lin *et al.*, 2015b). To clarify whether B-cell activation can decrease

EphA4 expression, EphA4 transcripts were measured after 1 to 3 days of incubation.

As shown in Figure 2 and Appendix X, EphA4 was downregulated not only by EBV infection but also by anti-CD40 antibody plus IL-4 treatment. This fit previous findings that EBV LMP1 functionally mimics constitutive active CD40 receptor associated with TRAFs (Soni *et al.*, 2007; Young & Rickinson, 2004). Meanwhile, treatments of LPS or poly I:C which triggered TLR4 or TLR3 signal pathway did not alter EphA4 expression.

To determine whether this effect is common in B cells infected by EBV, expression of EphA4 mRNA was detected by RT-Q-PCR in another eight LCL cell lines established in our lab (Tsai *et al.*, 2009). Compared to primary B cells, EphA4 transcripts were also detected at 10% to 50% normal levels in LCLs (Figure 3). To verify these observations, total RNA and protein were collected from two paired primary B cells and their corresponding LCLs. Consistent with the data above, EphA4 mRNA and protein expression were reduced in LCLs, suggesting that EBV infection influenced the expression of EphA4 at the transcriptional and translational levels (Figure 4).
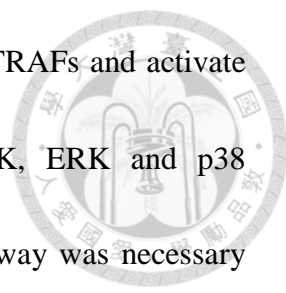
3.2 LMP1 is responsible for EphA4 suppression

Many EB viral products are able to regulate gene expression. To determine which EB viral gene was involved in EphA4 suppression, selected viral genes were expressed

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ectopically in the EBV-negative nasopharyngeal carcinoma cells TW01 through

transfection. Based on the results of Figure 5-8, we demonstrated that LMP1 was the

candidate protein that inhibits EphA4 expression. Other EBV gene products, including

Zta, EBNA2, EBER1, EBER2 and BARF0, had no effect on the EphA4 expression

level (Figure 6-8). This LMP1-mediated EphA4 downregulation was also observed in

a dose dependent manner at mRNA and protein levels in EBV-negative Burkitt's

lymphoma cells BJAB transduced with LMP1 lentivirus (Figure 9). Of note,

upregulation of EphA4 protein and mRNA expression were seen in LCLs using an

shLMP1 knockdown approach (Figure 10). Taken together, all the evidence indicated

that LMP1 was the viral protein responsible for EphA4 downregulation.

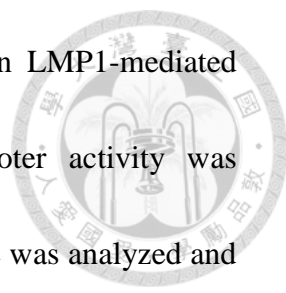**3.3 LMP1 suppresses EphA4 through ERK pathways**

Structurally, LMP1 resembles a CD40 receptor but its activity is

ligand-independent. LMP1 delivers signaling through its long C-terminal activation

regions (CTARs) (Soni *et al.*, 2007; Young & Rickinson, 2004). To determine which

CTAR domains were involved in EphA4 downregulation, lentiviruses containing

LMP1 full length (WT), CTAR1 deleted mutant (ΔCTAR1), CTAR2 deleted mutant

(ΔCTAR2), and both CTAR1 and CTAR2 deleted mutant (ΔCTAR1/2) were

transduced into BJAB cells. It seems that both CTAR1 and 2 of LMP1 are involved in

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this repression (Figure 11). Usually, these CTARs associate with TRAFs and activate

downstream PI3K/Akt, NF-κB and MAPK, including the JNK, ERK and p38

pathways (Dawson *et al.*, 2012). To dissect which signaling pathway was necessary

for LMP1-mediated EphA4 downregulation, LCLs were treated with MEK inhibitor

PD98059, JNK inhibitor SP600125, PI3K inhibitor LY294002 or NF-κB inhibitor

Bay11-7082. Expression of EphA4 was increased in LCLs treated with PD98059 but

not other inhibitors (Figure 12). Furthermore, LMP1-triggered EphA4 downregulation

through ERK pathway was confirmed by silencing both ERK1/2 by lentiviral

transduction of LCLs (Figure 13).

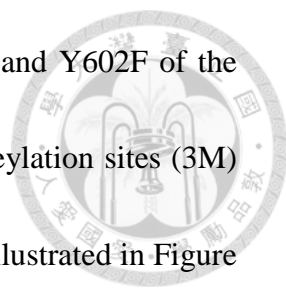**3.4 LMP1 represses EphA4 promoter activity through Sp1**

LMP1 downregulated the expression of EphA4 at the transcriptional and

translational levels (Figure 5-10) through the ERK pathway (Figure 12-13). ERK has

been reported to phosphorylate Sp1 at T453 and T739 to regulate the promoter

activity of targeted genes (Milanini-Mongiat *et al.*, 2002) and we speculated that

LMP1 may inhibit EphA4 promoter activity through Sp1. To test this hypothesis,

EphA4 protein was detected in Sp1-knockdown LCLs and its amount was augmented

(Figure 14). Meanwhile, EphA4 protein amounts were restored in

LMP1-overexpressing BJAB cells, when cells were simultaneously knocked down of

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Sp1 (Figure 15). These data suggested that Sp1 was involved in LMP1-mediated EphA4 downregulation. The effect of Sp1 on EphA4 promoter activity was investigated further. The *EPHA4* promoter (-1000 to +42) sequence was analyzed and four Sp1 binding sites were predicted (Figure 16). Clearly, EphA4 promoter activity was upregulated in Sp1-silenced LCLs (Figure 17) and in Sp1-knockdown, LMP1-expressing TW01 cells (Figure 18). Also, addition of the Sp1 inhibitor mithramycin, which interferes with Sp1 binding to the guanine-cytosine (G-C) rich sequence, to the LMP1 transfectants increased the EphA4 promoter activity (Figure 19). Furthermore, to determine whether LMP1 promotes Sp1 binding to the EphA4 promoter, a ChIP assay was performed in BJAB cells expressing LMP1. We showed that Sp1 bound to the EphA4 promoter at the region of -51 to -60 nt in BJAB cells, following LMP1 expression (Figure 20).

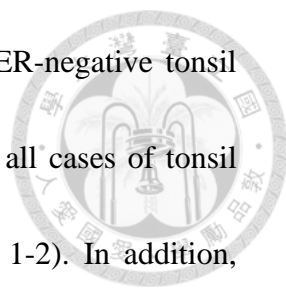## 3.5 The Eph JM domain, but not kinase domain, is involved in preventing LCL proliferation

Like other EphA family members, EphA4 contains an extracellular domain, transmembrane (TM) and juxtamembrane (JM) domains, and an intracellular kinase domain (Pasquale, 2010). To understand the biological function of EphA4 in B cells, EphA4 full length (WT) and mutants, including a kinase-dead mutant with V635M

(KD), mutations of the tyrosine auto-phosphorylation site Y596F and Y602F of the

JM region (2M), mutations of both kinase-dead and two-phosphoreylation sites (3M)

and delected juxtamembrane domain from a. a. 591-602 (ΔJM) as illustrated in Figure

21, were delivered to LCLs by lentivirus infection. Cell proliferation was inhibited

significantly in LCLs expressed EphA4 WT and KD but restored in LCLs expressed

EphA4 2M, 3M and ΔJM (Figure 22) indicated that JM region of EphA4 is important

for inhibiting LCL proliferation. To further verify the phenomenon, LCLs delivered

EphA4 WT, KD and 2M were reseeded for 0-5 days and cell proliferation was

inhibited significantly in the LCLs expressing EphA4 WT and KD but not in the

LCLs expressing EphA4 2M (Figure 23). The transduced LCLs expressed WT or

mutated EphA4 at physiologic level, compared to primary B lymphocytes (Figure 23,

right panel). Cell cycle analysis of LCLs with ectopic expression of EphA4 WT and

mutant forms showed no significant difference (Figure 24). These data showed that

EphA4 repressed LCL proliferation through its JM region, suggesting that the kinase

domain is not important for EphA4 repression of LCL proliferation.

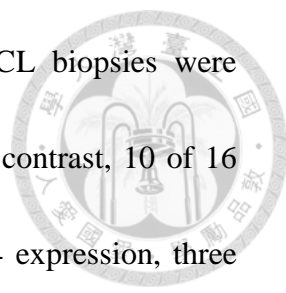## 3.6 EphA4 can be detected in tonsil biopsies but not in PTLD

EBV-positive PTLD exhibits similar biological features to LCLs. Thus, to

address whether EphA4 was downregulated in PTLD biopsies, we examined EphA4

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doi:10.6342/NTU201603335

expression levels in 14 EBER-positive PTLD biopsies and 5 EBER-negative tonsil

biopsies by IHC assay. The results of the IHC assay showed that all cases of tonsil

biopsies were positive for EphA4 staining (Figure 24 and Table 1-2). In addition,

EphA4 was predominantly expressed in the cytoplasm and cell membranes in tonsil

biopsies (Figure 25). On the other hand, 13 of 14 cases (92.9%) of PTLD were

negative for EphA4 expression (Figure 25 and Table 1-2). In addition, LMP1 was

responsible for downregulation of EphA4 in our studies (Figure 5, 9-11). Therefore,

the expression levels of LMP1 were measured in EBER-positive PTLD biopsies by

IHC assay and 12 of 14 cases (85.7%) of PTLD were positive for LMP1 staining

(Figure 25 and Table 1-2).

3.7 A reverse correlation between EphA4 and the survival rate of EBV-positive

DLBCL is revealed

According to 2016 WHO classification, EBV-positive DLBCL of the elderly,

which is equivalent to EBV-positive DLBCL, not otherwise specified (NOS), displays

a similar EBV viral protein expression as LCL (Dolcetti *et al.*, 2013; Swerdlow *et al.*,

2016). To confirm that EphA4 expression is correlated with EBV infection and LMP1

expression, 16 EBER-positive DLBCL and 11 EBER-negative DLBCL biopsies from

patients without HIV infection or any iatrogenic immunosuppression were examined.

The IHC assay indicated that all cases of EBV-negative DLBCL biopsies were positive for EphA4 staining (Figure 26, Table 1 and Table 3). In contrast, 10 of 16 cases (62.5%) of EBV-positive DLBCL were negative for EphA4 expression, three showed very weak, positive signals and three were stained positively (Figure 26, Table 1 and Table 3). That the presence of EBV was associated with reduced EphA4 expression was statistically significant (Table 4, p=0.0011, Fisher's exact test). On the other hand, 12 of 15 cases (80%) of EBV-positive DLBCL were positive for LMP1 staining (Figure 26, Table 1 and Table 3). LMP1 expression was also inversely correlated with EphA4 expression (Table 4, p=0.0375, Fisher's exact test).

Next, we asked whether there was correlation between EphA4 expression and patients' survival. So, we analyzed the survival data of patients with DLBCL from GEO datasets (accession number GSE4475). The profile of overall survival in patients was used to estimate whether patients with low EphA4 expression have worse prognosis. Based on the median expression value of EphA4, 123 patients were grouped into two clusters; 60 patients had high EphA4 and 63 patients had low EphA4. Patients with low EphA4 had worse overall survival (Figure 27, p=0.0414, log-rank test). Our results indicated that low EphA4 expression may potentially contribute to a poor prognosis for these DLBCL patients.

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Discussion

4.1 The roles of EphA2 in a ligand-dependent or ligand-independent manner

Physiologically, Eph receptors and their ligands are critical for many developmental processes. Pathologically, the Eph family is dysregulated in a variety of human diseases, especially cancers. During cancer formation, Eph can function in tumor suppression or tumor promotion, depending on the type of cancer, the interacting ligands and cross-talk with other RTKs. Among them, EphA2 is a good example. Limited expression of EphA2 is required for mammary gland development but its overexpression may enhance the malignancy of breast cancer (Kaenel *et al.*, 2012). In brain tumors, the presence of ephrin-A1 determines the direction of a reciprocal loop between EphA2 and Akt, which results in inhibition or promotion of the tumor grade (Miao *et al.*, 2009).

4.2 EphA4 involved in tumor progression

In addition to neuronal development, EphA4 has been found to be involved in tumor progression (Easty *et al.*, 1997; Kandouz, 2012; Narayan *et al.*, 2003; Oshima *et al.*, 2008; Pasquale, 2010; Saintigny *et al.*, 2012). For example, EphA4 is upregulated in colon cancer with liver metastasis (Oshima *et al.*, 2008). In invasive cervical carcinoma, EphA4 expression is decreased due to chromosomal deletions,

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with loss of heterozygosity (Narayan *et al.*, 2003). EphA4 mRNA is downregulated or

lost in metastatic melanoma (Easty *et al.*, 1997).

4.3 The transcriptional modification of EphA4

The regulatory mechanisms of EphA4 mRNA expression have been explored in

terms of transcription. In the cervical cancer cell line HeLa and U373 glioma cells,

EphA4 is reduced due to mRNA instability through regulation of its 3'untranslated

regions by HuR (Winter *et al.*, 2008). In hepatocellular carcinoma, microRNA-10a

targets EphA4 mRNA and overexpression of EphA4 inhibits cell migration and

invasion (Yan *et al.*, 2013). Moreover, a Pax3/FKHR oncogenic fusion protein has

been shown to bind directly to the promoter region of EphA4, thus increasing EphA4

transcription in SaOS-2 cells (Begum *et al.*, 2005). Furthermore, DNA methylation of

EphA4 is observed in acute lymphoblastic leukemia (Kuang *et al.*, 2010). We

analyzed the *EPHA4* promoter (-1000 to +42) and found there were some

transcription factors such as NF-•B, AP-1, AP-2, AP-4 and Sp1 were predicted and

might influence the EphA4 promoter activity. NF-κB was ruled out because EphA4

expression level was not altered after treating with NF-κB inhibitor Bay11-7082

(Figure 12). To identify which transcription factors mediated by LMP1 to inhibit

EphA4 promoter activity, a reporter assay was conducted by serial deletion of EphA4

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promoter. AP-1, AP-2 and AP-4 were also excluded since EphA4 -196 to +42 promoter activity was still repressed by LMP1 (Figure 28). Similar with the transcriptional regulation mentioned above, our results showed that EBV LMP1 inhibits EphA4 promoter activity through Sp1 (Figure 14-20).
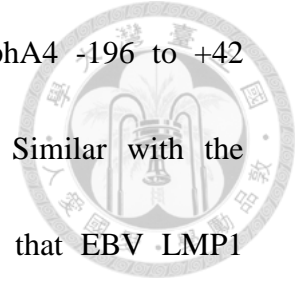
4.4 The roles of Sp1 in EphA4 promoter regulation

Sp1, which is expressed ubiquitously, can serve as a transactivator or repressor to regulate the promoter activities of target genes via phosphorylation (Chu, 2012). For example, FGF-2 stimulation enhances Sp1 binding to repress the promoter of PDGFR-• in smooth muscle cells through ERK mediated phosphorylation of Sp1 at T453 and T739 (Bonello & Khachigian, 2004). HER2 also has been reported to suppress the RECK promoter by ERK-mediated Sp1phosphorylation (Hsu *et al.*, 2006). In this study, LMP1 activated the ERK pathway to inhibit the EphA4 promoter through Sp1 binding (Figure 14-20). In addition, knockdown of ERK1/2 reduced the Sp1 phosphorylaton at T739 in LCLs (Figure 29) also confirmed that LMP1 utilized ERK-mediated Sp1 phosphorylation to repress the EphA4 expression. Moreover, our ChIP data indicated that LMP1 promoted Sp1 binding to the *EPHA4* promoter at the region of -51 to -60 nt (Figure 20) which was overlapped with the binding sites of Pax3/FKHR (Begum *et al.*, 2005). Thus, we provide this new insight into how a viral

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oncoprotein can regulate EphA4 promoter activity.

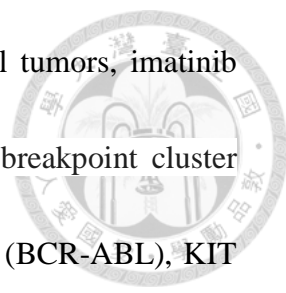## 4.5 The function of EphA4 in different cancer types

The function of EphA4 associated with cancer progression is controversial. As a tumor suppressor, EphA4 downregulates ERK phosphorylation to inhibit migration and invasion in non-small cell lung cancer cells (Saintigny *et al.*, 2012). However, EphA4 can act as tumor promoter; for example, EphA4 interacts directly with FGFR1 through its cytoplasmic domains, phosphorylates the docking protein, FRS2•, and enhances cell migration and proliferation (Fukai *et al.*, 2008; Yokote *et al.*, 2005). In our case, EphA4 prevents LCL from proliferation (Figure 22-23) suggesting that it probably plays an inhibitory role in EBV-positive B lymphoproliferative disorders. This is confirmed by the IHC results of PTLD and DLBCL (Figure 25-26). In this study, EphA4 was undetectable in the cells of EBER-positive PTLD biopsies but expressed in tonsil biopsies (Figure 25 and Table 1-2). EBV infection also caused downregulation of EphA4 in LCLs derived from tonsil compared to uninfected ones (Figure 30). In addition, the incidence of EBV in PTLD cases is over 90% (Dolcetti *et al.*, 2013), these data imply that EphA4 is involved in the pathogenesis of EBV-positive PTLD.

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4.6 The ligands of EphA4 in B lymphoctes

In addition to PTLD, our results indicated that cells in LMP1-positive DLBCL biopsies exhibited lower expression levels of EphA4 than those in LMP1-negative biopsies (Figure 26 and Table 1-3), verifying the inverse correlation of EphA4 and LMP1 expression in DLBCL patients. This LMP1-mediated EphA4 downregulation was also observed in a dose dependent manner in EBV-negative Hodgkin's lymphoma cells KMH2 (Figure 31). However, reduced EphA4 was not revealed in another EBV-negative Hodgkin's lymphoma cell line, Ramos (Figure 32). It has been reported that patients with EBV-positive DLBCL have worse overall survival and progression-free survival than their EBV-negative counterparts (Lu *et al.*, 2015; Swerdlow *et al.*, 2016), yet the underlying mechanism has not been fully understood. Here we provided observation to show that low EphA4 correlated with poor survival outcome for DLBCL (Figure 27). Also, some EphA4 ligands, such as ephrin-A1, A2, A3, A4 and B1 were downregulated post-EBV infection (Figure 33-37). The results of overexpression of EphA4 in LCL indicated that EphA4 prevention of B cell proliferation may be ligand-independent.

4.7 Therapeutic agents approved for targeting haematological tumors

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In regard to the tyrosine kinase inhibitors for haematological tumors, imatinib and nilotinib which target the oncogenic fusion protein, namely breakpoint cluster region protein- Abelson murine leukemia viral oncogene homolog (BCR-ABL), KIT and platelet-derived growth factor receptor (PDGFR) are approved to treat with chronic myelogeous leukaemia (CML) (Grimminger *et al.*, 2010). Similarly, bosutinib targeting not only SRC and ABL but AXL has been approved to treat with Philadelphia chromosome-positive CML (Graham *et al.*, 2014). Recently, ibrutinib targeting Bruton's tyrosine kinase, which transmits signals from B-cell antigen receptor, is approved for treatment of mantle cell lymphoma and B-cell chronic lymphocytic leukemia (Kim & Dhillon, 2015). In addition to small molecule drugs targeting tyrosine kinases, anti-CD20 mAb, rituximab is used to treat with PTLD (Oertel *et al.*, 2005; Straathof *et al.*, 2002). It is documented that patients with DLBCL receive R-CHOP therapy consist of rituximab, cyclophosphamide, doxorubicin hydrochloride, oncovin and prednisolone have better prognosis compared to CHOP only (Feugier *et al.*, 2005).
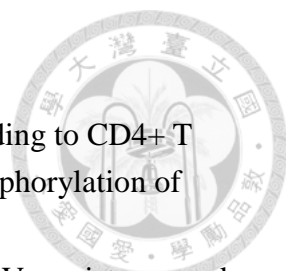
4.8 Conclusion

Taken together, LMP1-mediated EphA4 repression via ERK-Sp1 pathway accelerates B cell proliferation post-EBV infection (Figure 38). Our clinical results
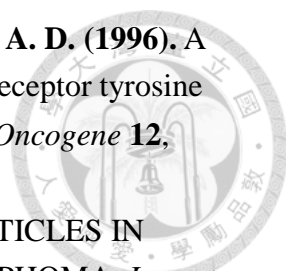
suggest that downregulation of EphA4 in patients with PTLD and DLBCL may

provide new insights into pathogenesis and a poor prognostic marker for

EBV-associated B cell malignancies. EphA4 may be considered as a potential therapy

target for PTLD and DLBCL.

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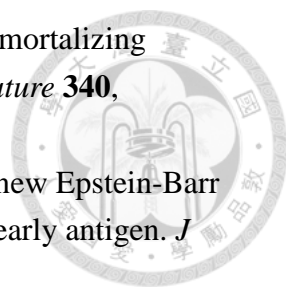
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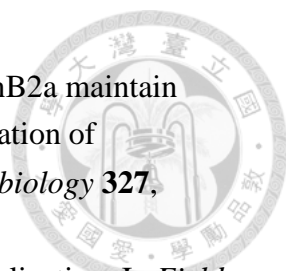
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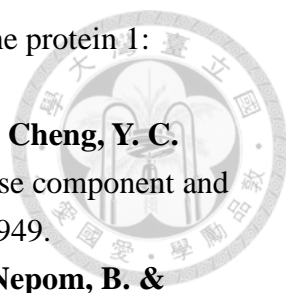
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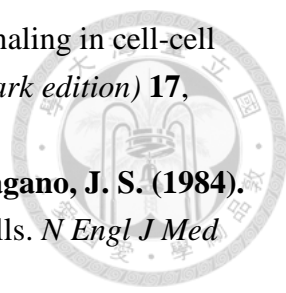
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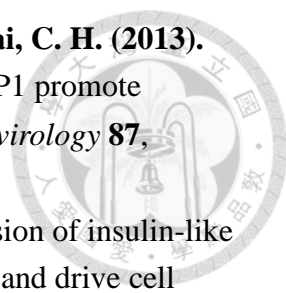
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Tables

Table 1

EBV products and EphA4 in tissue biopsies

	Case	EBER		LMP1[*]		EphA4	
		Negative	Positive	Negative	Positive	Negative	Positive
Tonsil	5	5/5	0/5	5/5	0/5	0/5	5/5
PTLD	14	0/14	14/14	2/14	12/14	13/14	1/14
DLBCL	27	11/27	16/27	14/26[*]	12/26[*]	10/27	17/27

[*]One case of LMP1 status was not determined in EBER-positive DLBCL.

Table 2

EBER, LMP1 and EphA4 expression in tonsil biopsies

Tonsil	EBER	LMP1	EphA4
Case 1	-	-	+
Case 2	-	-	+
Case 3	-	-	+
Case 4	-	-	+
Case 5	-	-	+

EBER, LMP1 and EphA4 expression in PTLD biopsies

PTLD	EBER	LMP1	EphA4
Case 1	+	+	-
Case 2	+	+	-
Case 3	+	+	-
Case 4	+	+	-
Case 5	+	+	-
Case 6	+	-	-
Case 7	+	+	-
Case 8	+	-	+
Case 9	+	+	-
Case 10	+	+/-[*]	-
Case 11	+	+	-
Case 12	+	+	-
Case 13	+	+	-
Case 14	+	+	-

[*] +/- denoted as weak expression.

Table 3

EBER, LMP1 and EphA4 expression in EBV-negative DLBCL biopsies

DLBCL	EBER	LMP1	EphA4
Case 1	-	-	+
Case 2	-	-	+
Case 3	-	-	+
Case 4	-	-	+
Case 5	-	-	+
Case 6	-	-	+
Case 7	-	-	+/-[*]
Case 8	-	-	+
Case 9	-	-	+
Case 10	-	-	+
Case 11	-	-	+

[*] +/- denoted as weak expression.

EBER, LMP1 and EphA4 expression in EBV-positive DLBCL biopsies

DLBCL	EBER	LMP1	EphA4
Case 1	+	+	-
Case 2	+	+	-
Case 3	+	+	-
Case 4	+	+	-
Case 5	+	+	+
Case 6	+	-	-
Case 7	+	+	+
Case 8	+	+	-
Case 9	+	-	+/-[*]
Case 10	+	+/-[*]	+/-[*]
Case 11	+	+	+
Case 12	+	-	-
Case 13	+	+	+/-[*]
Case 14	+	ND	-
Case 15	+	+	-
Case 16	+	+	-

[*] +/- denoted as weak expression. ND: not determined.

Table 4

Relationship between EphA4 and EBV in DLBCL

	EphA4		
	Positive	**Negative**	$p^{\dagger}$
EBER			
Positive	6/16	10/16	0.0011[‡]
Negative	11/11	0/11	
LMP1			
Positive	5/12	7/12	0.0375[‡]
Negative	12/14	2/14	

[†]*p*-value as determined by Fisher's exact test. [‡]Statistically significant.

A

B
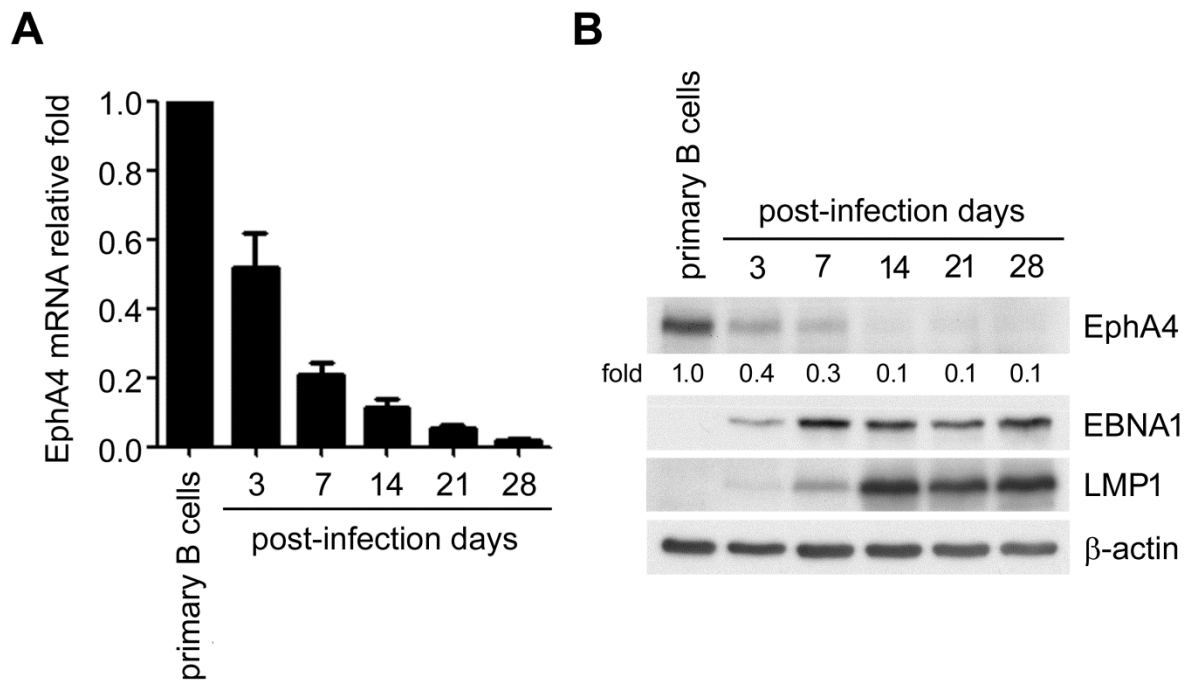
Figure 1. EphA4 expression is decreased post-EBV infection.

Peripheral CD19-positive B cells were seeded at 1×10^6 cells/mL and infected with EBV strain B95.8. Total RNAs and protein were harvested from primary B cells at the days indicated post-EBV infection. (A) Expression levels of EphA4 mRNA were measured by RT-Q-PCR. EphA4 mRNA relative folds were normalized to internal control MAGOH and standardized with uninfected primary B cells. (B) EphA4, EBNA1, LMP1 and β-actin proteins were measured by Western blotting. EphA4 protein relative folds were normalized to internal control β-actin and compared with uninfected primary B cells.

63
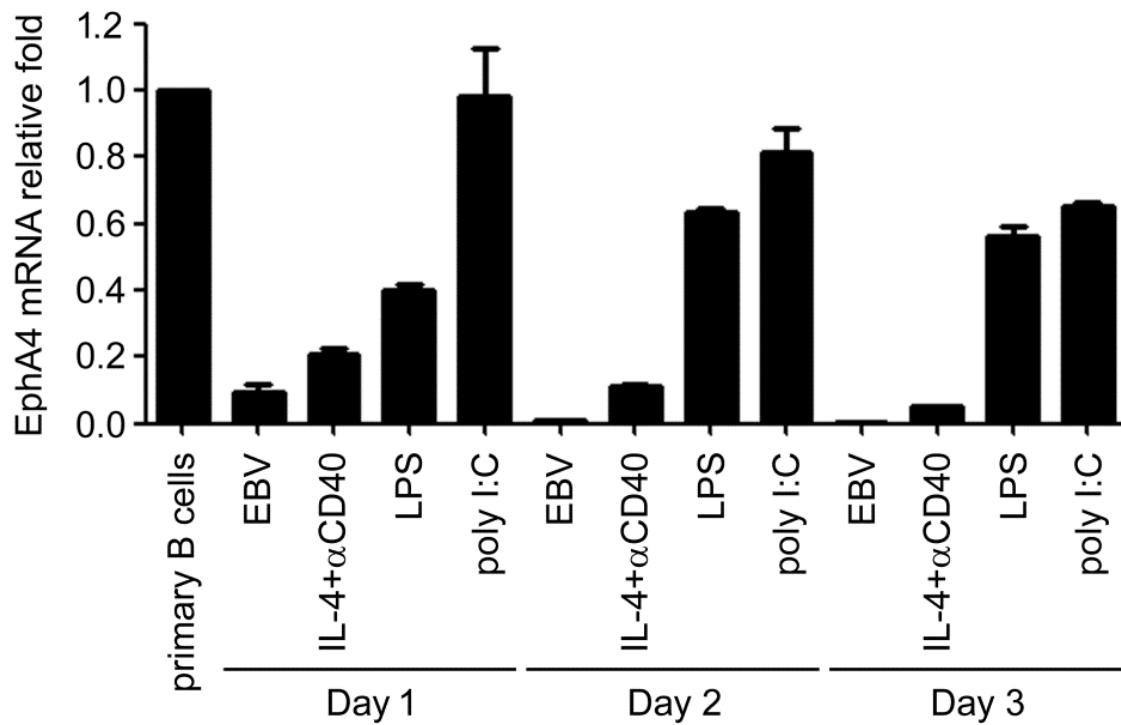
Figure 2. EphA4 is downregulated by EBV infection and by anti-CD40 antibody plus IL-4 treatment.

Total RNAs were extracted from primary B cells, EBV infection or B-cell stimulations including anti-CD40 antibody plus IL-4, LPS or poly I:C for 1 to 3 days. EphA4 transcripts were measured by RT-Q-PCR. EphA4 mRNA relative folds were normalized to internal control GAPDH and standardized with uninfected primary B cells.

64
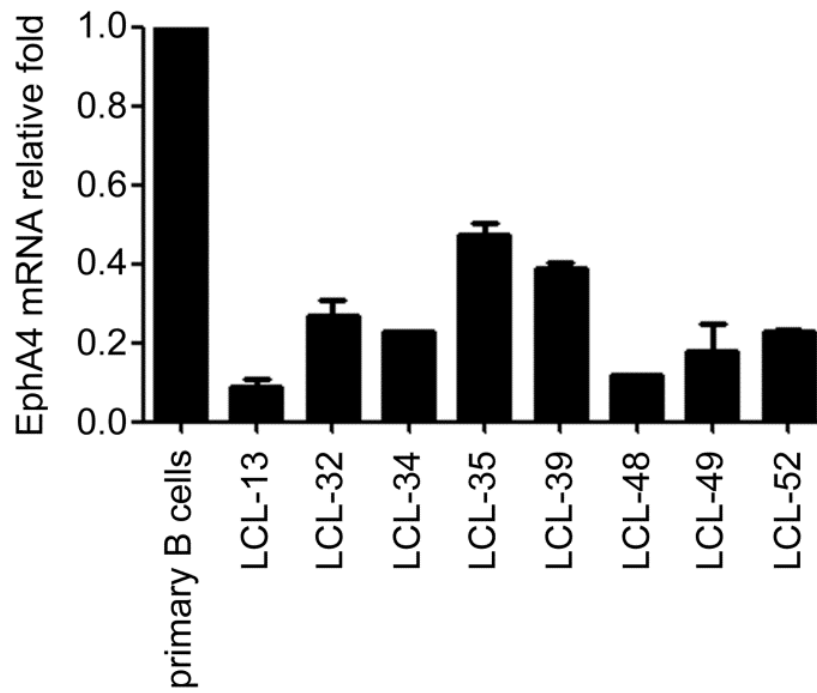
Figure 3. EphA4 expression is reduced in LCLs.

Total RNAs were extracted from primary B cells and eight LCL lines. Expression levels of EphA4 mRNA were measured by RT-Q-PCR. EphA4 mRNA relative folds were normalized to internal control MAGOH and standardized with uninfected primary B cells.

65
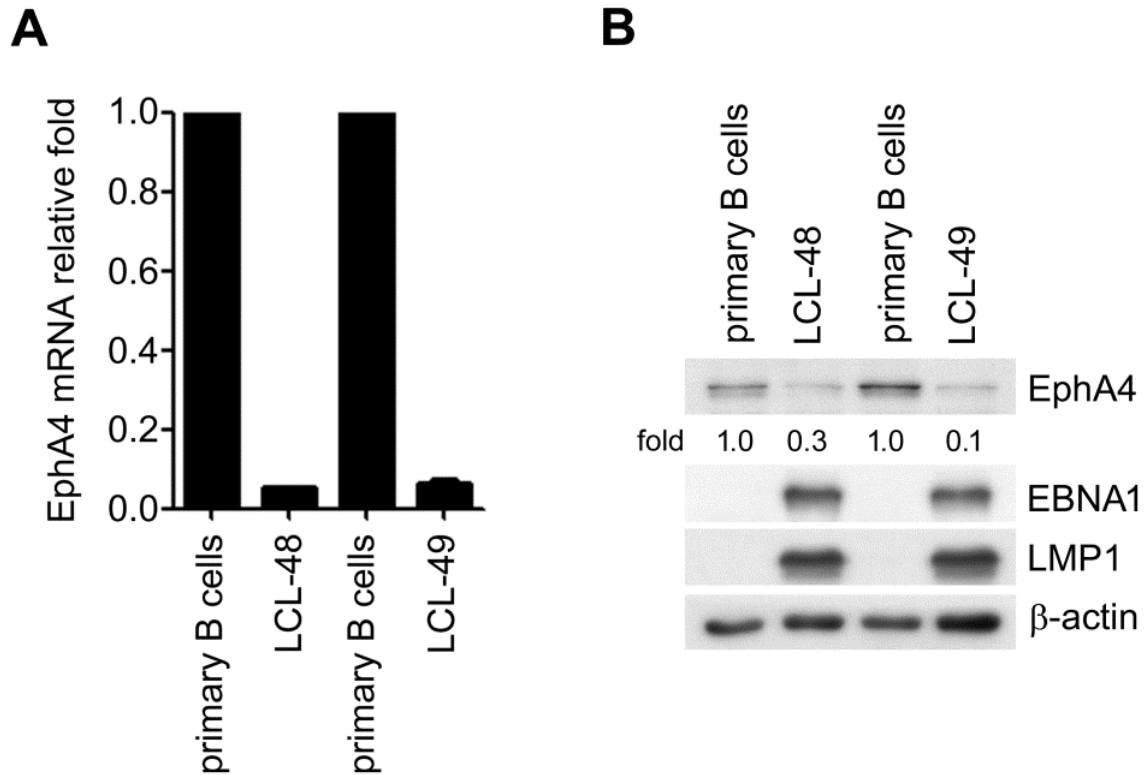
Figure 4. EphA4 expression is repressed in two-pared primary B cells after EBV infection.

Total RNAs and protein were harvested from paired uninfected B cells and LCLs generated from the peripheral blood mononuclear cells of two healthy donors. (A) Expression levels of EphA4 mRNA were measured by RT-Q-PCR. EphA4 mRNA relative folds were normalized to internal control MAGOH and standardized with uninfected primary B cells. (B) EphA4, EBNA1, LMP1 and β-actin proteins were measured by Western blotting. EphA4 protein relative folds were normalized to •-actin and compared with uninfected primary B cells.

66
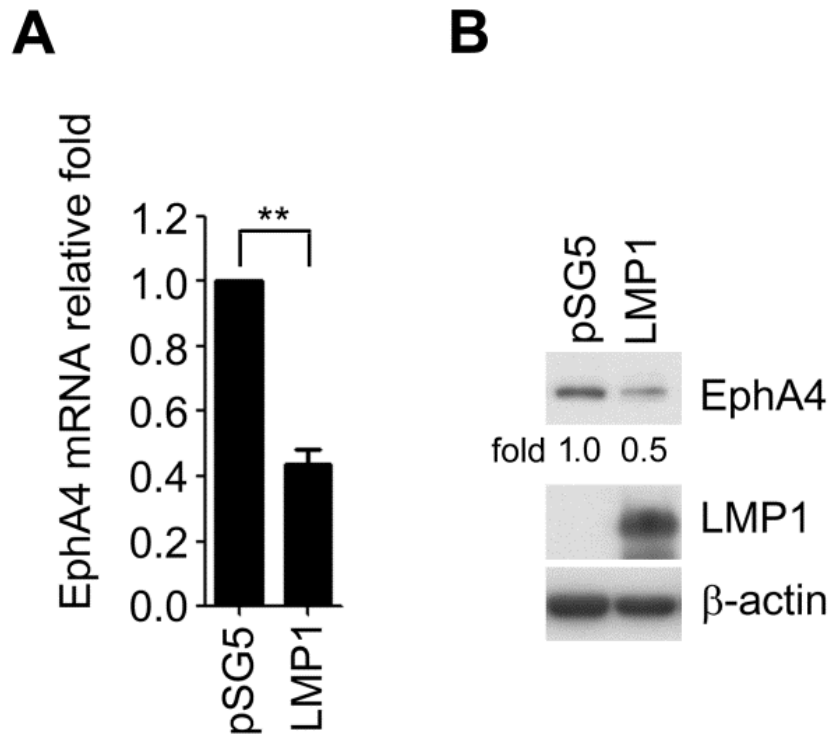
Figure 5. EphA4 is downregulated by LMP1 in nasopharyngeal carcinoma cell line, TW01.

EBV-negative TW01 cells were transfected with plasmids expressing the EBV viral gene LMP1. Total RNAs and protein lysates were obtained at day 3 post transfection. (A) EphA4 transcripts were detected by RT-Q-PCR. EphA4 mRNA relative folds were normalized to internal control GAPDH and then standardized with vector control. (B) Total proteins were harvested from the vector control and the LMP1-transfectant. EphA4 protein relative folds were normalized to internal control β-actin and standardized with vector control. Expression levels of LMP1 and β-actin protein were estimated by Western blotting. (**, $p < 0.01$, Student's t test).
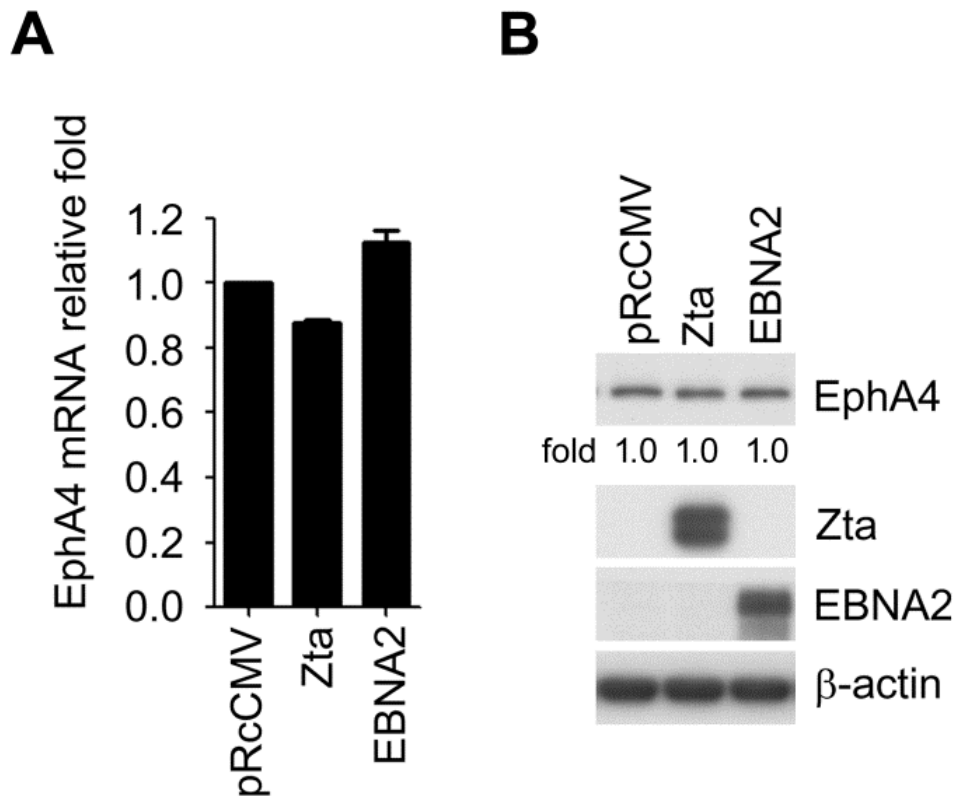
Figure 6. EphA4 expression is not altered in TW01 cells expressing Zta or EBNA2.

TW01 cells were transfected with plasmids harboring the EBV viral genes Zta or EBNA2. Total RNAs and protein lysates were obtained from each transfectant at day 3 post transfection. (A) EphA4 transcripts were detected by RT-Q-PCR. EphA4 mRNA relative folds were normalized to internal control GAPDH and then standardized with vector control. (B) Total proteins were harvested from the vector control and each transfectant. EphA4 protein relative folds were normalized to internal control β-actin and standardized with vector control. Expression levels of Zta, EBNA2 and β-actin protein were estimated by Western blotting.

68
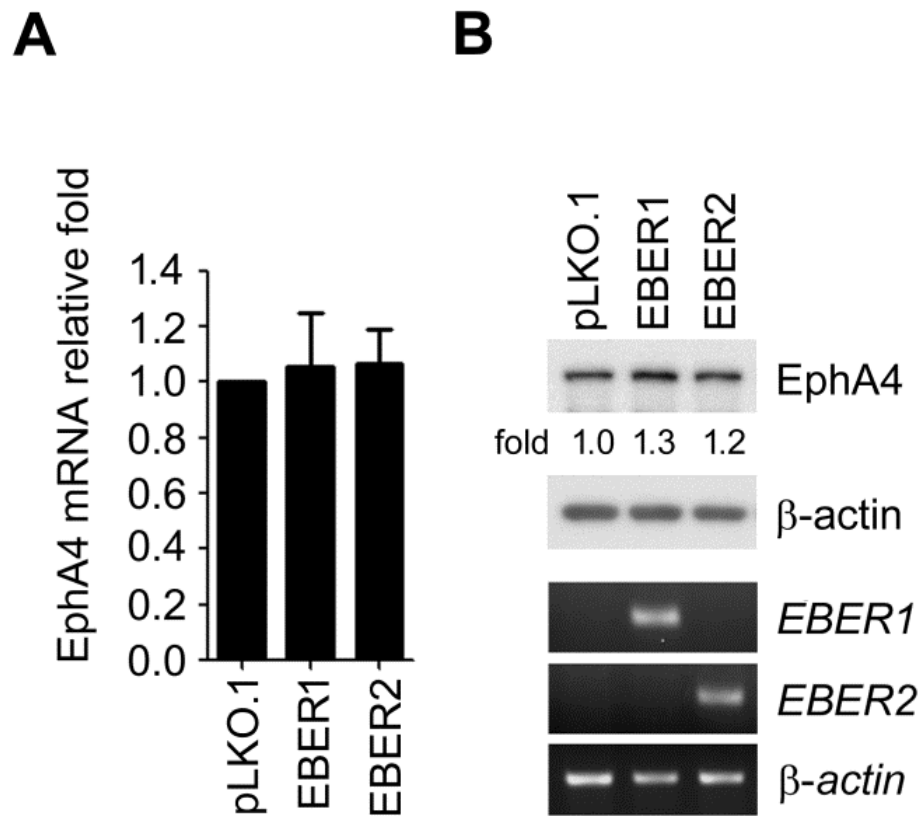
Figure 7. EphA4 expression levels are not changed by EBERs in TW01 cells.
TW01 cells were transfected with EBER1 and EBER2 plasmids respectively. Total RNAs and protein lysates were obtained from each transfectant at day 3 post transfection. (A) EphA4 transcripts were detected by RT-Q-PCR. EphA4 mRNA relative folds were normalized to internal control GAPDH and then standardized with vector control. (upper panels of B) Total proteins were harvested from the vector control and each transfectant. EphA4 protein relative folds were normalized to internal control β-actin and standardized with vector control. (lower panels of B) The *EBER1*, *EBER2* and *β-actin* transcripts were analyzed by RT-PCR.
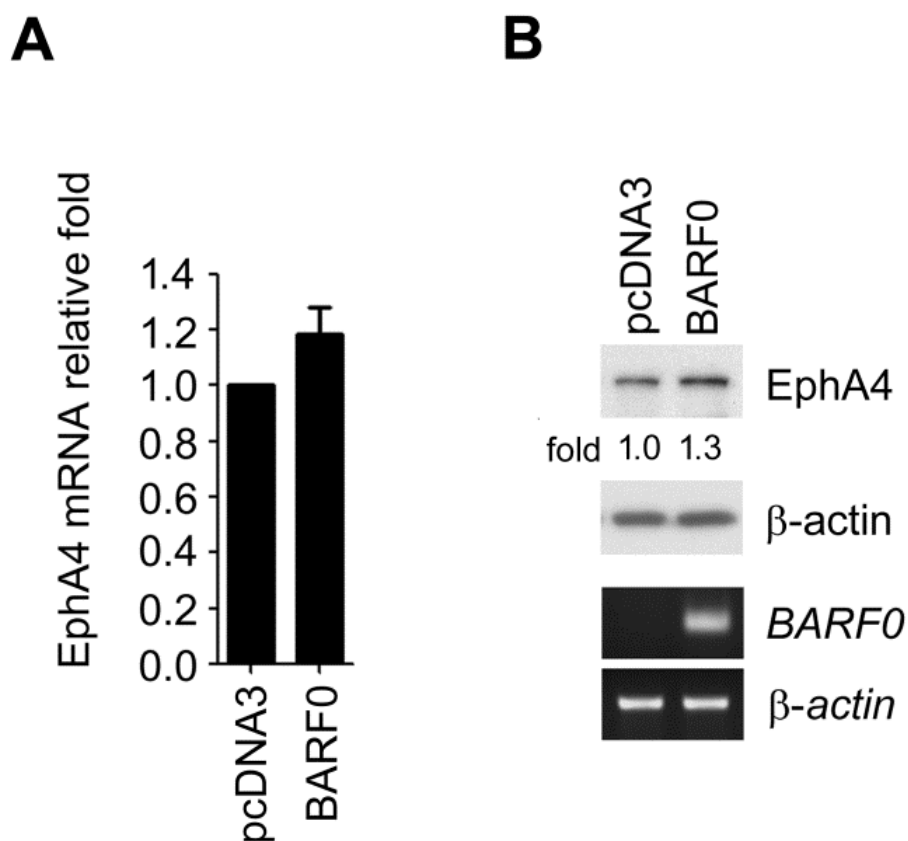
Figure 8. EphA4 expression is unchanged in TW01 cells expressing BARF0.
TW01 cells were transfected with plasmid expressing BARF0. Total RNAs and protein lysates were obtained from each transfectant at day 3 post transfection. (A) EphA4 transcripts were detected by RT-Q-PCR. EphA4 mRNA relative folds were normalized to internal control GAPDH and then standardized with vector control. (upper panels of B) Total proteins were harvested from the vector control and the BARF0-transfectant. EphA4 protein relative folds were normalized to internal control β-actin and standardized with vector control. (lower panels of B) The *BARF0* and *β-actin* transcripts were analyzed by RT-PCR.
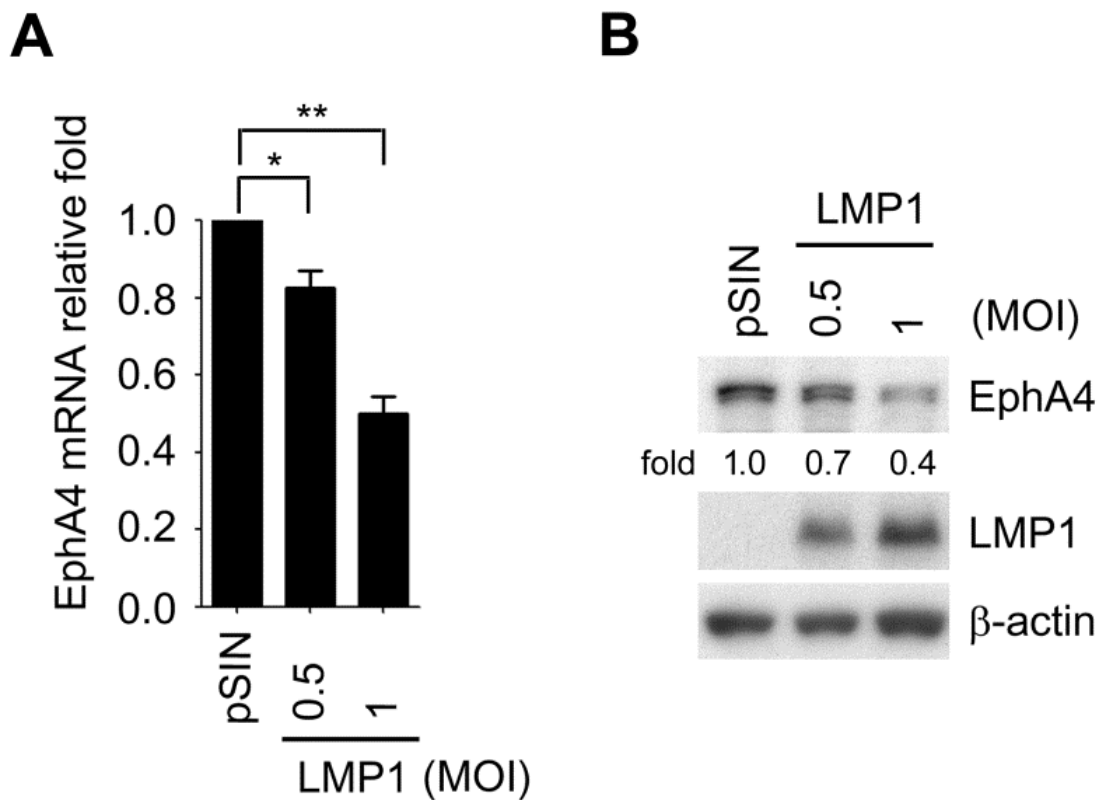
Figure 9. EphA4 is suppressed by LMP1 in a dose dependent manner in Burkitt's lymphoma cell line, BJAB.

EBV-negative BJAB cells were infected with LMP1 expressing lentivirus at MOI 0.5 or 1. (A) The expression levels of EphA4 mRNA were determined by RT-Q-PCR. EphA4 mRNA relative folds were normalized to internal control GAPDH and then standardized with the pSIN vector control. (B) EphA4, LMP1 and β-actin were detected by Western blotting. EphA4 protein relative folds were normalized to internal control β-actin and then standardized with pSIN vector control. (*, $p < 0.05$; **, $p < 0.01$, Student's t test).

71
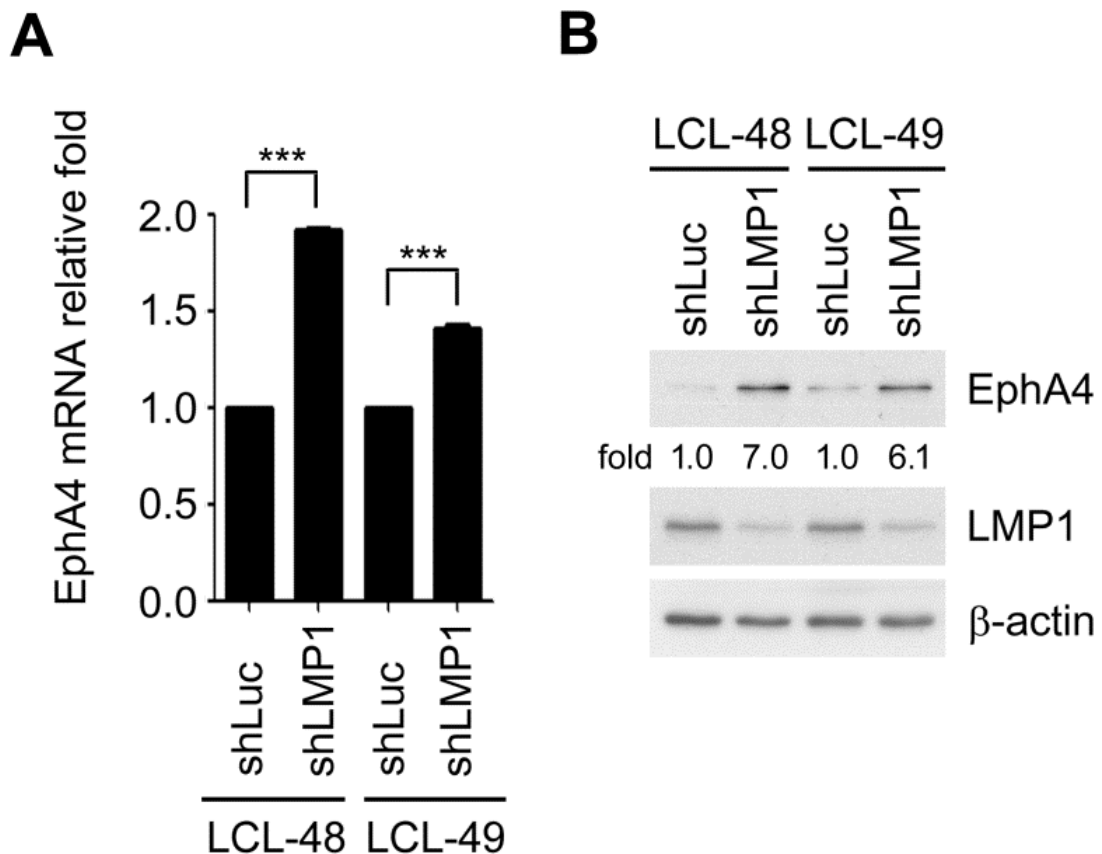
Figure 10. Knockdown of LMP1 reverses EphA4 expression in LCLs.

Knockdown of LMP1 in LCLs was performed by lentiviral transduction at the MOI of 1 for 5 days and infected cells were selected with 2 μg/mL puromycin for 2 days. (A) Expression levels of EphA4 mRNA were measured by RT-Q-PCR. EphA4 mRNA relative folds were normalized to internal control GAPDH and then standardized with vector control shLuc. (B) EphA4, LMP1 and β-actin were determined by Western blotting. EphA4 protein relative folds were normalized to β-actin and then standardized with vector control shLuc. (***, $p < 0.001$, Student's t test).
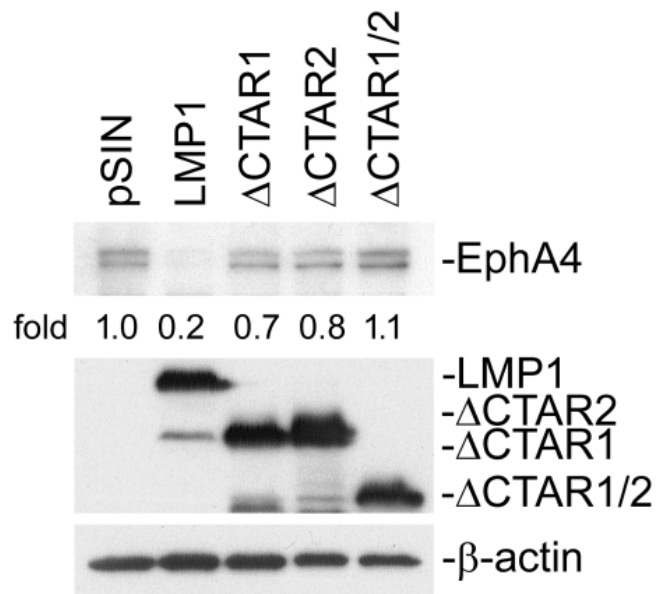
Figure 11. Both CTAR1 and CTAR2 contribute to EphA4 downregulation in BJAB cells.

BJAB cells were infected with pSIN, LMP1, ΔCTAR1, ΔCTAR2 or ΔCTAR1/2 expressing lentiviruses for 5 days. EphA4, LMP1 and β-actin were detected by Western blotting. EphA4 protein relative folds were normalized to β-actin and standardized with pSIN vector control.
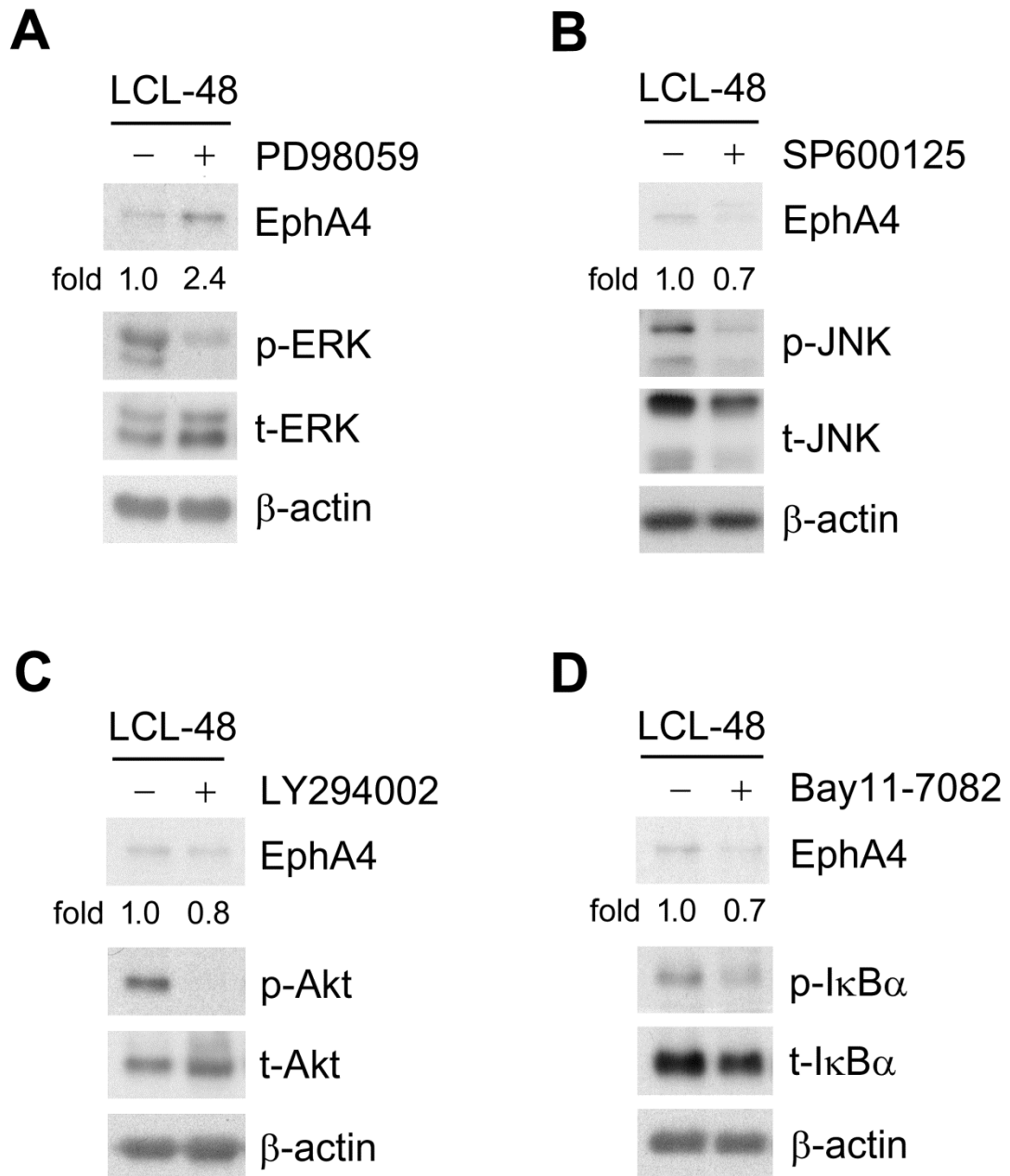
Figure 12. Inhibition of ERK pathway increases EphA4 expression in LCLs.
LCLs were treated with (A) 20 μM of MEK inhibitor PD98059, (B) 20 μM of JNK inhibitor SP600125, (C) 20 μM of PI3K inhibitor LY294002 or (D) 2.5 μM of NF-κB inhibitor Bay11-7082 for 48 hours. EphA4, phosphorylated (p-) and total (t-) proteins of ERK, JNK, Akt and IkBα were determined by Western blotting. β-actin served as an internal control. EphA4 protein relative folds were normalized to β-actin and standardized with DMSO solvent control.

74
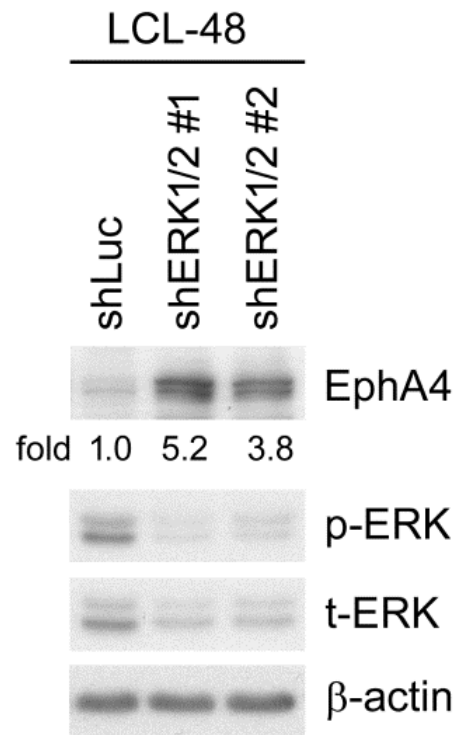
Figure 13. Knockdown of ERK1/2 reverses EphA4 expression in LCLs.
Knockdown of ERK1/2 in LCLs achieved using a lentivirus expressing shERK1 plus shERK2 for 3 days. Infected cells were selected with 2 μg/mL puromycin for 2 days. EphA4, phospho-ERK, total ERK proteins were then determined by Western blotting. EphA4 protein relative folds were normalized to β-actin and standardized with vector control shLuc.

LCL-48   LCL-49

shLuc shSp1 shLuc shSp1

EphA4

fold   1.0   7.4   1.0   3.0

Sp1
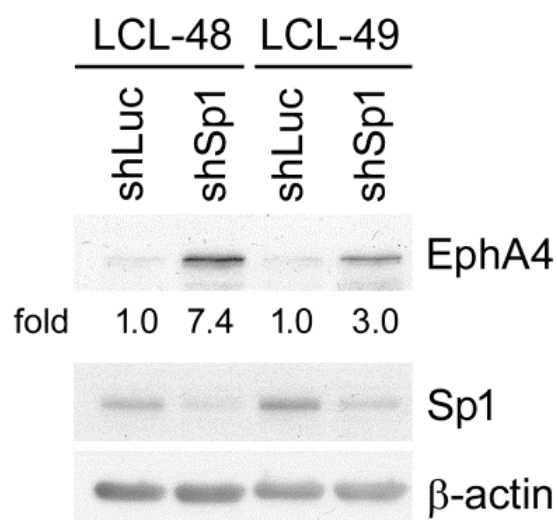
β-actin

Figure 14. Knockdown of Sp1 augments EphA4 expression in LCLs.

LCLs were infected with an shSp1 expressing lentivirus for 5 days and the infected cells were selected with 2 μg/mL puromycin for 2 days. EphA4, Sp1 and β-actin expression levels were detected by Western blotting. EphA4 protein relative folds were normalized to β-actin and standardized with vector control shLuc.

76
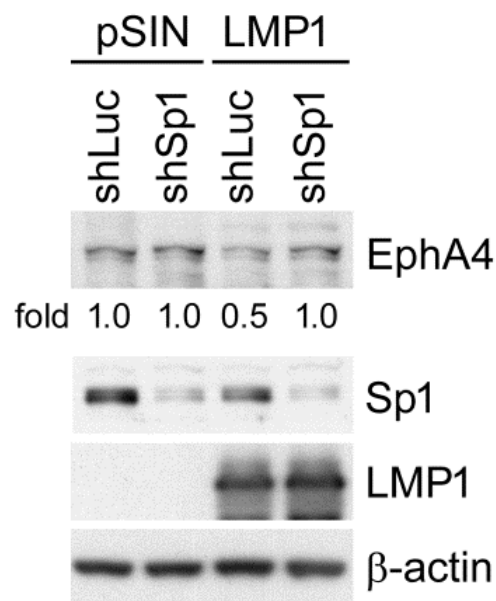
Figure 15. Knockdown of Sp1 restores EphA4 expression in BJAB cells expressing LMP1.

BJAB cells were infected simultaneously with LMP1 and shSp1 expressing lentiviruses for 3 days and infected cells were selected with 2 μg/mL puromycin for 2 days. EphA4, Sp1, LMP1 and β-actin were determined by Western blotting. EphA4 protein relative folds were normalized to β-actin and standardized with pSIN plus shLuc controls.

77
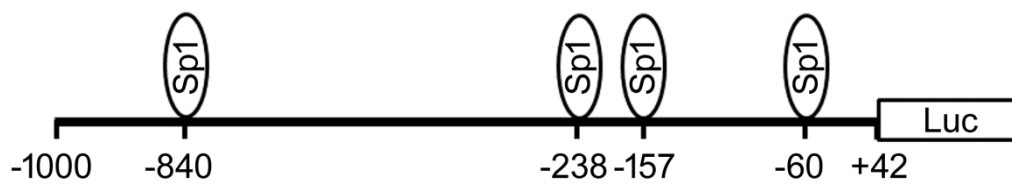
Figure 16. Schematic presentation of *EPHA4* promoter.

The *EPHA4* promoter which drove the luciferase gene in the reporter plasmid was illustrated. The *EPHA4* promoter contained 4 Sp1 binding sites indicated in the promoter sequence, from -1000 to +42 nt.
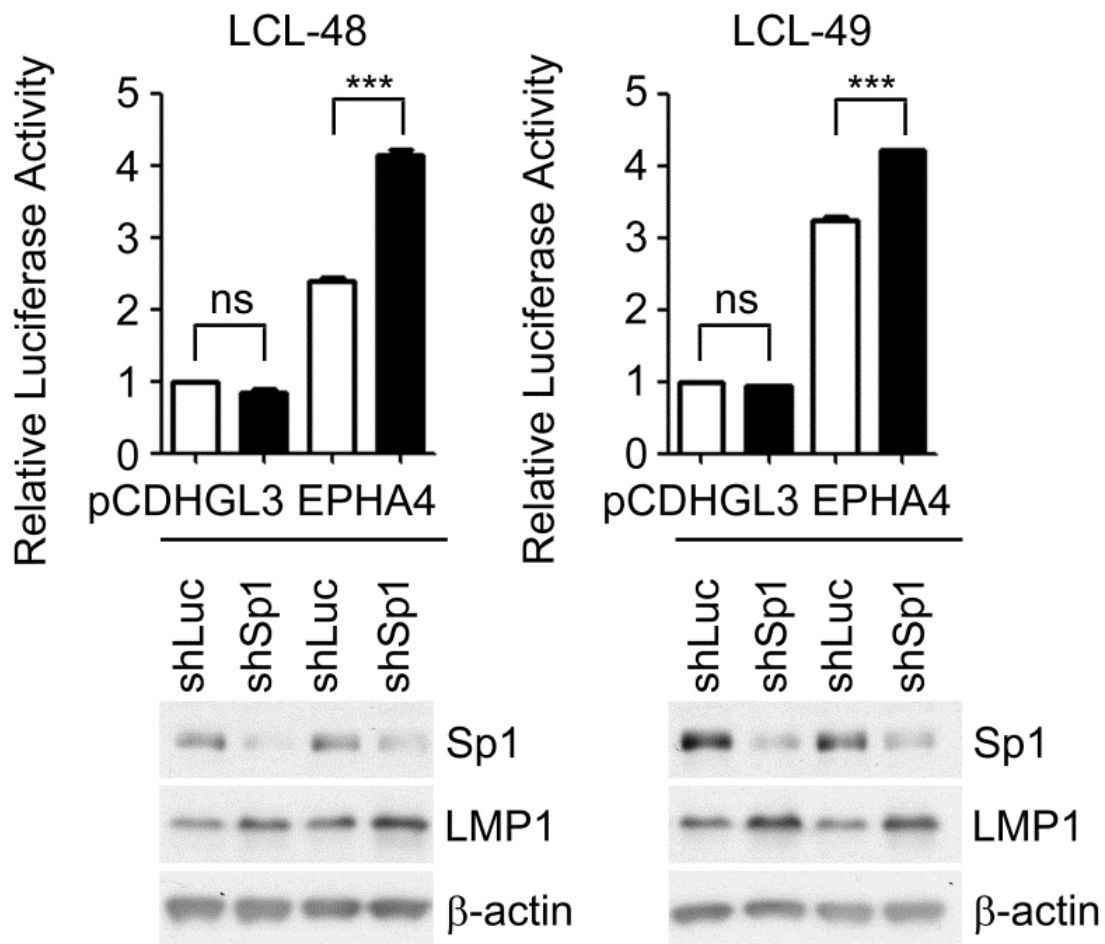
Figure 17. Knockdown of Sp1 reverses EphA4 promoter activity in LCLs.
LCLs were co-infected with lentiviruses of shSp1 and of GFP-tagged *EPHA4* promoter (-1000~+42) for 3 days and then the infected cells were selected with 2 μg/mL puromycin for 2 days. EphA4 relative luciferase activity was first normalized to GFP, followed by standardization with the vector pCDHGL3 (***, $p < 0.001$, Student's *t* test). Sp1, LMP1 and internal control β-actin were analyzed by Western blotting (lower panel).

79
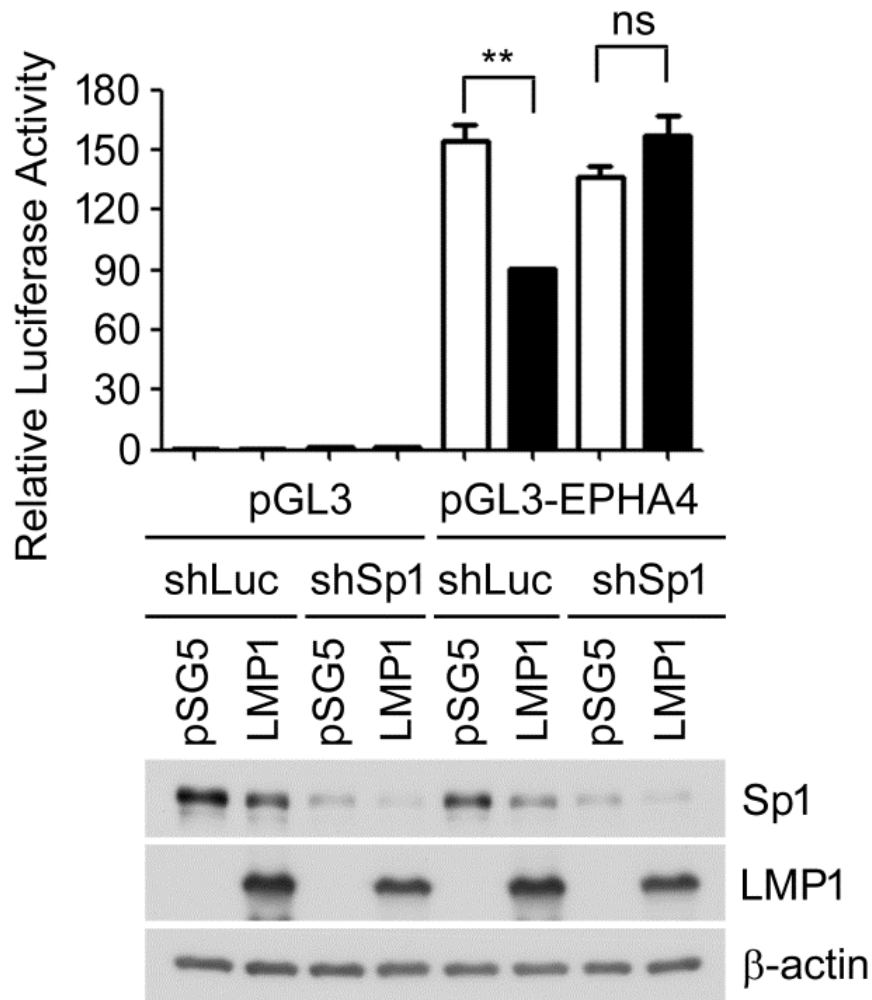
Figure 18. Knockdown of Sp1 restores EphA4 promoter activity in TW01 cells expressing LMP1.

TW01 cells were infected with an shSp1-expressing lentivirus for 3 days and the infected cells were selected with 2 μg/mL puromycin for 2 days. The Sp1-knockdown TW01 cells were co-transfected with LMP1 plasmid or its vector control pSG5, combined with reporter plasmids *EPHA4* promoter (-1000~+42) or vector control pGL3 and internal control pEGFPC1 plasmids for 2 days. EphA4 relative luciferase activity was first normalized to GFP, followed by standardization with the control vector pGL3 (**, $p < 0.01$, Student's *t* test). Expression of Sp1, LMP1 and β-actin proteins was analyzed by Western blotting (lower panel).

80
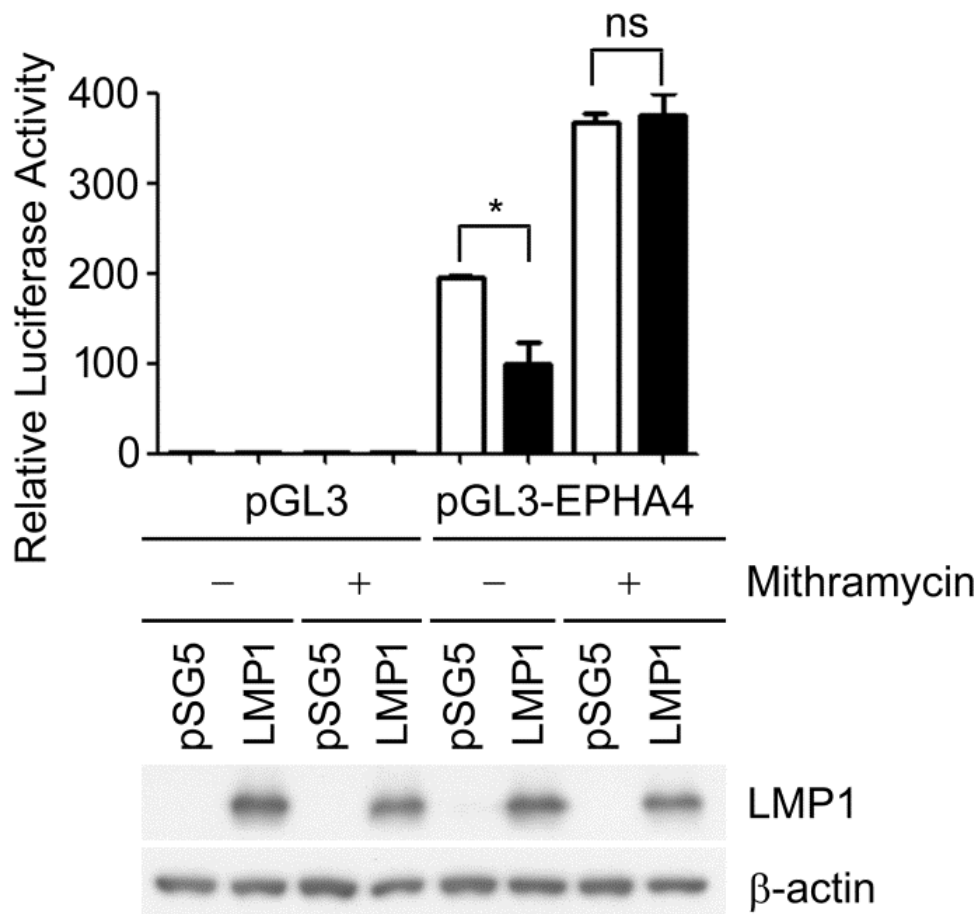
Figure 19. Inhibition of Sp1 restores EphA4 promoter activity in TW01 cells expressing LMP1.

TW01 cells were co-transfected with LMP1 or pSG5 plasmids, reporter plasmids of pGL3 vector control or *EPHA4* promoter (-1000~+42) and internal control pEGFPC1 plasmids. 24 hours post-transfection, 500 nM mithramycin was added to the cells for another 24 hours. EphA4 relative luciferase activity was first normalized to GFP, followed by standardization with the control vector pGL3 (*, $p < 0.05$, Student's *t* test). Expression levels of LMP1 and β-actin were measured by Western blotting (lower panel).
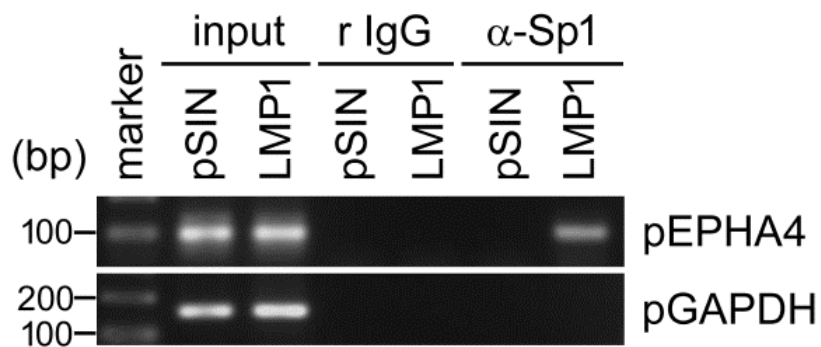
Figure 20. LMP1 promotes Sp1 binding to EphA4 promoter in BJAB cells.
BJAB cells were transduced with pSIN or LMP1 expressing lentiviruses for 5 days and a ChIP assay was performed. DNA-protein complexes were immunoprecipitated using anti-Sp1 Ab or isotype control rabbit IgG. *EPHA4* promoter and control *GAPDH* promoter DNA were detected in the immunoprecipitates by PCR. Total DNA was harvested from BJAB cells and used as the input control.

82
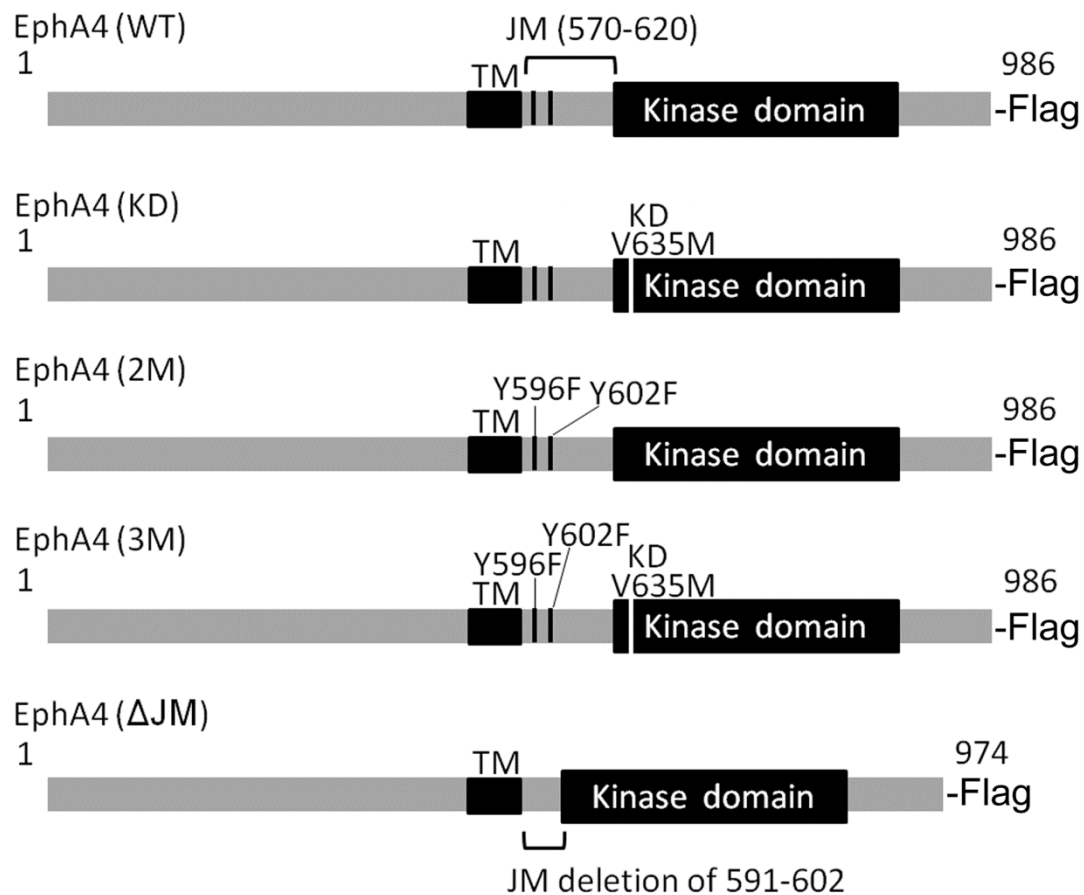
Figure 21. Schematic presentation of EphA4 WT and mutants.

Flag-tagged EphA4 WT, KD (kinase dead with V635M), 2M (JM region mutant with two tyrosine auto-phosphorylation sites Y596F and Y602F), 3M (kinase dead with V635M plus Y596F and Y602F), ΔJM (juxtamembrane domain deletion of a. a. 591-602) expression plasmids for lentivirus packaging were illustrated.

83
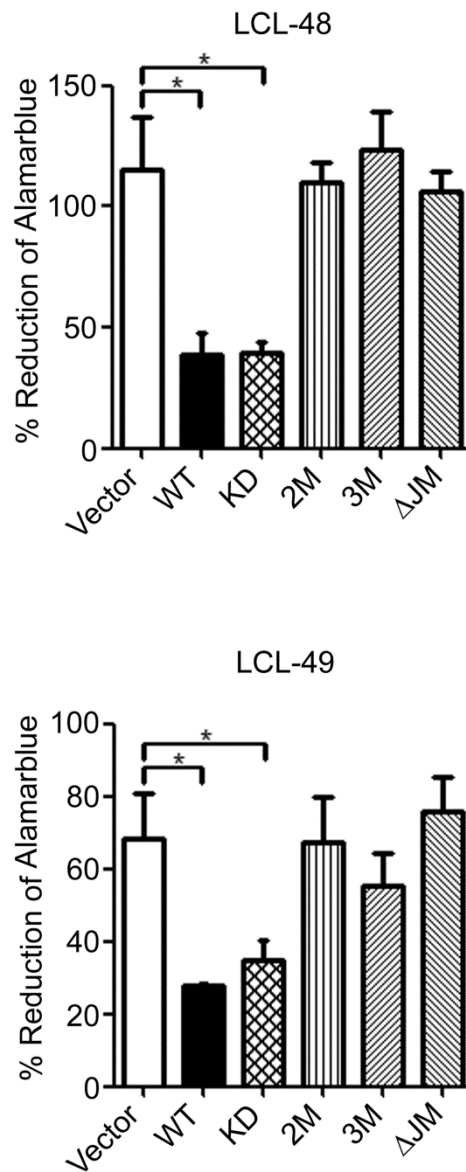
Figure 22. The EphA4 JM domain is required for prevention of LCL proliferation.

LCLs from two donors were infected with EphA4 WT, mutants or vector control expressing lentiviruses for 3 days and then reseeded at 1×10^4 cells per well in 96 well plates for 4 days. Cell proliferation assays were measured by AlamarBlue reduction. (*, $p < 0.05$, Student's t test).
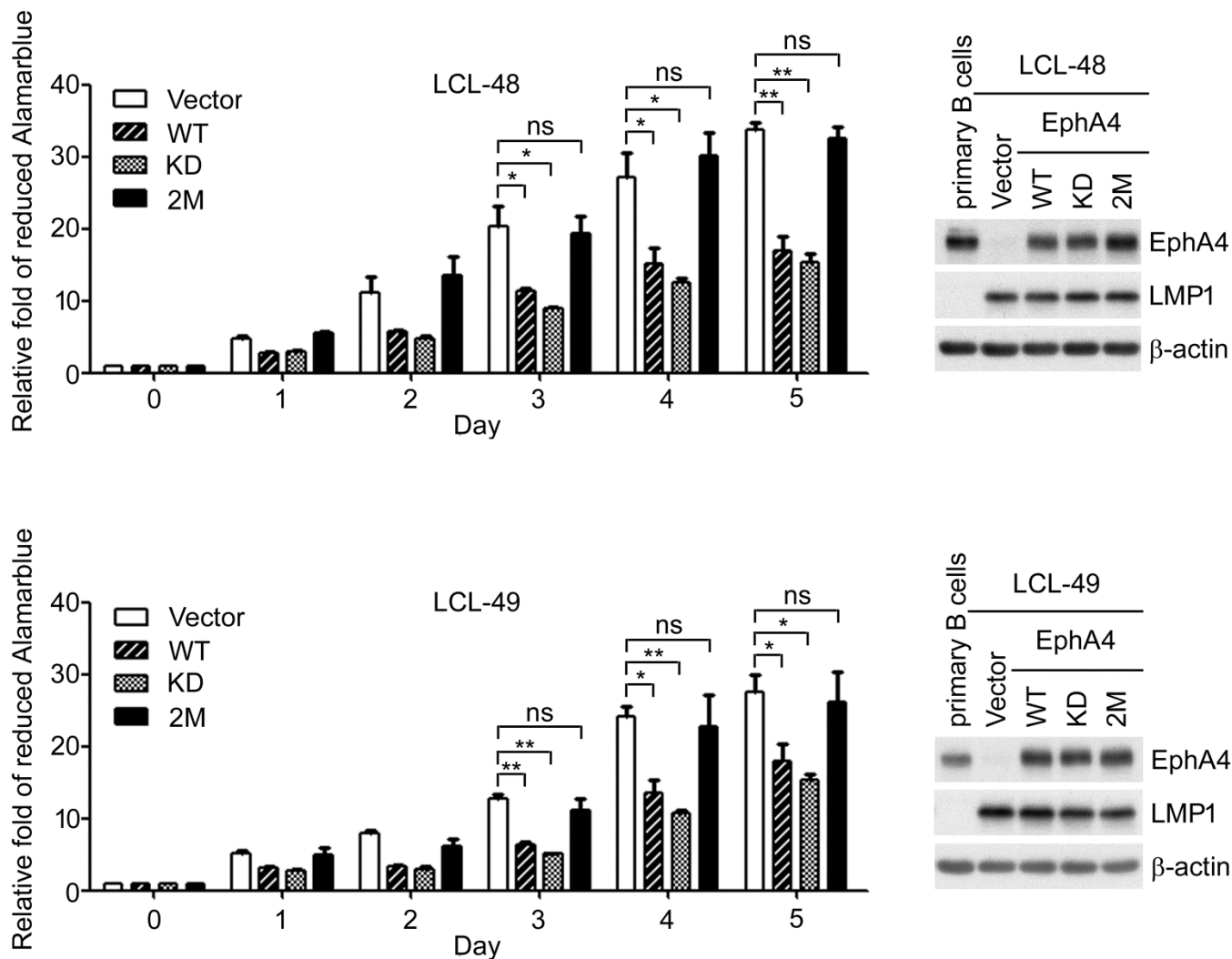
Figure 23. Two autophosphorylation sites of EphA4 JM region are important for inhibiting LCL proliferation.

LCLs from two donors were infected with EphA4 WT, mutants or vector control expressing lentiviruses for 2 days and then reseeded at 1×10^4 cells per well in 96 well plates for 5 days. Cell proliferation assays were measured by AlamarBlue reduction. Relative folds of proliferation were standardized with vector controls (*, $p < 0.05$; **, $p < 0.01$, Student's *t* test). Total proteins were obtained from primary B cells and LCLs expressing WT and mutant forms of EphA4 at day 5 post reseeding. EphA4, LMP1 and β-actin expression levels were detected by Western blotting (right panel). β-actin served as an internal control.

85
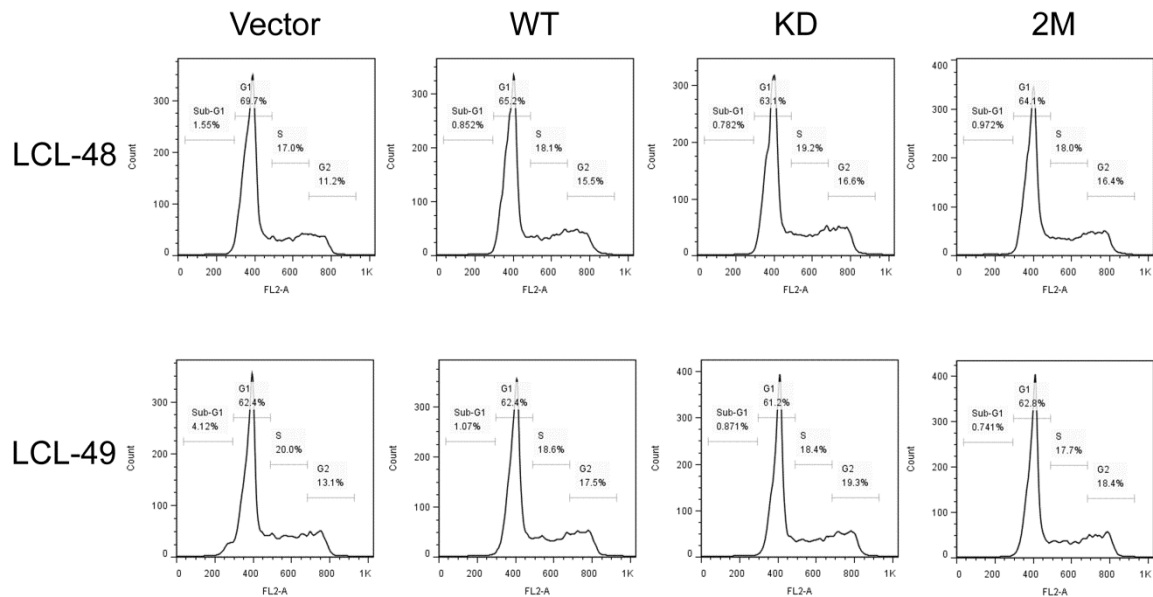
Figure 24. Ectopic expression of EphA4 do not affect cell cycle in LCLs.
LCLs from two donors were infected with EphA4 WT, mutants or vector control expressing lentiviruses for 2 days and then reseeded at a density of 1×10^5/mL cells in 6 well plates for 5 days. Cells were stained with 10 μg/mL propidium iodide for cell cycle analysis by flow cytometry.
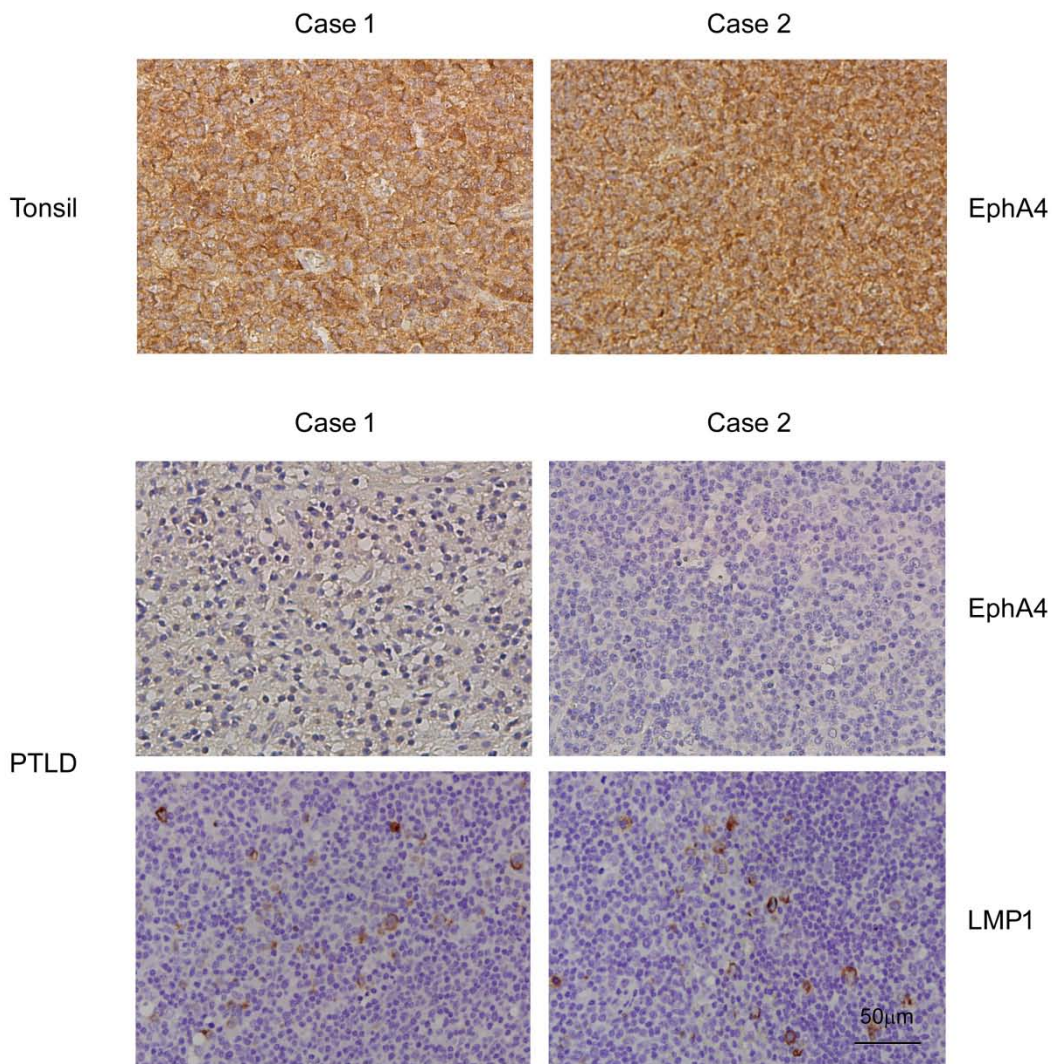
Figure 25. EphA4 is expressed in Tonsil but not expressed in PTLD biopsies.
Paraffin-embedded PTLD and tonsils sections were subjected to IHC assays and
hematoxylin was used for the nuclear counterstaining. Positive signals of EphA4 were
indicated as a brown color in tonsil biopsies but not in PTLD biopsies. LMP1
expression was also detected as a brown color by IHC assay in PTLD biopsies. The
nuclei of the cells are colored blue. Magnification x200 (Scale bar 50 μm). The IHC
data of EphA4 in Tonsil and PTLD biopsies were from Dr. Ya-Ching Chou.
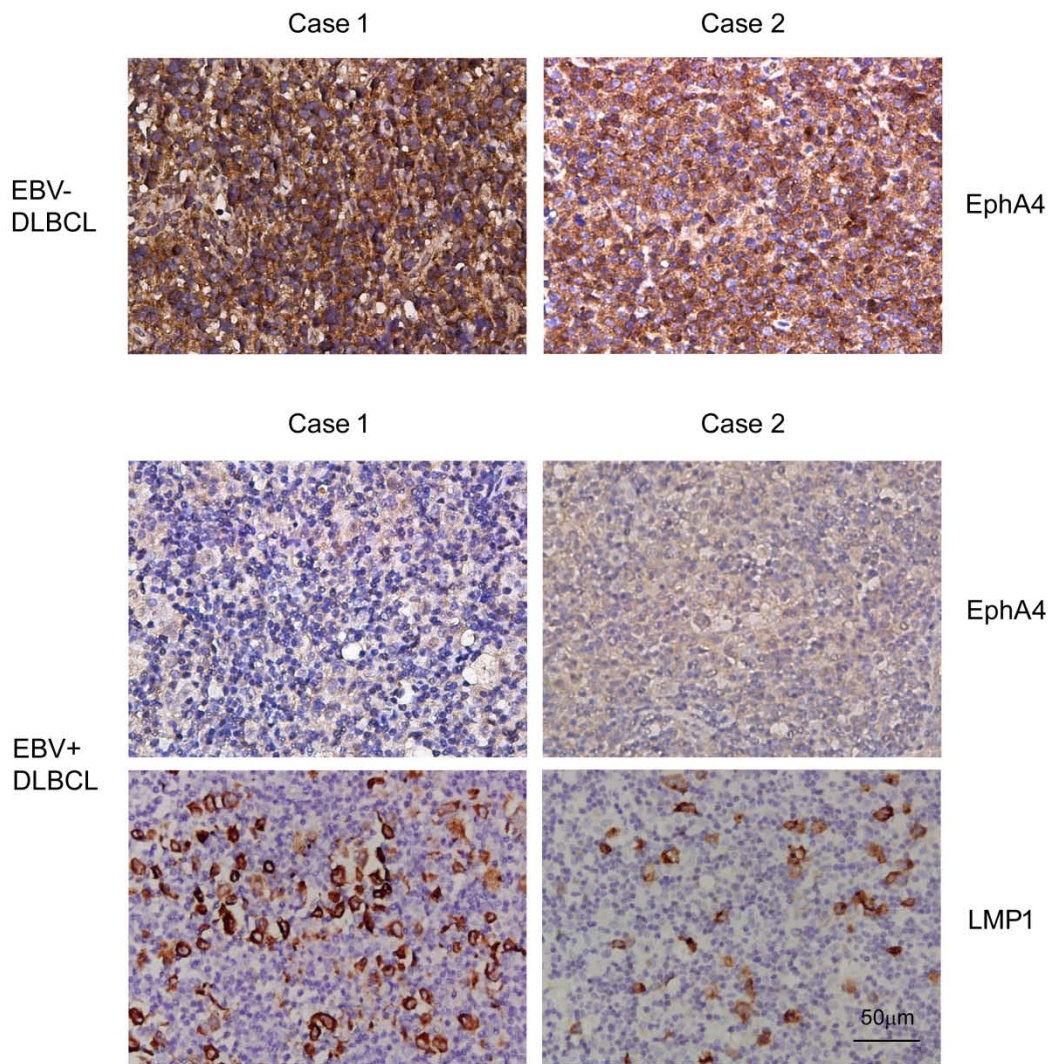
Figure 26. EphA4 expression is inversely correlated with EBV infection and LMP1 expression in DLBCL biopsies.

Paraffin-embedded DLBCL sections were stained for EphA4. Positive signals of EphA4 could be seen as a brown color in EBV-negative DLBCL, but not in EBV-positive DLBCL biopsies, by IHC. LMP1 expression was also detected in EBV-positive DLBCL biopsies. The nuclei were observed as a blue color and hematoxylin was used for the nuclear counterstain. Magnification x200 (Scale bar 50 μm).

88
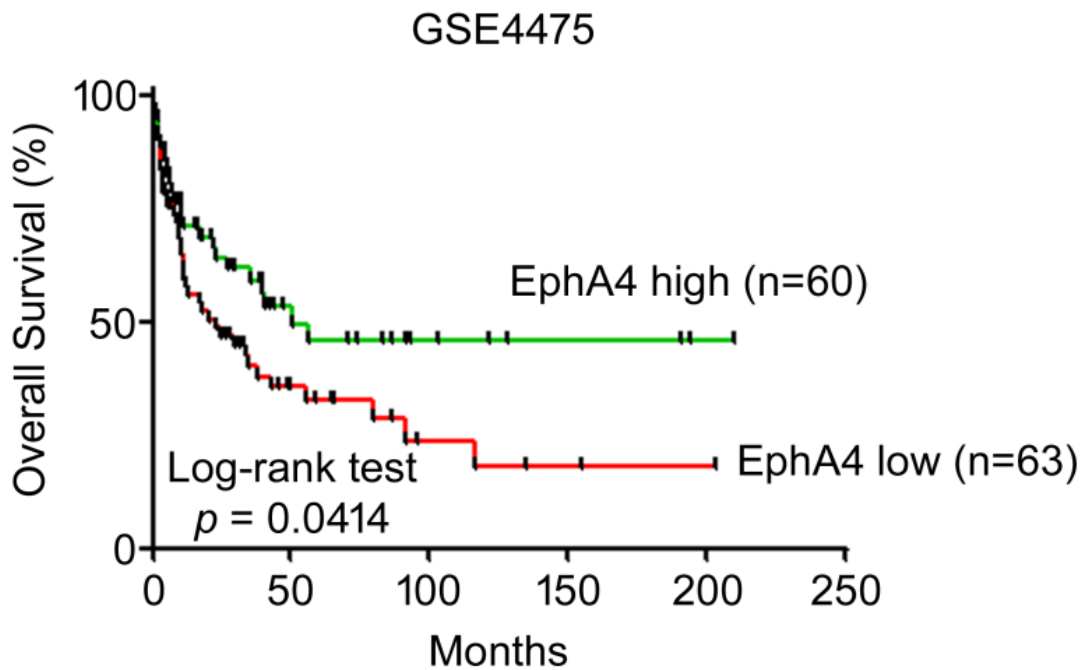
Figure 27. Low expression of EphA4 correlates with poor overall survival in patients with DLBCL.

The survival curve of DLBCL was obtained from the GEO datasets. Patients were divided into high (n=60) and low EphA4 (n=63) groups, according to the median expression level of EphA4. A Kaplan-Meier plot showed that patients with low EphA4 had worse overall survival (p=0.0414, log-rank test). The raw data were provided by Dr. Chi-Kuan Chen.
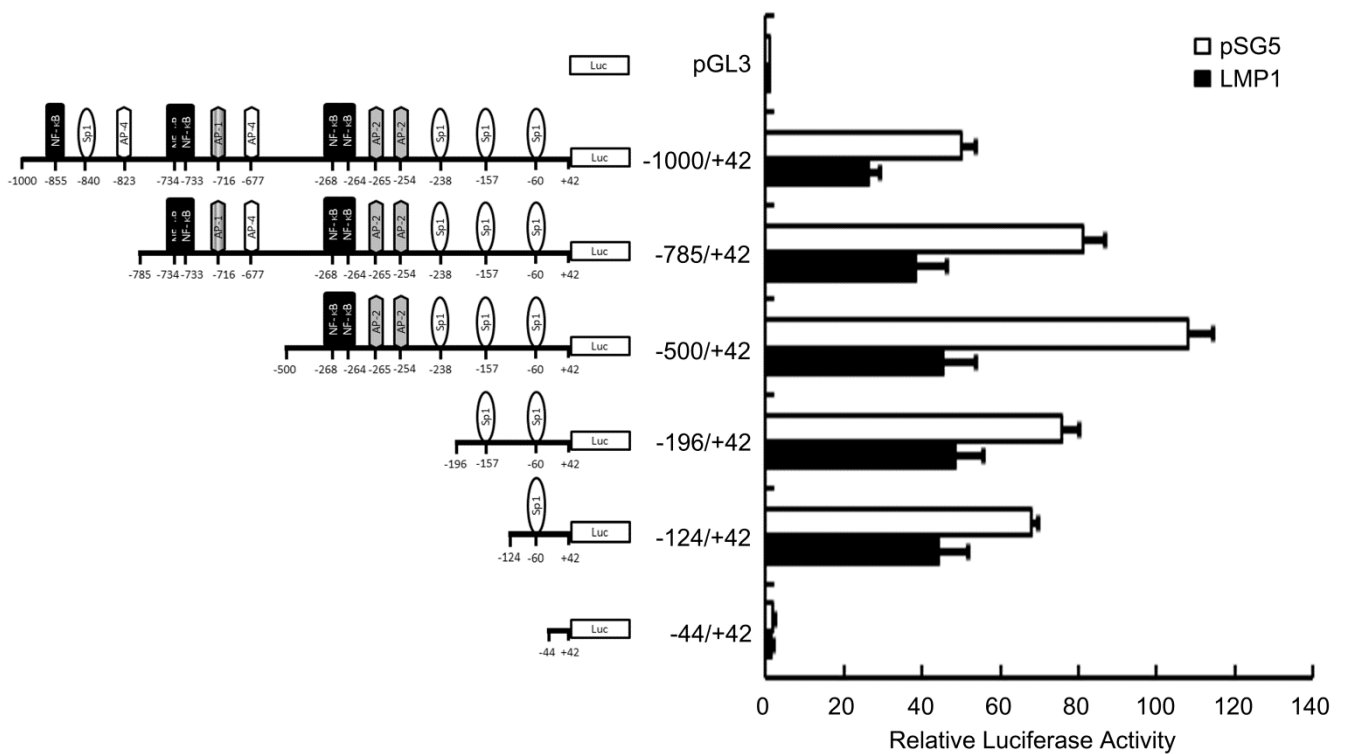
Figure 28. LMP1 downregulates EphA4 mRNA expression level through promoter regulation.

TW01 cells were cotransfected with effector plasmids LMP1 or pSG5, reporter plasmids pGL3 vector control or EphA4 promoter constructs with serial deletion (left panel) and internal control pEGFPC1 plasmids for 3 days. EphA4 relative luciferase activity was first normalized to GFP, followed by standardization with the control vector pGL3.
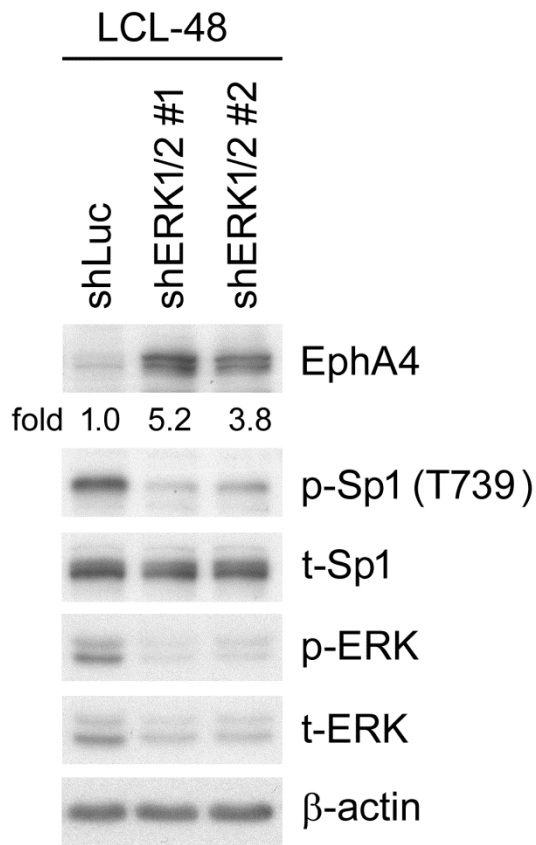
Figure 29. Knockdown of ERK1/2 decreases the Sp1 phosphorylation in LCLs.
Knockdown of ERK1/2 in LCLs was conducted by lentiviruses expressing shERK1 plus shERK2 for 3 days. Infected cells were selected with 2 μg/mL puromycin for 2 days. EphA4, phosphor-Sp1 (T739), total Sp1, phospho-ERK, total ERK proteins were then determined by Western blotting. EphA4 protein relative folds were normalized to β-actin and standardized with vector control shLuc.
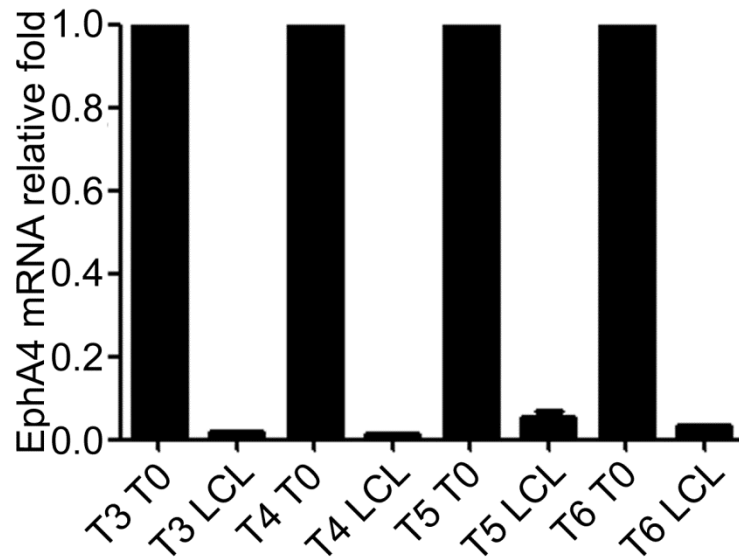
Figure 30. EphA4 is downregulated in human tonsil cells post EBV infection.
Total RNAs were harvested from paired uninfected cells and LCLs generated from
the tonsil mononuclear cells of four donors labeled as T3-T6. Expression levels of
EphA4 mRNA were measured by RT-Q-PCR. EphA4 mRNA relative folds were
normalized to internal control GAPDH and standardized with uninfected tonsil cell
T0.
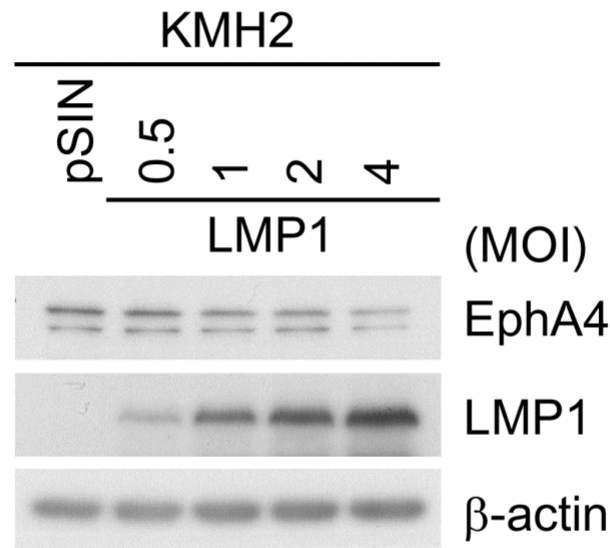
Figure 31. EphA4 is decreased by LMP1 in a dose dependent manner in Hodgkin's lymphoma cell line, KMH2.

EBV-negative KMH2 cells were infected with LMP1 expressing lentivirus at MOI 0.5, 1, 2 or 4. EphA4, LMP1 and β-actin were detected by Western blotting. EphA4 protein relative folds were normalized to internal control β-actin and then standardized with pSIN vector control.
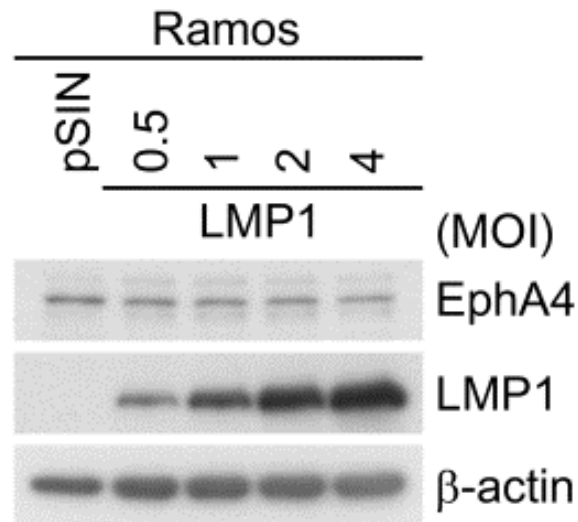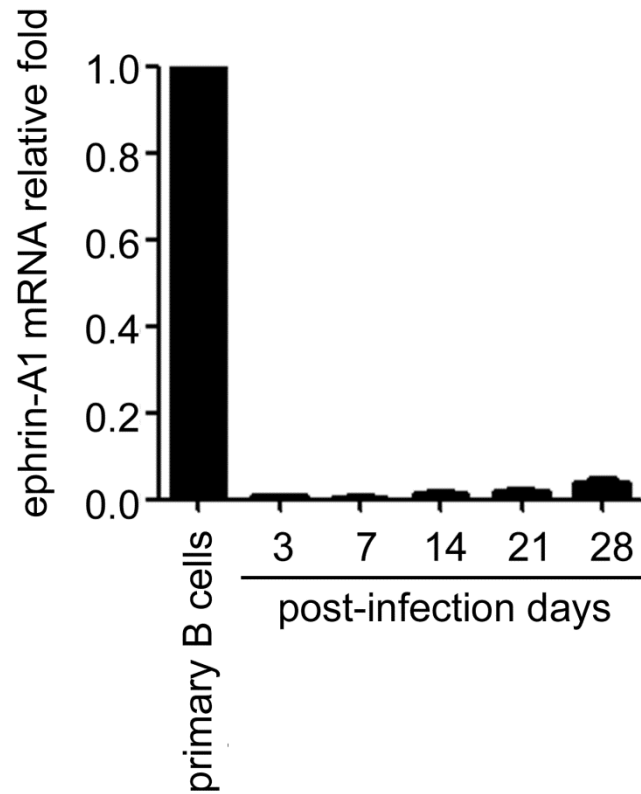
Figure 32. EphA4 is not repressed by LMP1 in Hodgkin's lymphoma cell line, Ramos.

EBV-negative Ramos cells were infected with LMP1 expressing lentivirus at MOI 0.5, 1, 2 or 4. EphA4, LMP1 and β-actin were detected by Western blotting. EphA4 protein relative folds were normalized to internal control β-actin and then standardized with pSIN vector control.

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Figure 33. Ephrin-A1 is decreased by EBV infection.
Peripheral CD19-positive B cells were seeded in 1×10^6 cells/mL and infected with EBV strain B95.8. Total RNAs were harvested from primary B cells at the days indicated post-EBV infection. Expression levels of ephrin-A1 mRNA were measured by RT-Q-PCR. Ephrin-A1 mRNA relative folds were normalized to internal control GAPDH and standardized with uninfected primary B cells.
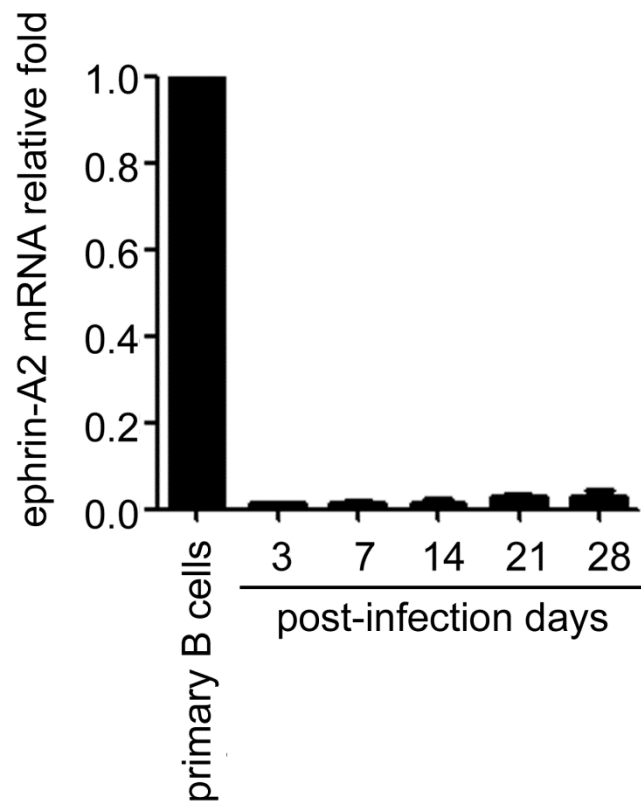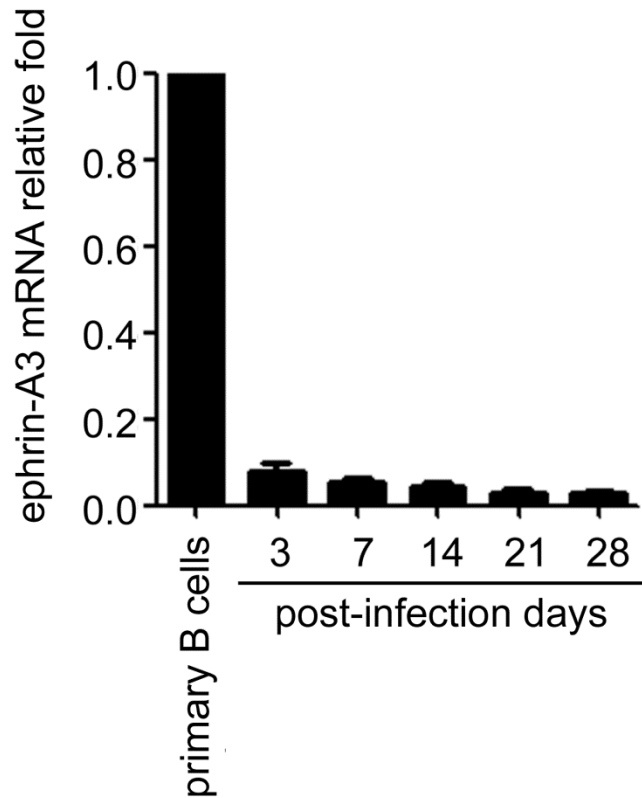
Figure 34. Ephrin-A2 is downregulated post EBV infection.
Peripheral CD19-positive B cells were seeded in 1×10^6 cells/mL and infected with EBV strain B95.8. Total RNAs were harvested from primary B cells at the days indicated post-EBV infection. Expression levels of ephrin-A2 mRNA were measured by RT-Q-PCR. Ephrin-A2 mRNA relative folds were normalized to internal control GAPDH and standardized with uninfected primary B cells.
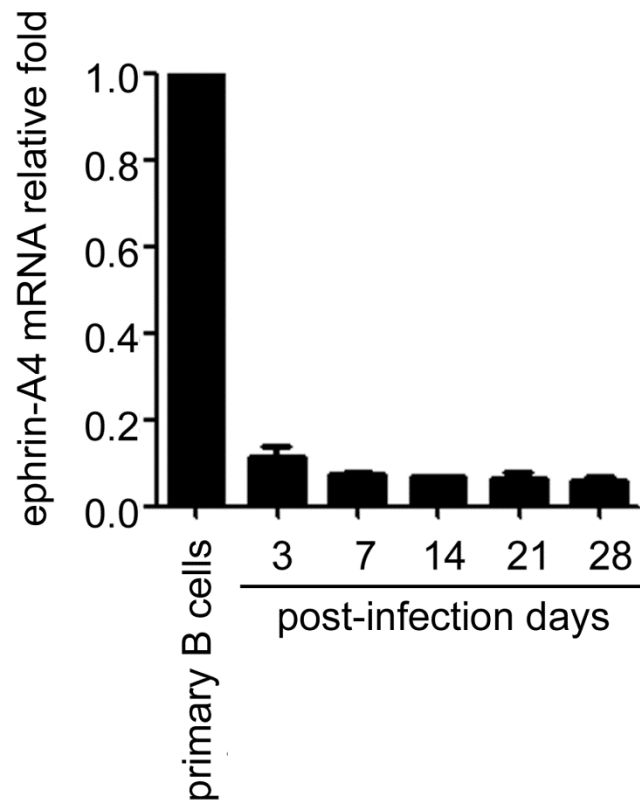
Figure 35. Ephrin-A3 is reduced after EBV infection.
Peripheral CD19-positive B cells were seeded at a density of 1×10^6 cells/mL and infected with EBV strain B95.8. Total RNAs were harvested from primary B cells at the days indicated post-EBV infection. Expression levels of ephrin-A3 mRNA were measured by RT-Q-PCR. Ephrin-A3 mRNA relative folds were normalized to internal control GAPDH and standardized with uninfected primary B cells.
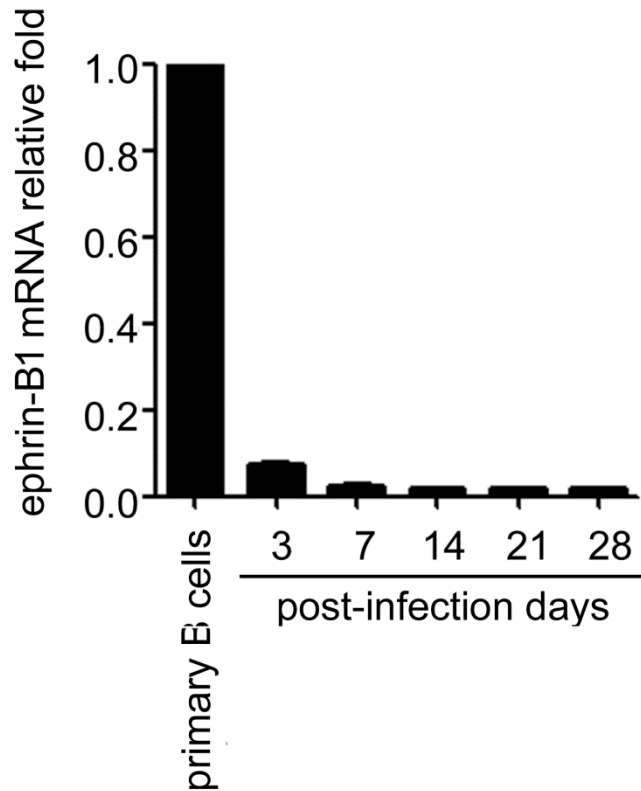
Figure 36. Ephrin-A4 is repressed post EBV infection.
Peripheral CD19-positive B cells were seeded in 1×10^6 cells/mL and infected with EBV strain B95.8. Total RNAs were harvested from primary B cells at the days indicated post-EBV infection. Expression levels of ephrin-A4 mRNA were measured by RT-Q-PCR. Ephrin-A4 mRNA relative folds were normalized to internal control GAPDH and standardized with uninfected primary B cells.
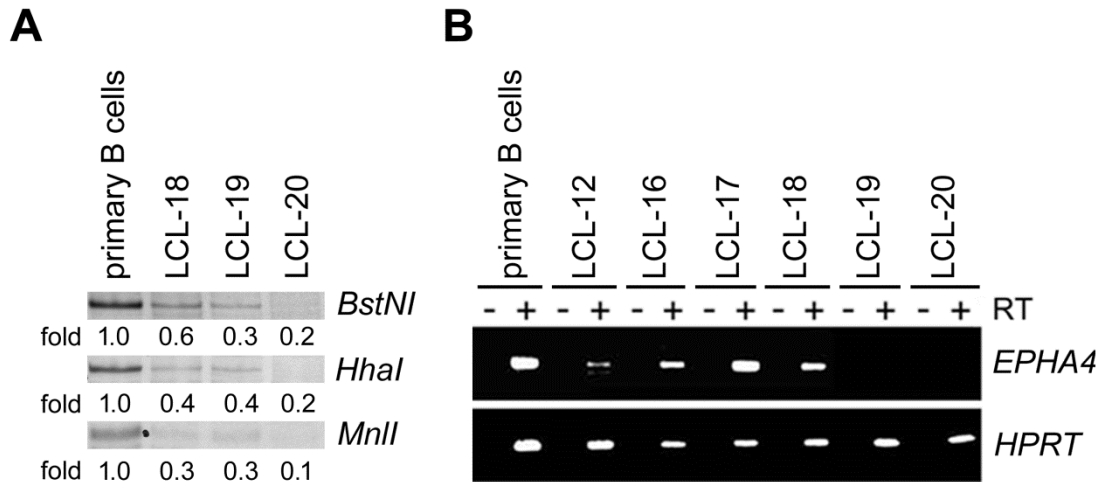
Figure 37. Ephrin-B1 is decreased by EBV infection.
Peripheral CD19-positive B cells were seeded at a density of 1×10^6 cells/mL and infected with EBV strain B95.8. Total RNAs were harvested from primary B cells at the days indicated post-EBV infection. Expression levels of ephrin-B1 mRNA were measured by RT-Q-PCR. Ephrin-B1 mRNA relative folds were normalized to internal control GAPDH and standardized with uninfected primary B cells.

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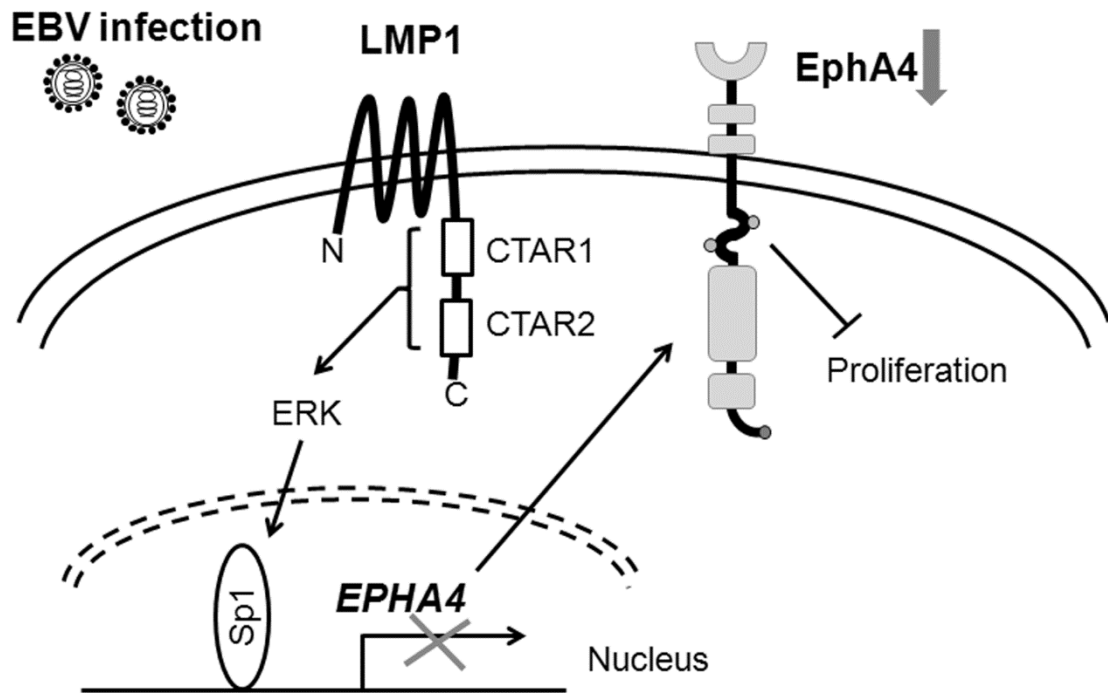
Figure 38. The model of LMP1-downregulated EphA4 expression and ectopic expression of EphA4 inhibit LCL proliferation.

LMP1 reduced EphA4 expression through ERK-Sp1 to inhibit EphA4 promoter activity in B cells and overexpression of EphA4 prevent LCL proliferation via its juxtamembrane region.
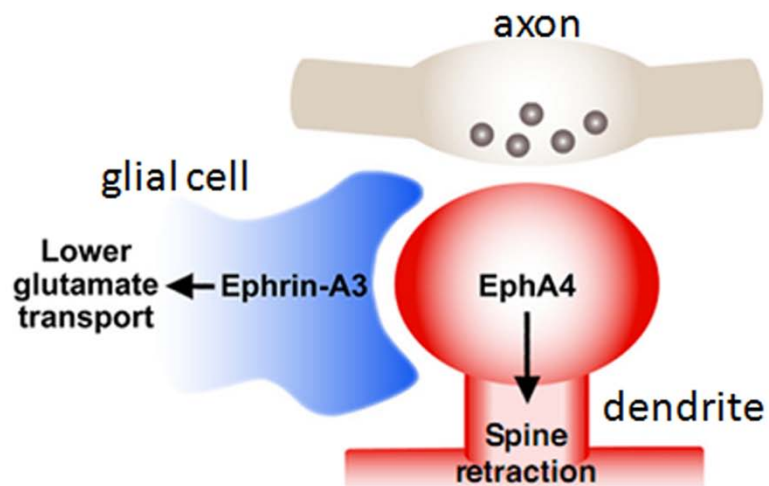
Appendix I

A

primary B cells | LCL-18 | LCL-19 | LCL-20

*BstN*I
fold 1.0 0.6 0.3 0.2

*Hha*I
fold 1.0 0.4 0.4 0.2

*Mnl*I
fold 1.0 0.3 0.3 0.1

B

primary B cells | LCL-12 | LCL-16 | LCL-17 | LCL-18 | LCL-19 | LCL-20

- +   - +   - +   - +   - +   - +   - +   RT

EPHA4

HPRT

Appendix I. EphA4 is reduced by EBV infection in B cells.

(A) Total RNAs were harvested from uninfected primary B cells, from peripheral blood, and EBV-immortalized LCLs from three different donors. cDNAs were generated using [^{33}P]-labeled degenerate primers for PTKs. EphA4-specific cDNAs were digested by three restriction enzymes, *BstN*I, *Hha*I and *Mnl*I. Relative fold EphA4 expression was compared to primary B cells. (B) Confirmation of the down-regulation of EphA4 transcripts in LCLs by RT-PCR. The amounts of EphA4 transcripts are detected by RT-PCR using specific EphA4 primer in primary B cells and in six LCLs (LCL-12, LCL-16, and LCL-17, LCL-18, LCL-19, and LCL-20). Detection of hypoxanthine phosphoribosyl transferase (HPRT) is served as an internal control. (data from Dr. Jean Lu).
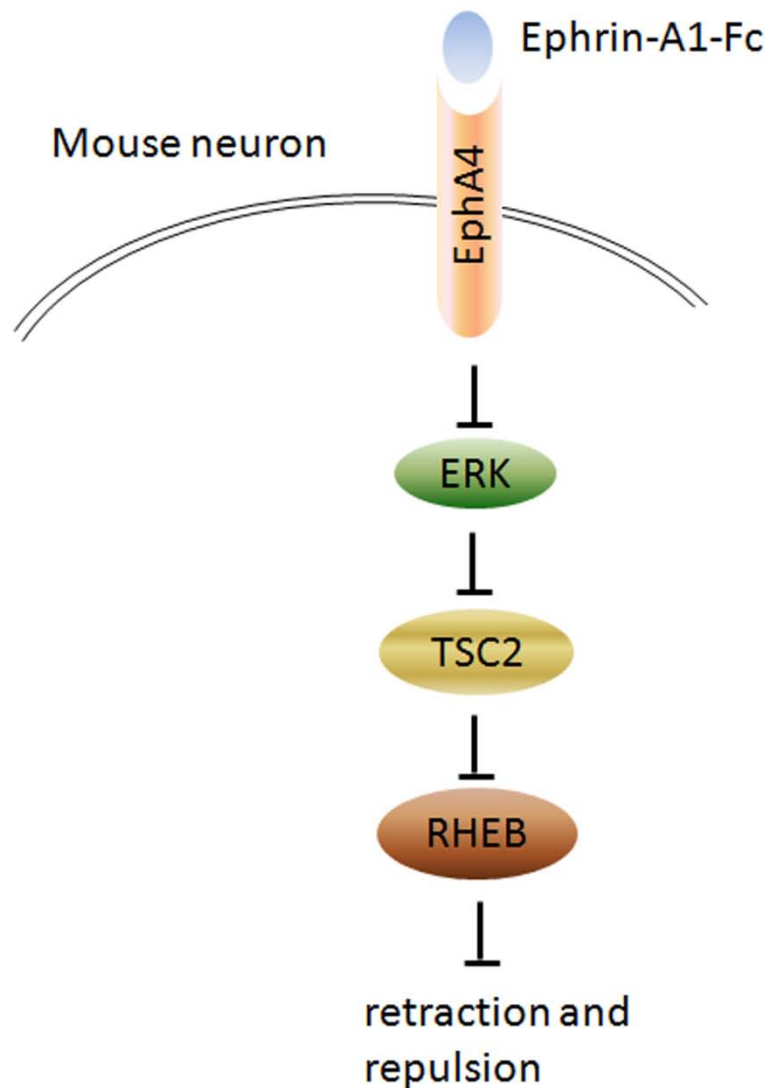
Appendix II. EphA4 regulates axon guidance.

EphA4/ephrin-A3 interaction activates EphA4 forward signaling in neuron which leads to dendrite retraction. Ephrin-A3 reverse signaling in glial cells which inhibits glutamate transports. (Carmona *et al.*, 2009)
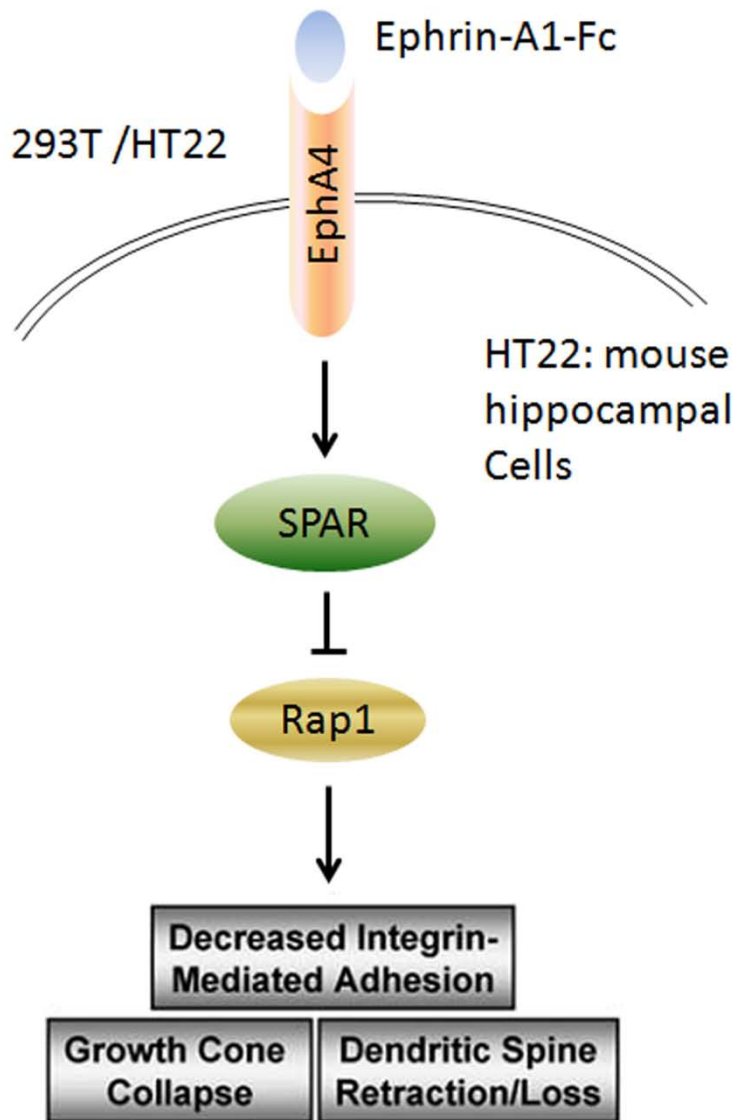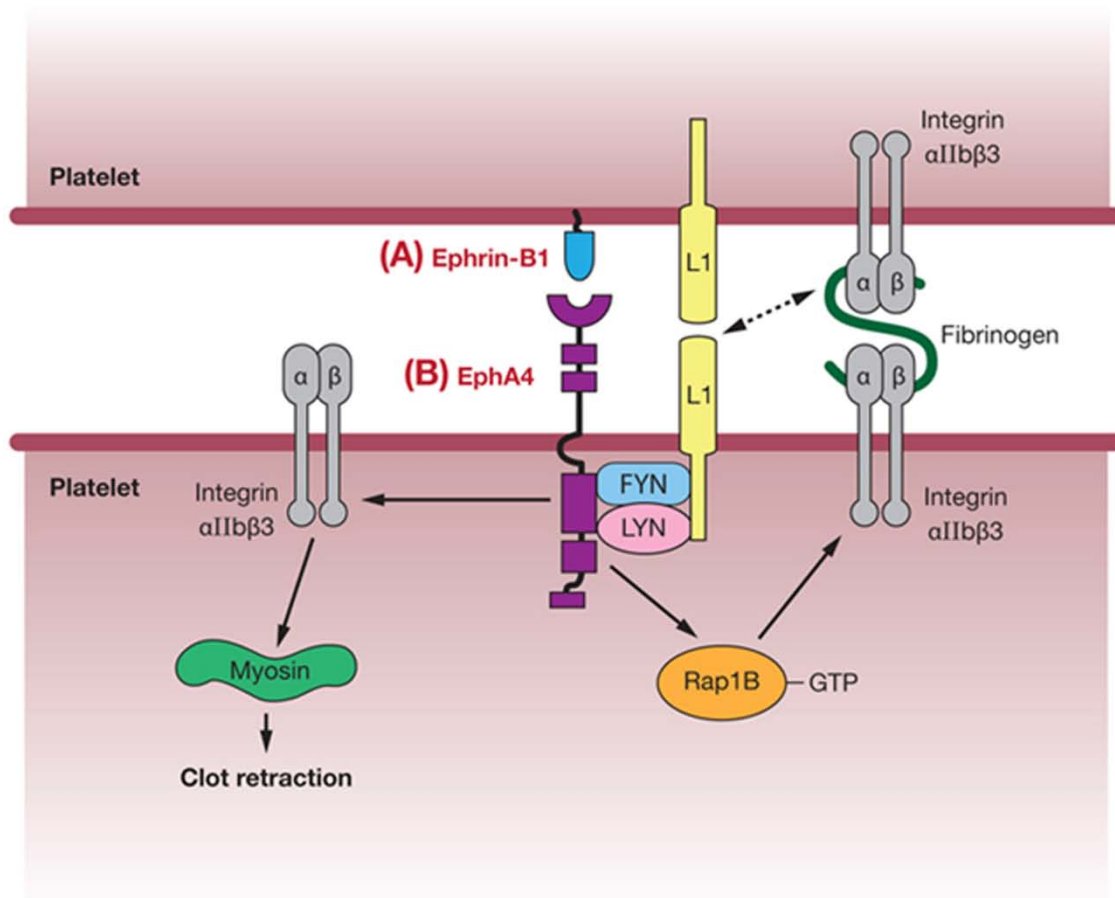
Appendix III

Appendix III. The ephrin-A1/EphA4 regulates SPAR-Rap1 pathway in neuron.
EphA4 activates spine-associated RapGAP (SPAR) to reduce Ras-related protein 1 (Rap1) activity, therefore inhibits integrin-mediated adhesion to the extracellular matrix and causes neuron dendrite spine retraction and growth cone collapse in the presence of its soluble ligand ephrin-A1-Fc. (Richter *et al.*, 2007)

Appendix IV. The ephrin-A1/EphA4 mediates ERK-TSC2-RHEB pathway to regulate axon guidance.

In ephrin-A1 stimulated neurons, EphA4 suppresses ERK phosphorylation and reduces the inhibition of Tuberous sclerosis complex 2 (TSC2) by ERK. Therefore, the enhanced TSC2 further inactivates Ras homolog enriched in brain (RHEB) and downstream mTOR pathway and finally induces neuron repulsion and growth cone collapse. (Nie *et al.*, 2010)

**Appendix V. EphA4 promotes platelet aggregation.**

The binding of EphA4/ephrin-B1 from adjacent platelets recruits Lyn, Fyn and the cell adhesion molecule L1 to promote integrin-mediated adhesion via Rap1B activation thus leads to platelet aggregation. (Singh *et al.*, 2012)
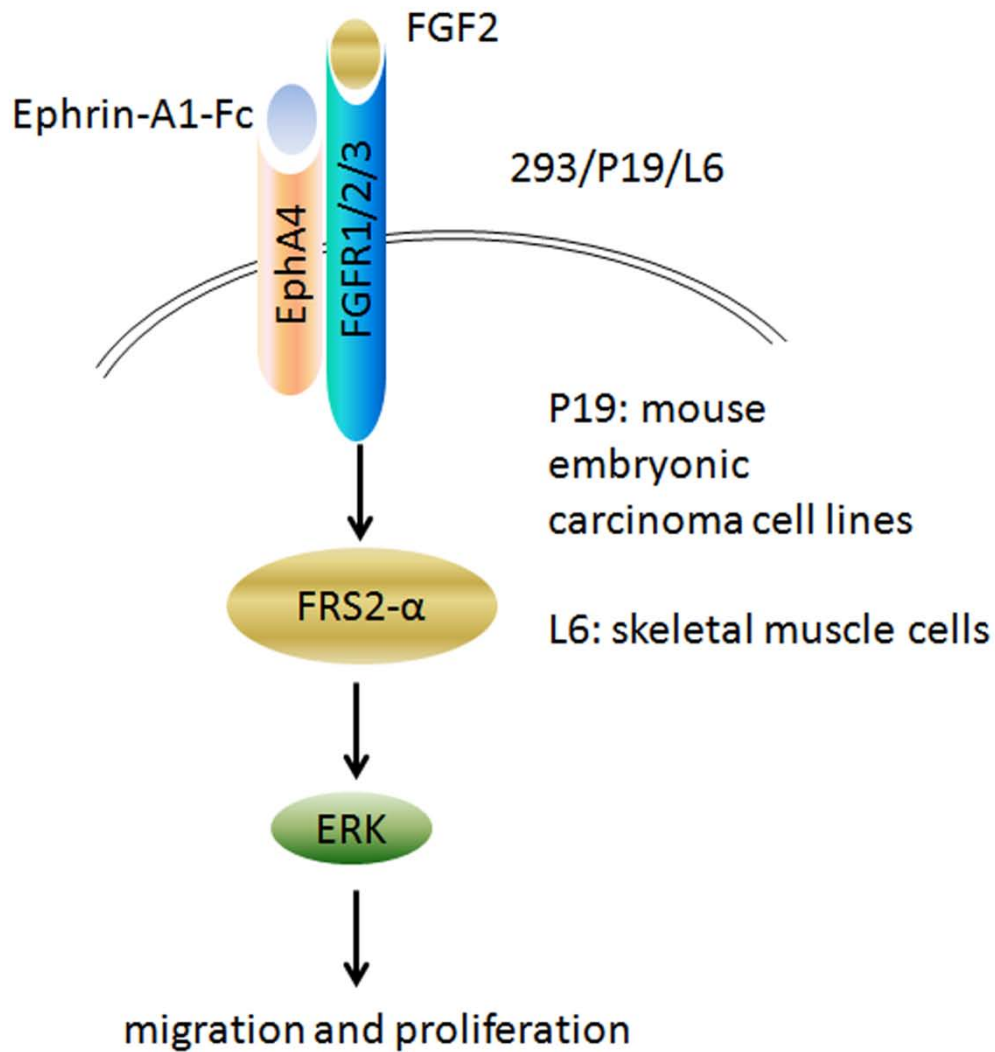
Appendix VI
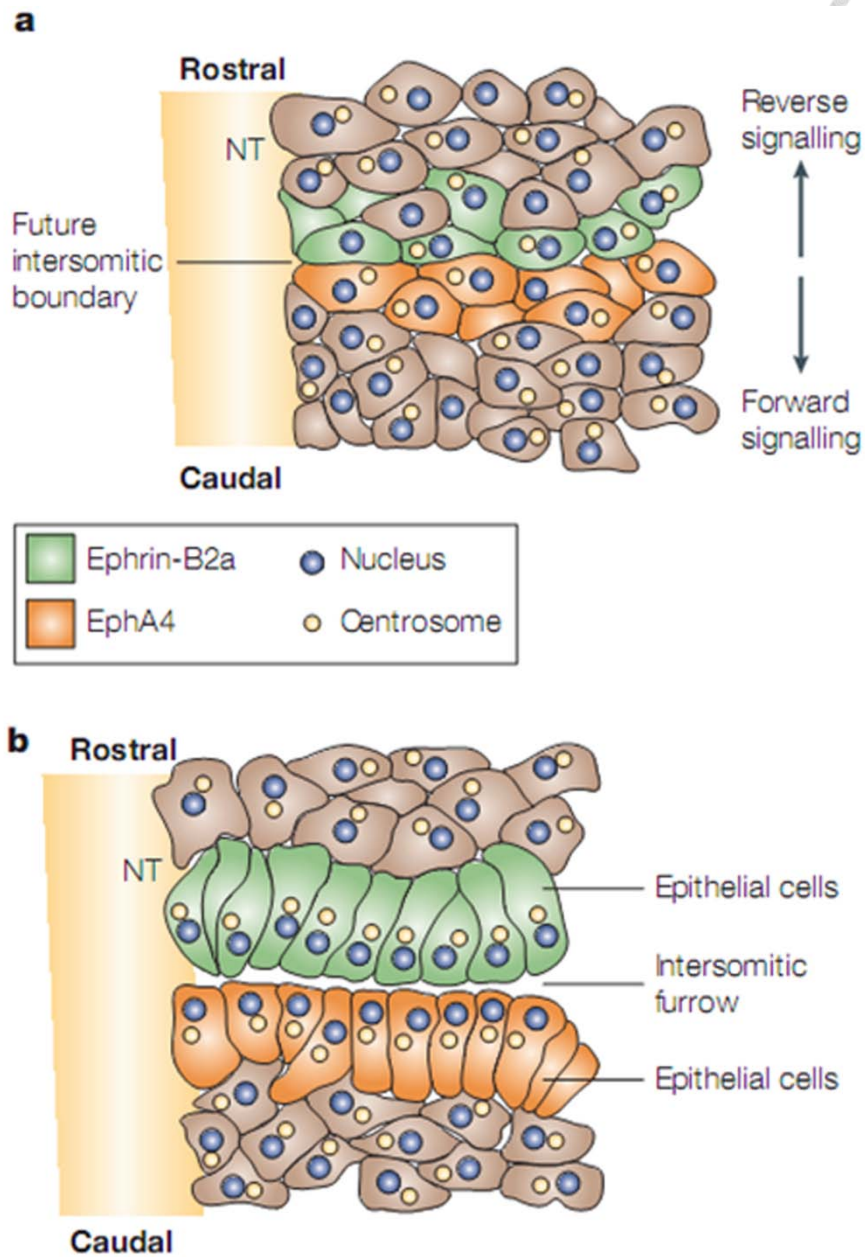
Appendix VI. EphA4 induces mesenchymal-to-epithelial transition (MET) during somite morphogenesis.

EphA4 and ephrin-B2 triggers MET during somite morphogenesis in zebrafish neural tube. (Pasquale, 2005)
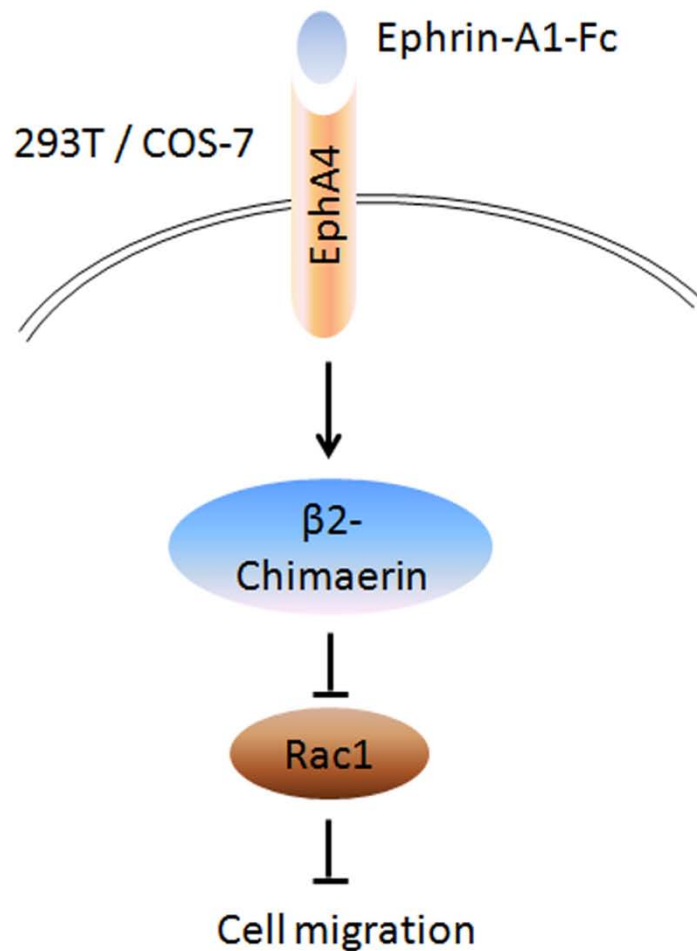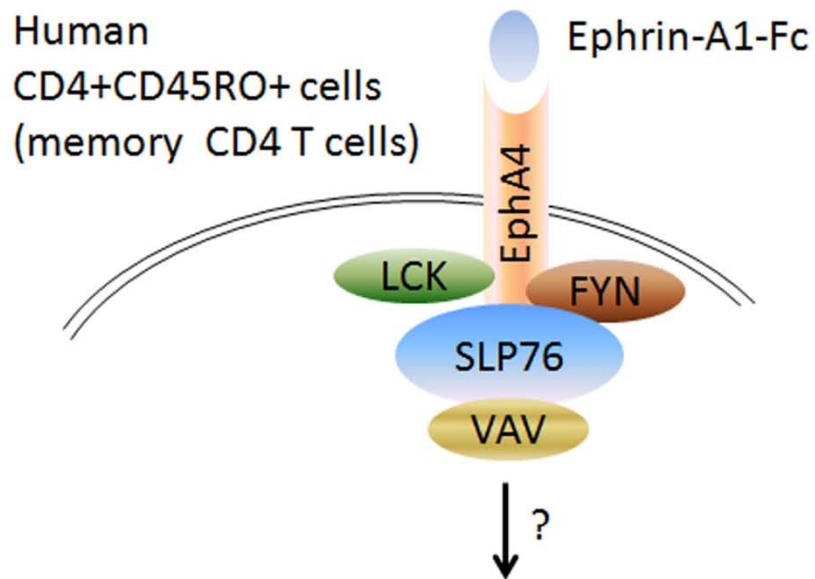
Appendix VII

Appendix VII. EphA4 is involved in tumor suppression.

Treating with ephrin-A1 to EphA4 expressing cells leads to β2-chimaerin binding to kinase domain of EphA4, inactivates Rac1 and further causes inhibition of cell migration in 293T and COS-7 cells. (Takeuchi *et al.*, 2009)

Appendix VIII

**Appendix VIII. EphA4 is involved in tumor promoting pathway.**

EphA4 is associated with FGFR1 via their cytoplasmic domains, phosphorylates docking proteins, FGFR substrate 2-α (FRS2-α) and promotes cell migration and proliferation. (Fukai *et al.*, 2008; Yokote *et al.*, 2005)

Appendix IX. The EphA4 promotes memory CD4 T cell migration.

EphA4 promotes human memory CD4 T cell (CD4+CD45RO+ cells) migration in the presence of ephrin-A1 and Lck, Fyn, Slp76 and Vav are involved in EphA4-induced signal pathway. (Holen *et al.*, 2010)

Appendix X
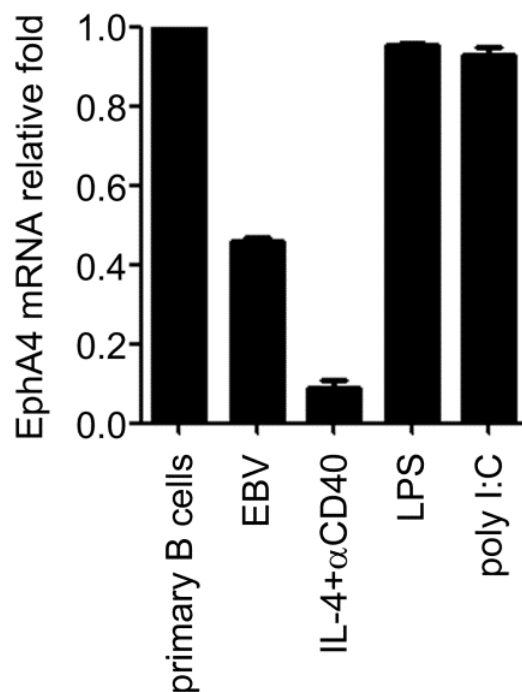
Appendix X. EphA4 expression is decreased by EBV infection and by treaanti-CD40 antibody plus IL-4.

Total RNAs were extracted from primary B cells, EBV infection or B-cell stimulations including anti-CD40 antibody plus IL-4, LPS or poly I:C for 3 days. EphA4 transcripts were measured by RT-Q-PCR. EphA4 mRNA relative folds were normalized to internal control MAGOH and standardized with uninfected primary B cells (data from Kai-Min Lin).

Appendix XI

shRNA

Gene	Targeting sequence (5' to 3')
shLuciferase (shLuc)	CTTCGAAATGTCCGTTCGGTT
shSp1	GGCAGATCTGCAGTCCATTAA
shERK1#1	CTATACCAAGTCCATCGACAT
shERK1#2	TCCCTGTCAAAGCTGTCACTT
shERK2	TATCCATTCAGCTAACGTTCT
shLMP1	GCTCATTATTGCTCTCTAT

Appendix XII

RT-PCR primer

Gene	Sequence (5' to 3')
BARF0	F- AGAGACCAGGCTGCTAAACA
	R- AACCAGCTTTCCTTTCCGAG
EBER1	F- ACCGGTAGGACCTACGCTGCCCTAGAGGT
	R- AAAACATGCGGACCACCAGCTGGT
EBER2	F- ACCGGTAGGACAGCCGTTGCCCTAGTGGT
	R- AAAAATAGCGGACAAGCCGAATACC
β-actin	F- TTCTACAATGAGCTGCGTGT
	R- GCCAGACAGCACTGTGTTGG

RT-Q-PCR primer

Gene	Sequence (5' to 3')	Probe*
EphA4	F- GATAGCAAGCCCTCTGGAAG	20
	R- CCAATCAGTTCGTAGCCAGTT	
GAPDH	F- TCCACTGGCGTCTTCACC	45
	R- GGCAGAGATGATGACCCTTTT	
MAGOH	F- AAAGAGGATGATGCATTGTGG	51
	R- TCTTCTCCAATGACGATTTCA	

*Probe (Universal ProbeLibrary for Human, Roche)

ChIP-PCR primer

promoter	Sequence (5' to 3')
EPHA4	F- TGTCTGCGCCGCCATTGGCC
	R- AGTTAGGAGAGCAGCGGGCTG
GAPDH	F- AGCTCAGGCCTCAAGACCTT
	R- AAGAAGATGCGGCTGACTGT

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