EPSTEIN-BARR VIRUS-ENCODED LMP-1, LMP-2A AND LMP-2B PROTEINS IN CELL CYCLE REGULATION

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ABSTRACT

Epstein-Barr virus (EBV) is a ubiquitous tumour-causing virus which infects more than 90% of world population asymptomatically. It is closely associated with nasopharyngeal carcinoma (NPC). LMP-1, -2A and -2B are three latent membrane proteins (LMPs) encoded by EBV which are frequently detected in NPC tumour. Recent studies suggest that these three proteins co-operate in the tumorigenesis of NPC but the mechanism is not clear. In this study, LMPs were cloned into pcDNA3.1 and transfected into HEK293T cell line to reveal their oncogenic mechanism via investigation on their involvement in the regulation of cell cycle and genes that are involved. The transcription of cell cycle arrest genes were examined via semi-quantitative reverse transcription-PCR and real time PCR. Cell cycle progression was examined via flow cytometry. LMPs were successfully cloned in pcDNA3.1 and expressed in HEK293T in single and co-expression manner. 14-3-3σ and Reprimo were upregulated in all LMP-1 expressing cells. Moreover, cell cycle arrest at G_2/M progression was detected in all LMP-1 expressing cells. Therefore, we conclude that LMP-1 may induce cell cycle arrest at G_2/M progression via upregulation of 14-3-3σ and Reprimo.

ABSTRAK

Epstein-Barr virus (EBV) adalah virus penyebab ketumbuhan yang biasa menjangkiti lebih daripada 90% penduduk dunia. Ia berkait rapat dengan karsinoma nasofarinks (NPC). LMP-1-2A dan 2B adalah tiga membran protein terpendam (LMPs) yang dikodkan oleh EBV yang kerap dikesan di dalam ketumbuhan NPC. Kajian baru-baru ini menunjukkan bahawa ketiga-tiga protein ini bekerjasama dalam tumorigenesasi NPC, namun mekanisme ini masih tidak jelas. Dalam kajian ini, LMPs diklonkan ke dalam pcDNA3.1 dan ditransfeksikan ke dalam titisan sel HEK293T untuk mendedahkan mekanisme onkogenik mereka melalui siasatan ke atas penglibatan mereka dalam peraturan kitaran sel dan gen yang terlibat. Transkripsi gen yang terlibat dalam penahanan kitaran sel telah diperiksa melalui PCR transkripsi berbalik semi-kuantitatif dan PCR masa nyata. Perkembangan kitaran sel diperiksa melalui sitometri aliran. LMPs telah berjaya diklon ke dalam pcDNA3.1 dan diekspresikan dalam HEK293T sel secara tunggal dan ekspresi bersama. Ekspresi 14-3-3σ dan Reprimo telah meningkat dalam semua sel yang mengekspresikan LMP-1. Selain itu, penahanan kitaran sel pada perkembangan G_2/M dikesan di dalam semua sel yang mengekspresikan LMP-1. Oleh itu, kami menyimpulkan bahawa LMP-1 boleh mendorong penahanan kitaran sel pada fasa G_2/M dengan meningkatkan ekspresi 14-3-3σ dan Reprimo.

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TABLE OF CONTENTS

CONTENTS	**PAGE**
Abstract	ii
Abstrak	iii
Acknowledgements	iv
Presentations and Publication	v
Table of Contents	vi
List of Tables	ix
List of Figures	x
Abbreviation	xii
Chapter 1: Introduction	1
1.1 Discovery of Epstein - Barr virus (EBV)	1
1.2 EBV virus: Structure and genome map	3
1.3 Life cycle of EBV	7
1.4 Stages of EBV infection	10
1.4.1 Lytic infection	10
1.4.2 Latent infection	10
1.4.2.1 Pattern of EBV latency	11
1.5 LMP-1	12
1.5.1 LMP-1 structure	12
1.5.2 Oncogenic roles of LMP-1	13
1.5.3 Paradox effects of LMP-1	16
1.6 LMP-2	17
1.6.1 LMP-2 structure	17
1.6.2 Roles of LMP-2	18
1.7 Interaction of LMP-1, LMP-2A and LMP2B	21
1.8 Cell cycle regulation	22
1.9 Objectives and Aims	23

Chapter 2: Materials and Methods 24
2.1Cell lines and cell cultures 24
2.1.1 Maintenance of cell lines 24
2.1.2 Cryopreservation and reviving cells 25
2.1.3 Counting cells 25
2.2 RNA extraction 26
2.3 Gene Cloning 27
2.3.1 cDNA Synthesis 27
2.3.2 Polymerase Chain Reaction (PCR) 28
2.3.3 Gene insertion to pcDNA3.1 vector 29
2.3.4 Boiling PCR for transformants 31
2.3.5 Plasmid Extraction 32
2.3.6 Sequencing of Inserted Genes 33
2.4 Expression of LMP-1, LMP-2A and LMP-2B 34
2.5 Analysis of genes expressions in LMP-1, LMP-2A and LMP-2B expressing cells 35
2.5.1 Analysis of RNA expression 35
2.5.1.1 RNA extraction 35
2.5.1.2 cDNA synthesis 35
2.5.1.3 PCR 35
2.5.1.4 Semi-quantitative RT-PCR 35
2.5.1.5 Real Time Quantitative PCR 38
2.5.2 Analysis of protein by Western blotting 39
2.5.2.1 Protein extraction 39
2.5.2.2 Bradford assay 40
2.5.2.3 SDS-PAGE Electrophoresis 41
2.5.2.4 Immunoblotting 43
2.6 Flow cytometry for cell cycle Analysis 45
2.7 Data Analysis 45

Chapter 3: Results 46
3.1 Cloning and sequence analysis of LMP-1, 2A and 2B derived from B95.8 cells 46
3.2 Transfection optimization 48
3.3 Expression of LMP-1, LMP-2A, LMP-2B in HEK293T cell line 50
3.4 Reduction in cell density in all LMP-1 expressing cells 52
3.5 Upregulation of *14-3-3σ* and *Reprimo* in LMP-1 expressing cells from semi-quantitative RT-PCR analysis 54
3.6 Upregulation of *14-3-3σ* and *Reprimo* in LMP-1 expressing cells from real-time quantitative PCR assay 57
3.7 14-3-3σ and Reprimo proteins were upregulated in all the LMP-1 expressing cells 61
3.8 Overexpression of LMP-1 arrests cells in G_2/M phase 62

Chapter 4: Discussion 66
4.1 Expression of LMPs 66
4.2 Overexpression of LMP-1 Induces Cell cycle Arrest 68
4.3 LMP-1 may have Induced Cell Cycle Arrest via Upregulation of 14-3-3α and Reprimo 70
4.4 Limitations 72

Chapter 5: Conclusion 74
5.1 Conclusion 74

References 75
Appendices 94
Appendix A: pcDNA3.1 vector 94
Appendix B: Sequences of LMP-1, LMP-2A and LMP-2B in EBV B95.8 strain 95

List of Tables

Table	Subject	Page
1.1	EBV-associated malignancies (Crawford, 2001).	2
1.2	Patterns of EBV latency in different diseases.	11
2.1	Genes-specific primers used in PCR.	28
2.2	Components of PCR mixture and amplification conditions.	29
2.3	List of sequencing primers.	33
2.4	Components and conditions for semi-quantitative RT-PCR.	36
2.5	List of Primers used for semi-quantitative RT-PCR.	37
2.6	Components of the running gel and stacking gel.	42
2.7	List of Antibodies used in Western blotting.	44
3.1	Cell Cycle distribution of transfected cells and HEK293T cells.	65

List of Figures

Figure	Subject	Page
1.1	Schematic diagram of EBV.	4
1.2	*Bam*HI restriction endonuclease map of prototype B95.8 EBV genome.	5
1.3	EBV episome.	6
1.4	Schematic *in vivo* infection of EBV.	9
1.5	Schematic structure of LMP-1 and its functional domains; as well as their associated proteins (Li and Chang, 2003).	13
1.6	Schematic structure of LMP-2A and its functional domains; as well as their associated proteins. (Young and Rickinson, 2004).	18
2.1	Multiple-cloning site (MCS) and Insertion site of pcDNA3.1 directional cloning vector.	30
3.1	RT-PCR and cloning of LMP-1, 2A and 2B derived from B95.8 cell line.	47
3.2	Verification of gene insertion into pcDNA3.1 with T7 and BGH primers.	48
3.3	Transfection of HEK293T cells with the amount of pcDNA3.1-CFP (µg) to Fugene HD (µl) atthe ratio of 1:5.	49
3.4	Transcriptionof LMP-1 and LMP-2 in HEK293T.	50
3.5	Detection of LMPs protein expression in HEK293T after transfection with various pcDNA3.1 constructs.	51
3.6	Effects of LMPs expression on cell viability and morphology.	53
3.7	Semi-quantitative comparison of mRNA expression levels of *p53, p27, p21, Reprimo* and *14-3-3σ* in LMPs expressing cells.	56
3.8	Melting curve profiles for PCR amplifications of *GAPDH, β-actin, 14-3-3σ* and *Reprimo*.	59
3.9	The relative mRNAs expressions of *14-3-3σ* and *Reprimo* in LMPs expressing cells.	60

3.10	Western blotting analysis of 14-3-3σ and Reprimo in all the transfected cells.	61
3.11	Flow cytometry analysis of HEK293T cells.	64

List of Abbreviation

%	Percentage
℃	Degree Celcius
µg	Mircogram
µl	Microliter
µM	Micromolar
AP	Alkaline Phosphatase
BCR	B-cell receptor
BL	Burkitt’s lymphoma
bp	Base pair
CD	Cluster of differentiation
Cdk	Cyclin dependent kinase
cDNA	Complementary DNA
CTAR	C-terminal activator region
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic Acid
DTT	Dithiothreitol
dNTP	Deoxynucleotide triphosphate
EBER	EBV-encoded RNA
EBNA	EBV nuclear antigen
EBV	Epstein-Barr Virus
EDTA	Ethylenediamine tetraacetic acid
ERK	Extracellular-regulated kinase
et al.,	*et alii* (and others)
EtBr	Ethidium Bromide
FACS	Fluorescence-activated cell sorting

FBS	Fetal bovine serum
g	Gram
HD	Hodgkin’s diseases
HIV	Human Immunodeficiency Virus
HL	Hodgkin’s Lymphoma
HLA	Human leukocyte antigen
hr	Hour
HRP	Horseradish Peroxidase
IM	Infectious Mononucleosis
IR1	Internal repeats
ITAM	Immunoreceptor tyrosine-based activation motif
JAK	Janus kinase
JNK	c-Jun N-terminal kinase
kb	Kilobase-pair
kDa	kilo Dalton
l	Litre
LB	Luria Bertani
LCL	Lymphoblastoid cell lines
LMP	Latent membrane protein
M	Molar
MAPK	Mitogen-activated protein kinase
mg	Miligram
min	Minute
ml	Mililitre
mM	Milimolar
mRNA	messenger RNA

NFκB	Nuclear factor kappa B
ng	Nanogram
NPC	Nasopharyngeal carcinoma
OD	Optical Density
ORF	Open reading frames
PAGE	Polyacrylamide gel electrophoresis
PBST	Phosphate buffered saline
PCR	Polymerase Chain Reaction
PTK	Protein tyrosine kinases
PVDF	Polivynilidene fluoride transfer
RIP	Receptor interacting-protein
RNA	Ribonucleic Acid
Rpm	Revolutions per minutes
RPMI	Roswell Park Memorial Institute Medium
RT	Reverse transcribed
RTPCR	Realtime PCR
S	Second
S.O.C	Super optimal broth with catabolite repression
SAPK	Stress-activated protein kinase
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
STAT	Signal transducer activators of transcription
Taq	*Thermos aquaticus*
TBE	Tris-Borate-EDTA (buffer)
TCR	T-cell receptor
TEMED	*N,N,N'N'*-tetramethylenediamine

TES	Transformation effector site
TGF	Transforming growth factor
TNFR	Tumour necrosis factor receptor
TPA	Phorbol esters [12-O-tetradecanoylphorbol-13-acetate
TR	Tandem direct repeat
TRADD	TNFR-associated death domain protein
TRAF	TNFR-associated protein
Tris	Tris (hydroxymethyl) aminomethane
Tris-HCL	Tris (hydroxymethyl)aminomethane hydrochloric acid
UK	United Kingdom
USA	United States America
V	Volt
v/v	Volume/volume
w/v	Weight/volume

1.1 Discovery of Epstein-Barr Virus (EBV)

In 1957, Denis Burkitt, a surgeon who is working in Kampala, Africa, was called for a consultation on a child with tumors on both sides of the upper and lower jaws. This observation leads him to the first discovery of "African Lymphoma" and now known as Burkitt's lymphoma (BL). In order to determine the cause of this malignancy, Burkitt and his colleagues collected data from all over Africa. Finally, Burkitt suggested that a viral infection was involved in the aetiology of this tumor (Burkitt and O'Conor, 1961; Burkitt, 1972; Tselis, 2006).

A meeting with Dr. Anthony Epstein in 1961, Burkitt agreed to send the biopsy samples from BL patients to UK for further investigation. When Epstein and his colleagues at the Middlesex Hospital, London, studied about the BL cell lines under electron microscopes, the virus particles were observed with similarity to morphology of herpex simplex virus (Epstein *et al.*, 1965). This new identified virus was then grouped as a member of *herpesviridae* family and named Epstein - Barr virus (EBV) after discovery by Michael Epstein and Yvonne Barr, who together with Bert Achong in year 1964, which turns out to be the first candidate of human tumor virus (Tselis, 2006).

Several human malignancies are now proved to be related to the oncogenic properties of EBV, including nasopharyngeal carcinoma (NPC) in southern China, including Hong Kong, Hodgkin's Lymphoma (HL), as well as Burkitt's Lymphoma (BL) in Africa and Papua New Guinea (Raab-Traud, 1996; Crawford, 2001; Tsuchiya, 2002; Rickinson and Kieff, 2007). EBV associated malignancies were summarized in table 1.1. EBV is a ubiquitous tumor causing virus, infected more than 90% of the adult in world population and achieves life-long infection in B lymphocytes asymptotically (Crawford, 2001).

Table 1.1: EBV-associated malignancies (Crawford, 2001).

Tumor	Cell of origin	Approximate EBV association	Cofactors/risk factors
Burkitt's lymphoma	Centroblast	African 96% Sporadic 10-70% AIDS 30-40%	Malaria, c-myc deregulation, HIV
B-Lymphoproliferative disease	B Lymphoblast	90%	Immunosuppression, HIV
Hodgkin's Lymphoma	Centrocyte	40-80%	Infectious Mononucleosis (IM)
T-cell lymphoma	T lymphocyte	10%	Chronic IM, immunosuppression
Nasopharyngeal Carcinoma	Squamous epithelial cell	100%	Genetic & dietary factors
Gastric carcinoma	Epithelial cell	10%	Unknown

1.2 EBV virus: Structure and genome map

EBV is a double stranded DNA virus which is belonged to the gammaherpesvirus family (Raab-Traub, 2002) that encodes nearly 100 viral proteins which play the important role in the lytic production of EBV virions and suppression of host immune system (Hiraki *et al.*, 2001). Like other gammaherpesviruses, its linear, double stranded DNA which is wrapped with an inner core followed by a capsid which consists of 162 capsomeres with about 100 nm in diameter; a tegument which surround the nucleocapsid; and an outer envelope with external glycoprotein spikes for binding the virion the cell surface receptor (Figure 1.1) (Epstein *et al.*, 1965; Kieff and Rickinson, 2007). The major EBV capsid is formed by combination of proteins with 155 kDa major capsid protein, 30 kDa minor capsid protein, 18 kDa small capsid protein, 40 kDa minor capsid protein binding protein, and 68 kDa portal protein, all in the expected ratios, based on 12 portal molecules per virion (Kieff and Rickinson, 2007). The EBV tegument is a combination of the 350 kDa large tegument protein (BPLF1), 140 kDa large tegument protein binding protein (BOLF1), 15 kDa myristylated protein (BBLF1), 32 kDa myristylated protein binding protein (BGLF2), 58 kDa capsid associated protein (BVRF1), 58 kDa packaging protein (BGLF1), 27 kDa palmitylated protein (BSRF1), and 47 kDa TS kinase (BGLF4), which are similar to other Herpesvirus teguments. In addition, EBV has a 140 kDa major tegument protein (BNRF1), 19 kDa BLRF2, 72 kDa BRRF2, 54 kDa BDLF2, 42 kDa BKRF4, which are also similar to other gammaherpesvirus. Besides, cellular proteins such as actin, HSP70, Cofilin, β-tubulin, enolase, and Hsp90 also can be found in EBV tegument, which are probably related to cytoplasmic re-envelopment. EBV has a range of envelope glycoproteins which are known as EBV gp350 (BLLF1), gH (BXLF2), gB-N, gB-C, and full-length gB (BALF4), gp42 (BZLF2), gM (BBRF3), gp78 (BILF2), gN (BLRF1), gp150 (BDLF3), and gL (BKRF2), respectively. However, the major envelope protein

gp350/220, which is exclusive to EBV and also played an important role in membrane adhesion, thus it allows the virus enter to host cells (Hutt-Fletcher, 2007; Maruo *et al.*, 2001).

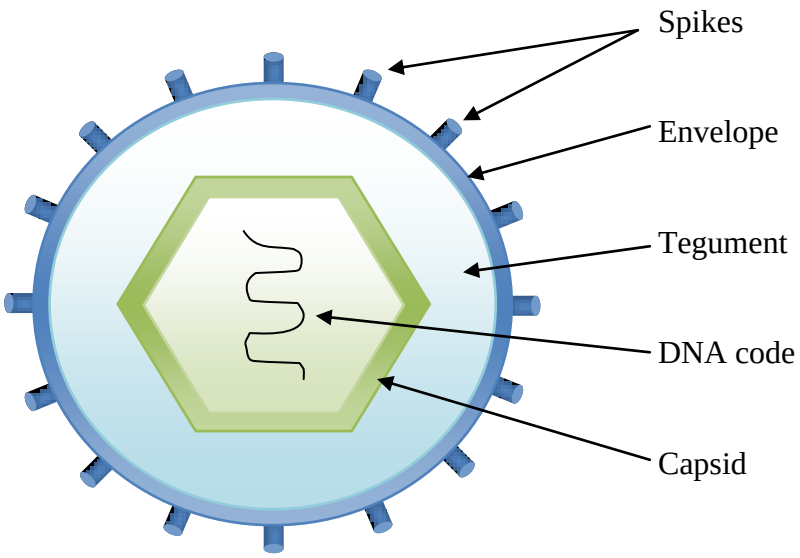

Figure 1.1: Schematic diagram of EBV. The EBV particle consists of four basic components: Inner DNA core, a capsid, an amorphous tegument, and an outer envelope with external glycoproteins spikes.

In 1984, B95.8 prototype laboratory strain of EBV is fully sequenced using *Bam*HI fragment library (Baer *et al.*, 1984). Thus, sequences like genes, open reading frames (ORFs) and promoters is referred to the corresponding fragment. For example, an envelope glycoprotein gp110 which is named as BALF4 due to its ORF (F) on the *Bam*HI A (BA) fragment, and its position is fourth leftward (Figure 1.2).

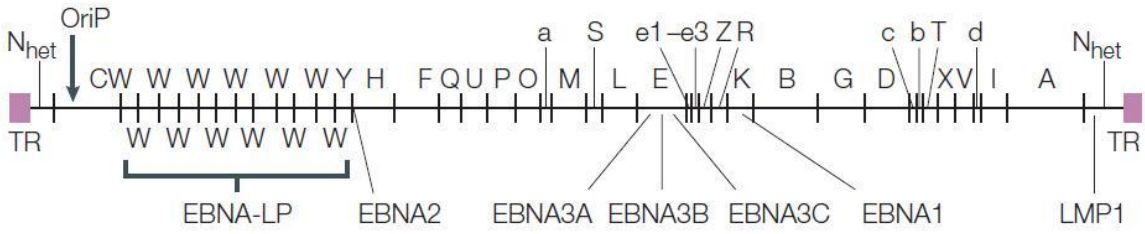

Figure 1.2: *Bam*HI restriction endonuclease map of prototype B95.8 EBV genome. The fragments are named according to their size from the largest to the smallest in alphabetical order. TRs are shown in grey color at both termini of the genome (Young and Rickinson, 2004).

EBV genome consists of approximately 184 kbp DNA in length with 60 percent of guanine or cytosine; 0.5 kbp tandem direct repeats (TR) of the same sequence at both termini; six to 12 tandem reiterations of 3 kbp internal repeats (IR1) and two short and long unique sequence domains (U_S and U_L). After infection, the EBV achieves latent phase in proliferative cells by joining its TRs to produce a circular DNA (episome) (Figure 1.3). Each EBV episome in progeny infected cell generally tends to have a same number of TRs as parental genome. During viral DNA replication, the unique cleavage and joining events of the single viral genome, different numbers of TRs are introduced to the termini of the viral genome. Therefore, the numbers of TRs in latently infected cells are useful to determine the common progenitor (Kieff and Rickinson, 2007).

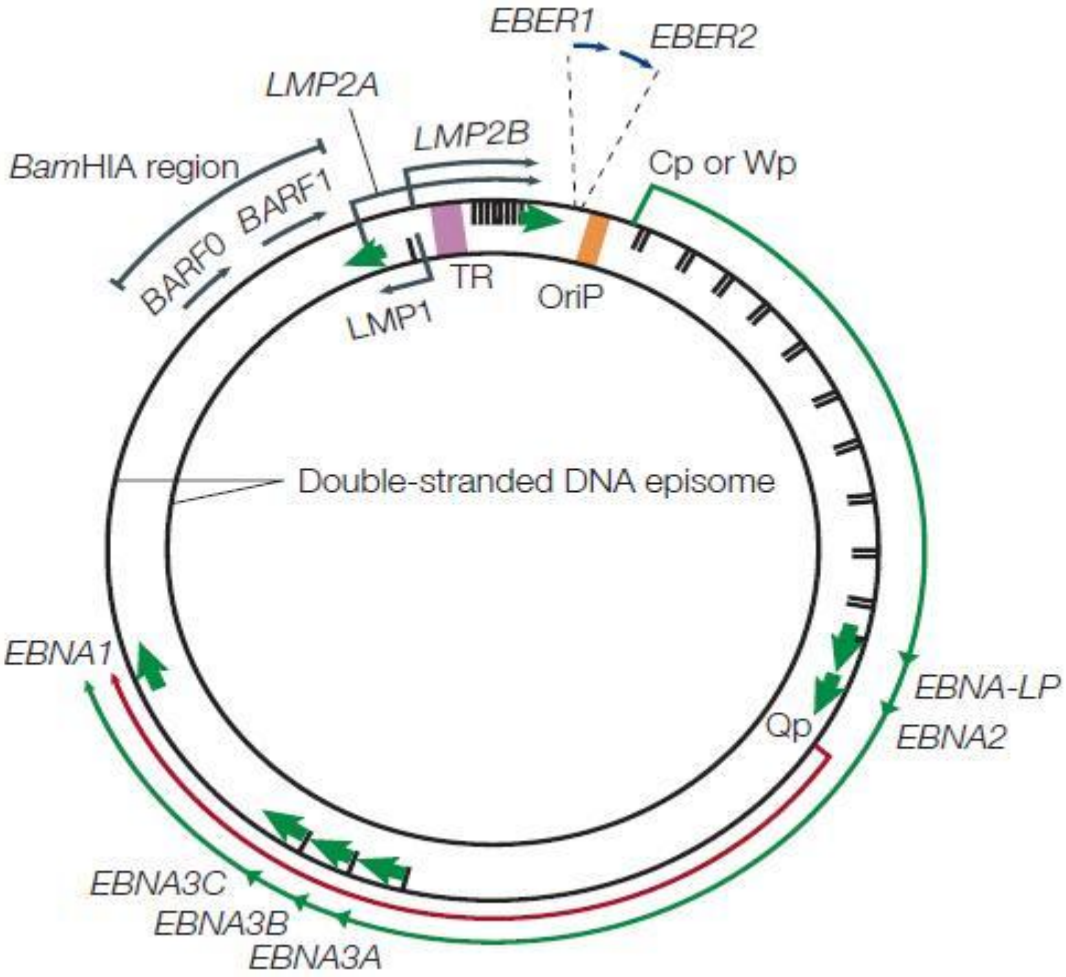

Figure 1.3: EBV episome. The origin of the plasmid replication is *oriP* which is shown in orange. The latent genes expressions are shown in black arrow. The gene transcription initiation is started at promoter C (Cp) or Wp and Qp. (Young and Rickinson, 2004).

Two types of EBV exist in most populations which are designated as type 1 EBV and type 2 EBV. Type 1 is the most common in most population but type 2 is nearly as common as type 1 in equatorial Africa and New Guinea (Kieff and Rickinson, 2007). The differences between these two types are in the gene encoded for EBV nuclear proteins (Dambaugh *et al.*, 1984; Kieff and Rickinson, 2007; Sample *et al.*, 1986).

1.3 Life cycle of EBV

EBV infects almost all individuals in human population in the world and achieves life-long infection in human but the biology of EBV infection *in vivo* (Figure 1.4) is still ambiguous. Primary infection usually occurs during childhood and is asymtopmatic, but in some development countries which may delay until adulthood or adolescence and resulting in appearance of clinical syndrome of infectious mononucleosis (IM) (Crawford *et al.*, 2006; Young and Rickinson, 2004).

EBV is orally transmitted and its primary site of infection is generally at oropharyngeal site. High titers of infectious virus are found in the acute IM patients. Thus, oropharyngeal site is believed to be the location for viral replication because this region can ensure the productions of new virions in oropharyngeal secretions and are easier to transfer to the new susceptible hosts during persistent lytic infection (Young and Rickinson, 2004).

After infection at oropharyngeal site (possibly in the mucosal epithelium), EBV establishes lytic replication and spread out the viral particles throughout the lymphoid tissues. EBV begins with infect B lymphocytes through binding of gp350 to the CD21 receptor on the cell surface and through the binding of gp42 to human leukocyte antigen (HLA) as a second co-receptor. Next, EBV establishes a latent growth-transforming infection (latency III) and majority of these proliferating cells will be removed by the emerging latent-antigen-specific primary-T-cell response but some of the cells escape and achieve latency 0 by downregulating viral antigen expression. Thus, it establishes a stable reservoir of resting viral-genome positive memory B cells. For EBV infected naïve B cells, EBV transforms naïve B cells into memory by mimicking the physiological process of antigen-driven memory B cell development in lymphoid tissues during transit through the germinal center which involving somatic immunoglobulin-gene hypermutation process. However, the process still waiting to be proven due to the

discovery of EBV-infected B cells in tonsils from patients with IM which localize to extrafollicular area but not germinal centres (Young and Rickinson, 2004).

For the persistent infection, the EBV infected cell is recruited into germinal-centre, which follows by different latency activation. However, some of the infected B-cell might reenter the reservoir as memory cells and the remaining cells will differentiate into plasma-cell which move to mucosal site in the oropharynx and activate the lytic cycle. The virions produced might initiate lytic replication in permissive epithelial cells and allow the production of infectious virus in oropharynx. Some virions also might reinfect the naïve and memory B cell to achieve growth-transforming latency III infection and replenish the reservoir but are more likely to be removed by the well established memory T-cell response (Young and Rickinson, 2004).

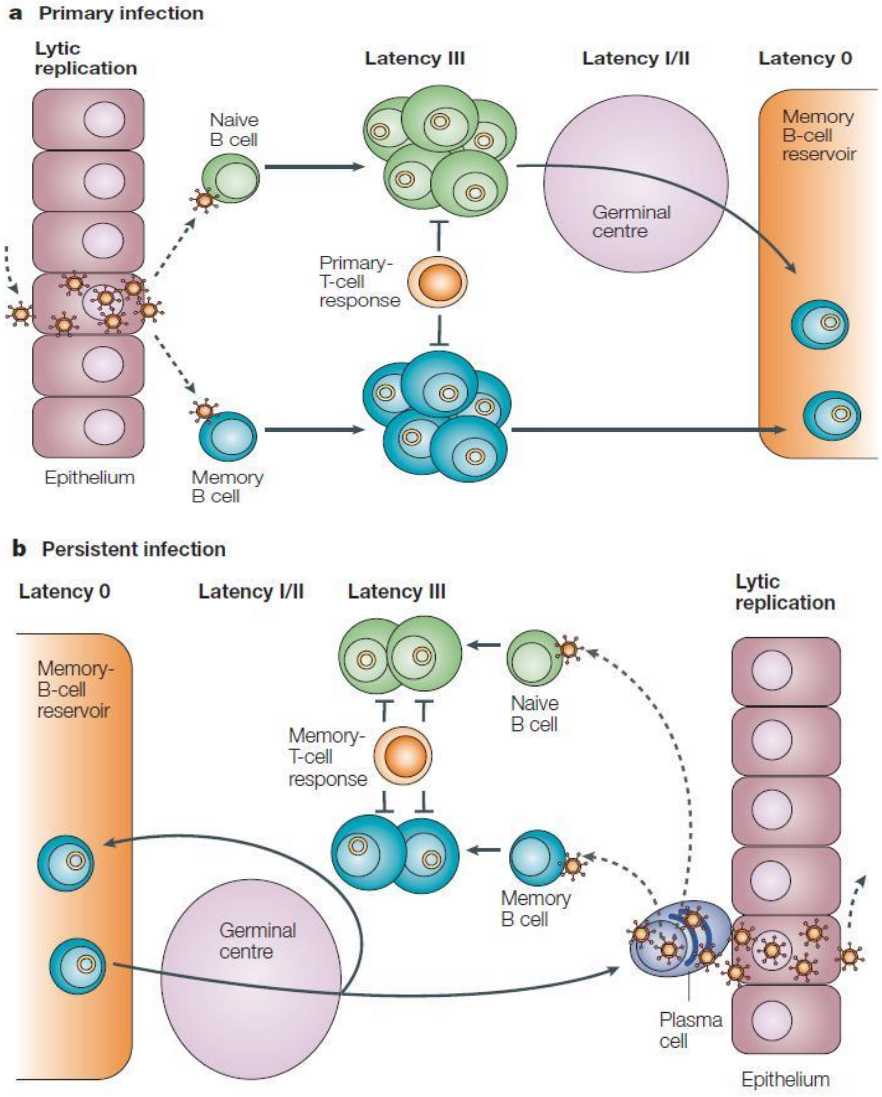

Figure 1.4: Schematic *in vivo* infection of EBV. Diagram showed the (**a**) primary infection and the (**b**) persistent infection of EBV (Young and Rickinson, 2004).

1.4 Stages of EBV infection

1.4.1 Lytic infection

The *in vitro* infection of human resting B cells with EBV, which establishes a Lymphoblastoid cell lines (LCL) and which is widely use as a tool for examining the EBV infection and lytic replication. Lytic infection is usually studied by inducer such as TGF-beta, phorbol esters [12-O-tetradecanoylphorbol-13-acetate (TPA)], butyric acid etc (Kieff and Rickinson, 2007). During the lytic replication, EBV expresses about 90 proteins which are classified as immediate-early, early and late proteins. Immediate-early proteins are expressed just after infection in the presence of protein synthesis inhibitors and early proteins are expressed in the presence of viral DNA synthesis inhibitors, but for late proteins which are not transcribed in the presence of these inhibitors. Immediate-early proteins play a role in regulating the gene expression in the virus; early proteins are important for virus DNA replication, and late proteins are structural proteins which compose the EBV virion (Cohen, 2006).

1.4.2 Latent infection

There are nearly 100 EBV genes in EBV genome, however there are only a few genes expressed in latent infection. The expression of only a few genes in EBV latent infected cells is mainly to reduce recognition of viral antigen by cytotoxic T-cells (Hiraki *et al.*, 2001). There is only a limited set of latent genes including six EBV nuclear antigens (EBNA1,-2,-3A,-3B,-3C and LP); three latent membrane proteins (LMP1, LMP2A and LMP2B); two non coding EBV-encoded RNA (EBER1 and EBER2); and transcripts from the *Bam*HI A region of EBV genome (Hiraki *et al.*, 2001; Cohen, 2006; Kieff and Rickinson, 2007). Latent genes have been shown to have significant transformation ability in EBV infected B-cells (Cohen, 2006).

1.4.2.1 Pattern of EBV latency

In different EBV-related pathological situations, different set of EBV genes are expressed and represent as different latency. There are four types of latency in EBV latent infection (Cohen, 2006; Hiraki *et al.*, 2001; Kieff and Rickinson, 2007; Rowe *et al.*, 1992) (Table 1.2).

Table 1.2: Patterns of EBV latency in different diseases.

Latency	EBNA1	EBNA2	EBNA3	LMP1	LMP2	EBER	Diseases
I	+	-	-	-	-	+	Burkitt's Lymphoma (BL)
II	+	-	-	+	+	+	Hodgkin's Disease, Peripheral T cell Lymphoma & Nasopharyngeal Carcinoma (NPC)
III	+	+	+	+	+	+	Lymphoproliferative disease, X-linked lymphoproliferative Disease & Infectious mononucleosis (IM)
Other	±	-	-	-	+	+	Healthy carrier

Latency I:

In type I latency, only restricted genes such as EBNA-1 and EBER are expressed. This pattern of latency can be found in tissue of Burkitt's lymphoma and gastric carcinoma.

Latency II:

In type II latency, EBNA-1, LMP-1, LMP-2 and EBER are expressed. This type of latency is seen in tissues from Hodgkin's disease, Peripheral T cell Lymphoma and Nasopharyngeal Carcinoma (NPC) patients.

Latency III:

In type III latency, all EBV latent genes are expressed as well as EBERs and some *Bam*HI A RNAs. Latency III is associated with Lymphoproliferative disease, X-linked lymphoproliferative Disease and Infectious mononucleosis (IM).

Other:

In healthy carrier, most of the time LMP-2 and EBERs are expressed but only sometime EBNA is expressed.

1.5 LMP-1

1.5.1 LMP-1 structure

The Epstein–Barr virus latent membrane protein 1 (LMP-1) is an integral membrane protein with a molecular weight of approximately 63 kDa which consists of three major segment or domains. It begins with a 24 amino acids N-terminal cytoplasmic domain which is responsible for maintaining the orientation of the LMP-1 in the cells (Coffin *et al.*, 2001). Next, it follows by a six transmembrane hydrophobic domains with 161 amino acids which responsible in the oligomerization of LMP-1 molecules (Gires *et al.*, 1997) and activation of the small GTPase Cdc42 which initiates the cytoskeletal reorganization (Puls *et al.*, 1999). The last segment is a long cytoplasmic C-terminal with 200 amino acids which is rich in acidic residues (Kieff and Rickinson, 2007); it can be subdivided into three essential C-terminal activation regions (CTARs) 1-3 (Li and Chang, 2003). Hereby, the schematic structure of LMP-1 and its functional domains; as well as their associated proteins are presented in figure 1.5. Each segments of the LMP-1 have its own specific functions and all three domains of the LMP-1 are required for transformation of Rat-1 fibroblasts (Moorthy and Thorley-lawson, 1993).

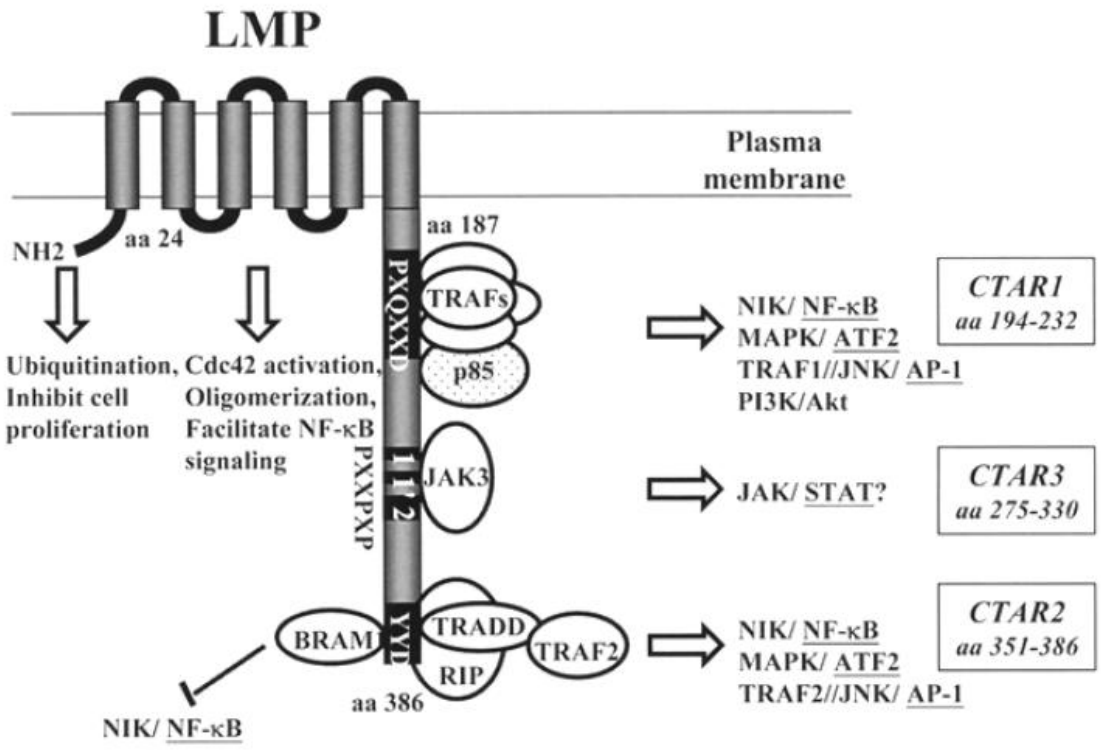

Figure 1.5: Schematic structure of LMP-1 and its functional domains; as well as their associated proteins (Li and Chang, 2003).

1.5.2 Oncogenic roles of LMP-1

LMP-1 is a well known oncoprotein that commonly causes tumorigenesis by activating multiple signaling pathways in a ligand independent manner. The short N-terminus domain of LMP-1 involves in rapid turnover and localization of the protein (Aviel *et al.*, 2000; Coffin *et al.*, 2001). The first 12 a.a. of LMP-1 funtions as a ubiquitin binding site (Aviel *et al.*, 2000).

Furthermore, there are leucine-heptad-like motifs (LLXXLLX) located within the first and sixth transmembrane domains (figure 1.5), involved in self-association and protein-protein interaction (Kaykas *et al.*, 2002). Besides, the transmembrane domains are also responsible for the oligomerization of LMP-1 molecules (Gires *et al.*, 1997) and

activation of the small GTPase Cdc42 which initiates the cytoskeletal reorganization (Puls *et al.*, 1999).

LMP-1 expression in nude mice results in the B-cell lymphoma (Kulwichit *et al.*, 1998). LMP-1 induces a signaling response in cells that mimics a constitutively active form of the B-cell-surface molecule CD40 (Uchida *et al.*, 1999). Besides, LMP-1 protein also has some functional similarity with some other tumour necrosis factor receptor (TNFR) family member like TNFR1 (Mosialos *et al.*, 1995). LMP-1 acts as a constitutively activated form of TNFR in a ligand independent manner (Gires *et al.*, 1997; Kilger et al., 1998). Three functional domains at the C-terminus of LMP-1 that trigger the signaling pathway are named transformation effector site (TES) or C-terminal activator region (CTAR). LMP-1 interacts with many proteins that also interact with TNFR family members, such as TNFR-associated proteins (TRAFs) bind to CTAR1 (Devergne *et al.*, 1996), the TNFR-associated death domain protein (TRADD) (Izumi and Kieff, 1997) and the receptor interacting-protein (RIP) bind to CTAR2 (Izumi *et al.*, 1999) to activate TNFR-associated pathway.

LMP1 activates NFκB pathway through its CTAR1 or CTAR2. CTAR1 interacts with TRAF1, TRAF2, TRAF3 and TRAF5 to activate NFκB pathway (Devergne *et al.*, 1996; Kieff and Rickinson, 2007). There is a similarity in protein sequences among the CTAR1 and the sites in CD40 and CD30 that are essential for NFκB pathway activation (Devergne *et al.*, 1996; Miller *et al.*, 1998). Consequently, mimicry of LMP-1 to the constitutively activated TNFR can have a similar effect as CD40 on transformation of B-lymphocyte and gene activation (Devergne *et al.*, 1996).

LMP-l C-terminus contains a proline rich region within 33-bp repeat in between CTAR1 and CTAR2 which mediates activation of JAK3. Janus kinase (JAK) mediated

activation of signal transducer activators of transcription (STAT) involves in diverse cellular processes such as proliferation, apoptosis and cell marker expression. Currently, this proline rich region has been named as CTAR3. LMP-1 activates PI3K/Akt with its CTAR1 region which is important to promote cell survival and induce actin filament remodeling (Dawson *et al.*, 2003).

LMP-1 also activates mitogen-activated protein kinase (MAPK) pathway such as extracellular-regulated kinase (ERK), p38 and c-Jun N-terminal kinase (JNK)/ Stress-activated protein kinase (SAPK). MAPK plays an important role in coordinating cellular activities from gene expression, mitosis, and metabolism to survival, apoptosis and differentiation (Roux *et al.*, 2004).

ERK-MAPK is activated directly via CTAR1 of LMP-1 and which is important in LMP1-induced transformation of Rat-1 fibroblasts (Mainou *et al.*, 2007). Indeed, a recent study has identified LMP-1 induced cell motility via ERK-MAPK activation, as LMP-1 expressing cells exhibit high rate of haplotactic migration compared to non-LMP1 expressing cells (Dawson *et al.*, 2008). LMP-1 activated ERK-MAPK contribute to the oncogenicity through its ability to promote cell migration and invasive properties.

In addition, recent study has demonstrated that LMP-1 activated p38/MAPK acts as an autoregulatory loop in LMP-1 upregulation which may be important for LMP-1 induced cell survival (Johansson *et al.*, 2010).

1.5.3 Paradox effects of LMP-1

LMP-1 is a well-known classical oncoprotein and is proved to transform rodent fibroblast and promote tumorigenesis (Wang *et al.*, 1985). On the contrary, studies on LMP-1 suggest that overexpression of LMP-1 exhibits toxicity to the cells (Hammerschmidt *et al.*, 1989). According to Le Clorennec *et al.* (2008), LMP-1 induces type II ligand independent autoactivation of CD95/Fas mediated Caspase-8 dependent apoptosis. This is thought to cause the cytotoxicity effect of LMP-1. On the other hand, LMP-1 may interrupt cell proliferation when introduced into B cell lines. Overexpression of LMP-1 impairs the *in vitro* growth, clonability and tumerigenicity in B cell lines (Torsteinsdóttir *et al.*, 1989; Cuomo *et al.*, 1992). According to Floettmann *et al*. (1996), induction of LMP-1 expression completely inhibits cell proliferation for 4-5 days by the induction of G_2 or M phase cell cycle arrest. This phenomenon of cell stop growing is also known as cytostasis. The cells that stop growing usually will be followed by either cytotoxicity and cell killing or escape from the stasis and repopulation (Rixe and Fojo, 2007). However, how LMP-1 causes cytostasis and arrests cells at the G_2/M phase are poorly understood. Previous studies show that mRNA and protein of p53 are overexpressed in NPC and the consequences of this accumulation may be related to EBV infection (Deng *et al.*, 2003; Gulley *et al.*, 1998; Murono *et al.*, 1999). A recent study showed that LMP-1 induced cell cycle arrest in HNE1 cells which are NPC derived cell line through activation of NFκB (Deng *et al.*, 2003). However, the exact mechanism of LMP-1 induced cell cycle arrest remains unclear.

1.6 LMP-2

1.6.1 LMP-2 structure

Latent membrane protein 2 can be divided into 2 subtypes, LMP-2A and LMP-2B or TP-1 and TP-2 (Laux *et al.*, 1989). The structures of these two proteins are similar; both have 12 transmembrane domains and a 27-amino-acid cytoplasmic carboxyl terminus (Young and Rickinson, 2004). LMP-2A and LMP-2B are transcribed independently by 2 different promoter sequences which are separated by 3kb across the fused terminal repeat of EBV genome (Sample *et al.*, 1989). The mRNA transcripts of LMP-2A and LMP-2B are 2.3kb and 2.0kb respectively. LMP-2B shares the same bidirectional promoter with LMP-1 (Laux *et al.*, 1989). The sizes of the proteins are predicted to be 50 kDa for LMP-2A and 40 kDa for LMP-2B (Sample *et al.*, 1989). These 2 proteins share 8 common 3'exons, but both have a unique 5' exon. The eight common exons encode 12 transmembrane spanning domains and the C-terminal of LMP-2A and LMP-2B.

The unique LMP-2A N-terminal consists of 119 amino acids hydrophilic cytoplasmic domains which mainly responsible for LMP-2A functions (Sample *et al.*, 1989). N-terminal of LMP-2A contains an immunoreceptor tyrosine-based activation motif (ITAM) which consists of paired tyrosine and leucine residues and modulates signal transduction of the BCR and the T-cell receptor (TCR) (Fruehling and Longnecker, 1997). LMP-2A alters normal BCR signal transduction in B cells through reducing levels of Lyn and blocking of tyrosine phosphorylation as well as calcium mobilization following BCR cross-linking (Rovedo and Longnecker, 2007). This association accounts for the ability of the LMP2A ITAM to block BCR signal transduction, thereby preventing the induction of lytic EBV replication in B cells (Miller *et al.*, 1995). LMP-2 C-terminal is a cysteine rich sites and its 12 transmembrane domains contain clustering signal which are believed to be essential for LMP-2

functions (Katzman and Longnecker, 2004; Matskova *et al.*, 2001). Hereby, the schematic structure of LMP-2A and its functional domains; as well as their associated proteins are presented in figure 1.6.

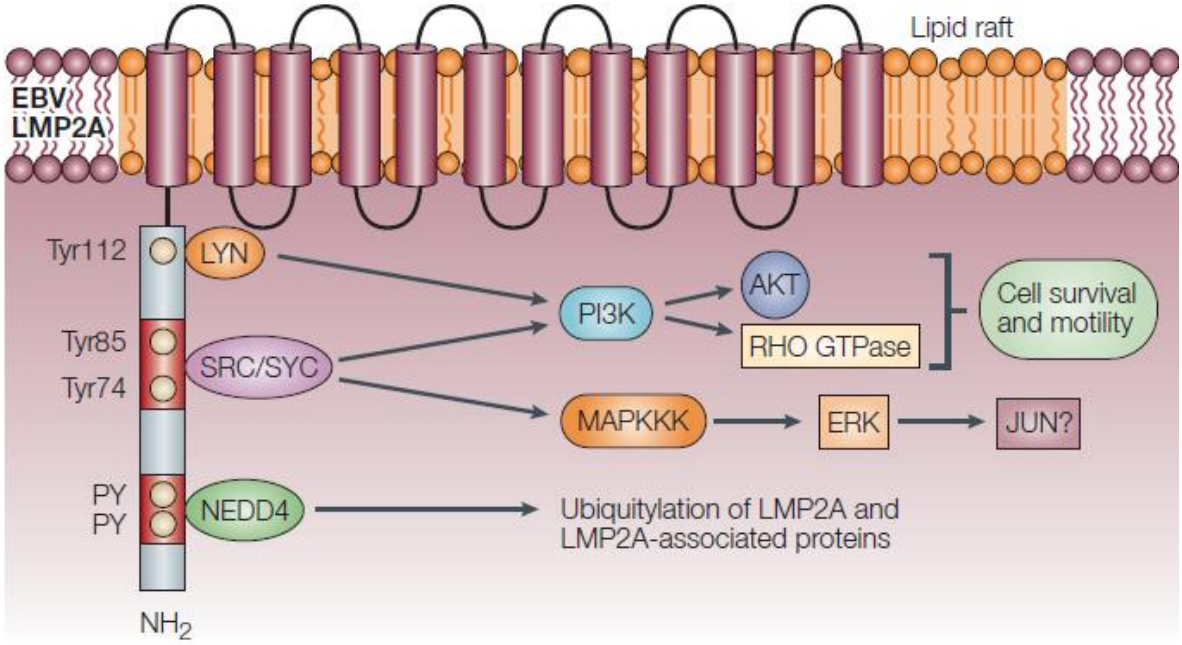

Figure 1.6: Schematic structure of LMP-2A and its functional domains; as well as their associated proteins. (Young and Rickinson, 2004).

1.6.2 Roles of LMP-2

In contrast to LMP-1, much less is known about LMP-2A/2B functions. The 12 hydrophobic transmembrane domains of LMP-2 involve in localization of LMP-2 to the cell plasma membrane (Kieff and Rickinson, 2007). The cysteine rich C-terminal of LMP-2A is palmitoylated which is a process of post-translational modification, although the function is unnecessary for normal LMP-2A function (Higuchi *et al.*, 2001; Katzman and Longnecker, 2004; Matskova *et al.*, 2001). LMP-2 associates with lipid raft which may be important for initial contact of src family tyrosine kinases (Higuchi *et al.*, 2001). Deletion studies prove that transmembrane domains and C-terminal domain are dispendable for *in vitro* B lymphocyte infection and growth transformation (Longnecker *et al.*, 1993a; Longnecker *et al.*, 1993b).

LMP-2A N-terminal contains eight tyrosine residues and three of which are essential for BCR signaling blockade via phosphorylation (Fruehling *et al.*, 1996; Fruehling *et al.*, 1998; Fruehling and Longnecker, 1997). A mutation study shows that ITAM motif of LMP2A increases cellular phosphorylation and thus involves in signal transduction (Scholle *et al.*, 2001).

LMP-2A interrupts BCR signaling and functions. A study shows that LMP-2A negatively regulates BCR signal through excluding it out from lipid raft and then targeting Src family tyrosine kinases (Lyn and Syk) for ubiquitin-dependant degradation (Dykstra *et al.*, 2001; Ikeda *et al.*, 2000). LMP-2A avoids protein tyrosine kinases (PTKs) from binding to BCR and thus prevents its activation. Moreover, LMP-2A blocks BCR activated calcium mobilization, tyrosine phoshorylation and gene transcriptional activation thus prevents EBV lytic activation and promotes cell growth of B-cells (Fruehling *et al.*, 1996; Fruehling *et al.*, 1998; Fruehling and Longnecker, 1997; Miller *et al.*, 1995; Winberg *et al.*, 2000).

LMP-2A exploits ubiquitin-protein ligase for degradation of LMP-2A and its associated proteins Lyn for ubiquitination thus for internalization and degradation (Ikeda *et al.*, 2000). Ubiquitin dependent degradation is an important process for regulating LMP-2A activity. Itchy, a Nedd ubiquitin ligase, binds and regulates LMP-2A activity (Ikeda *et al.*, 2000). When tyrosine residues are not phosphorylated, ubiquitin ligase will bind to the proline PY motifs of LMP-2A with its WW domains thus activates degradation (Ikeda *et al.*, 2000). LMP-2A employs ubiquitin ligase for removing the excess LMP-2A. Recently, it has been proven that these processes involve in regulating Notch and Wnt pathway (Portis *et al.*, 2004). In addition, LMP-2A might exploit Notch and Wnt pathway to regulate its function and maintain B-cell differentiation, activation and survival (Portis *et al.*, 2004).

In a recombinant EBV study, LMP-2A has been shown to down-regulate NFκB and STAT pathways in human carcinoma cell lines. There are down-regulation of NFκB and STAT3 activity due to LMP-2A suppression of LMP-1 expression thus reduces IL-6 expression but not in LMP-2A negative cells (Stewart *et al.*, 2004). The down regulation of NFκB and STAT activities are likely to contribute to diverse carcinoma and EBV-associated malignancies. For example, suppression of NFκB is able to induce epidermal hyperplasia which will contribute to the development of NPC (Seitz *et al.*, 1998).

LMP-2A activates PI3K/Akt pathway thus enhances cell growth and anti-apoptotic effect in B-cells, lymphoma, gastric carcinoma and epithelial cells (Portis and Longnecker, 2004; Scholle *et al.*, 2000; Fukuda *et al.*, 2001). Inhibition of PI3K/Akt triggers apoptosis in LMP-2A expressing Burkitt's lymphoma and gastric carcinoma cell lines (Fukuda and Longnecker 2004). LMP-2A has been shown to activate PI3K/Akt via inhibition of TGF-β1 to induce caspase activity (Fukuda and Longnecker 2004; Chen *et al.*, 1998). Activation of PI3K/Akt is important in the development and progression of EBV-associated malignancy. In addition, activation of Ras/PI3K/Akt by LMP-2 will lead to unique genetic changes thus contributes to aggressive tumorigenicity (Scholle *et al.*, 2000).

LMP-2A is shown to activate MAPK pathway in EBV-infected cell lines *in vitro* (Chen *et al.*, 2002; Portis and Longnecker, 2003; Panousis and Rowe, 1997). A transgenic mice study of LMP-2A shows that an activation of ERK/MAPK and PI3K/Akt to enhance proliferation and cell survival (Anderson and Longnecker, 2008). Another report also claims that LMP-2A activates ERK/MAPK and JNK/MAPK in NPC cell lines and thus promotes cell migration and invasion (Chen *et al.*, 2002). Moreover, studies in Syk-null DT40 chicken B cells show that Syk is essential for activation of the ERK1 and JNK1 (Jiang *et al.*, 1998). Although many studies prove that

LMP-2A activates MAPK pathway but the exact mechanisms still need to be studied. Although there are many research studied on EBV LMP-2A but little is currently known about the function of LMP-2B. As LMP-2B lacks of N-terminal of LMP-2A, LMP-2B is unlikely to share similar function as LMP-2A. Although many researches were conducted to study LMP-2 functions, but there are lack of report for involvement of LMP-2 in cell cycle arrest.

1.7 Interaction of LMP-1, LMP-2A and LMP2B

EBV-encoded LMP-1, LMP-2A and LMP-2B are frequently co-expressed in many EBV-associated malignancies such as NPC, HD and immunoblastic lymphoma (Brooks *et al.*, 1992; Deacon *et al.*, 1993; Niedobitek *et al.*, 1997; Rickinson and Kieff, 2007). These proteins share some similarity in their protein structure and all involve in regulating signaling pathway. Since these proteins share some common identity therefore they might co-localize to a same cellular compartment (Longnecker and Kieff, 1990; Lynch *et al.*, 2002; Rovedo and Longnecker, 2007). Due to their co-localization and pathways inducing ability, there might be some form of interaction between these proteins and combinatorial roles played by these LM proteins.

A study shows that LMP-2B modulates the activity of LMP-2A by preventing it from phosphorylation. LMP-2B contains similar C-terminal domain of LMP-2A, therefore it may prevent homodimerization of LMP-2A and subsequently inhibit its phosphorylation (Rovedo and Longnecker, 2007). LMP-2B discrupts LMP-2A homodimers and recruits deubiquitinating enzyme which will in turn restore normal BCR signaling. Therefore, LMP-2B might act as a "rheostat" for LMP-2A and BCR signaling (Rechsteiner et al., 2008).

In addition, another study shows that LMP-2A augments the signaling of LMP-1 to enhance NFκB and AP-1 pathway through extending LMP-1 turnover. Using a

coexpression study, LMP-2A has proven to modulate LMP-1 activities and that is not the consequence of direct interactions between the two proteins. Furthermore, LMP-2A, but not LMP-2B, modulates LMP-1 signalling pathway. LMP-2A increases the expression level of LMP-1 as well as increases its half-life (Dawson *et al.*, 2001).

1.8 Cell cycle regulation

The life cycle of a dividing cells can be divided into 4 stages; cell division occurs during mitosis (M); DNA and chromosomal replication phase (S) and gap 1 (G_1) and gap 2 (G_2) which separate the S and M phases. Additionally, during G_1 phase, cells can exit cell cycle and enter (quiescent) G_0 phase (Garrett, 2001).

Movement of each phase of cell cycle is controlled or regulated by a number of positions known as checkpoint. These checkpoints monitor cellular environment and make sure appropriate conditions have been fulfilled before it may enter each phase of cell cycle. These phases are controlled or monitored by a group of kinase complexes which consist of cyclin and cyclin dependent kinases (cdk) (Davy and Doorbar, 2007). These complexes are in turn regulated by multiple pathways that response to external stimuli and internal conditions of cells. A major function of these checkpoints is to maintain genome integrity throughout the cell cycle. The first of these checkpoints occurs at G_1/S phase transition that is mainly to scan for DNA damage. The cells may also arrest in S phase due to DNA damage or incomplete DNA replication. Next, G_2/M checkpoint, which monitors DNA replication and again screens for DNA damage just like G_1/S checkpoint. This followed by spindle checkpoint, which is to make sure functional mitotic spindle has been formed correctly (Garrett, 2001). Besides cyclin and cdk, there are groups of gene known as cell cycle regulatory genes that can regulate normal cell cycle such as *p53, p21, reprimo, Gadd45* and *14-3-3σ*. Uncontrolled gene

expression of these regulators can interrupt normal cell cycle (Chang *et al.*, 2003; Hata *et al.*, 2005; Hermeking *et al.*, 1997; Ohki *et al.*, 2000; Wang and El-Deiry, 2006).

A variety of viruses have been proved to be associated with G_2/M arrest, including DNA viruses, RNA viruses and retroviruses which hijack a normal cell cycle for their life cycle but the methods used by these viruses to achieved this arrest appear to be very diverse (Davy and Doorbar, 2007). Further study may be required to determine the mechanism of this phenomenon.

1.9 Objectives and Aims

The aim of this project was to investigate the following hypothesis:

"The interactions of LMP-1, LMP-2A and LMP-2B will cause changes in mRNA expression of cell cycle regulatory genes and interrupt normal cell cycle progression when over-express these latent membrane proteins thus cause physiological changes to the cells."

In order to test this hypothesis, three main objectives were undertaken:

1. To clone and express LMP-1, LMP-2A and LMP-2B in single and co-expression manner in HEK293T cell line.
2. To examine the effects of LMP-1, LMP-2A and LMP-2B in mRNA expression of cell cycle genes.
3. To examine the correlation of LMP-1, LMP-2A and LMP-2B with cell cycle progression.

2.1Cell lines and cell cultures

2.1.1 Maintenance of cell lines

Materials:

1) Roswell Park Memorial Institute Medium (RPMI-1640) (GIBCO/BRL, Grand Island, NY)
2) Dulbecco's Modified Eagle Medium (DMEM) (GIBCO/BRL, Grand Island, NY)
3) Fetal bovine serum (FBS) (GIBCO/BRL, Grand Island, NY)
4) Penicillin/streptomycin (10000 IU/ml penicillin and 10000 µg/ml streptomycin) (GIBCO/BRL, Grand Island, NY).
5) TrypLE^{TM} Express (stable Trypsin Replacement (GIBCO/BRL, Grand Island, NY)
6) Dimethyl Sulfoxide (DMSO) (Calbiochem/Merck KGaA Darmstadt, Germany)
7) 0.4% (w/v) trypan blue (Sigma, USA)
8) Phosphate Buffered Saline (PBS): 58 mM Na_2HPO_4, 17 mM NaH_2PO_4, 68 mM NaCl

Methods:

B95.8 and HEK293T line obtained from ATCC® , USA (VR-1492™ and CRL-11268™ respectively). B95.8 cell line was established by infection of EBV to marmoset monkey (*Saguinus Oedipus*) blood leukocytes (Miller and Lipman, 1973). B95.8 cell line was used as a source for LMP-1, LMP-2A and LMP-2B genes. B95.8 was maintained in RPMI-1640 supplemented with 10% (v/v) FBS, 100 IU/ml penicillin and 100 µg/ml streptomycin.

HEK293T cells which are widely used in molecular biology research for many years due to its viability and high transfection efficiency was maintained in DMEM supplemented with 10% (v/v) FBS, 100IU/ml penicillin and 100 µg/ml streptomycin.

All cells were maintained in an incubator (NuAire Inc., USA) at 37℃ in 5% CO_2 and sub-cultured in 1:10 ratio in the same condition unless for specific application.

2.1.2 Cryopreservation and reviving cells

To store cells for long term, viable cells at the log phase of growth were trypsinized and centrifuged at 800 rpm for 5 minutes and resuspended in cryopreservation medium (RPMI-1640 supplemented with 20% FBS and 10% DMSO) at about $1x10^6$ cells/ml. One ml of cryopreservation medium was aliquoted to each cryovial (Nalgene). The vials were placed in a NALGENE^{TM} Cryo 1℃ freezing container and stored at -20℃ for 3 hours before freezing at -80℃ for overnight. It was transferred into the liquid nitrogen tank on the next day.

For reviving cells, a vial of cells was thawed immediately in a 37℃ water-bath. Eight ml of RPMI-1640 was used to wash the suspension cells in a 15 ml falcon tube followed by centrifugation (Sorvall RT6000) at 800 rpm for 5 minutes. The resulting pellet was resuspended in 5ml of RPMI-1640 supplemented with 20% FBS and transferred to T-25 culture flask (Nunclon, Denmark). The cells were incubated at 37℃ in a 5% CO_2 environment. The growth rate of the culture was checked daily using inverted microscope (CK Olympus, Japan).

2.1.3 Counting cells

Cell viability and concentration were determined using trypan blue incorporation. Fifty µl of cell suspension was mixed with 450 µl of 0.4% (w/v) trypan blue. Ten µl of the mixture was added to the chamber on haemacytometer. The unstained cells were counted by light microscope. The concentration of cells was determined with the formula below:

Cell concentration (cells/ml) = n cells (counted viable cells) × 10^4 × dilution factor

2.2 RNA extraction

Materials

1) RNeasy Protect Kit® (Qiagen, Germany)
2) β-mercaptoethanol (Sigma, USA)
3) QIAshredder® Homogenizer (Qiagen, Germany)
4) Agarose gel, SeaKem® LE (Cambrex, USA)
5) Ethidium bromide (Sigma, USA)

Methods:

RNA was extracted from cells that have been stabilized in RNAlater® using the RNeasy Protect Kit® according to manufacturer's protocol. Briefly, cells were lysed in 600 µl lysis buffer (Buffer RLT + 1% (v/v) β-mercaptoethanol) and the lysates were homogenized through QIAshredder® spin-column homogenizer at 13000 rpm (Sigma 1-14 minicentrifuge, USA) for 2 minutes. Then, 1 volume of 70% ethanol (600 µl) was added to the flow-through and mixed. The flow-through was then transferred to an RNeasy mini column and centrifuged at 11000 rpm for 15 seconds. The flow-through was discarded followed by washing with buffer RW1 (700 µl) and twice with RPE (500 µl). The washing tubes were centrifuged at 11000 rpm for 15 seconds except the second wash of RPE at 13000 rpm for 2 minutes and the flow-through was discarded after each step. Finally, the RNeasy column was placed in a new tube and the RNA was eluted from the membrane with 50 µl of RNase-free water. The quality of the extracted RNA was checked using1% agarose gel stained with ethidium bromide. The concentrations of the RNA were quantified using spectrophotometer (Shimazu, Japan).

2.3 Gene Cloning

2.3.1 cDNA Synthesis

Materials:

1) SuperScript^{TM} III First-Strand Synthesis System for RT-PCR (Invitrogen, USA)

Methods:

Total RNA from B95.8 cells was reverse transcribed (RT) to generate cDNA of *LMP-1*, *LMP-2A* and *LMP-2B*. Reverse transcription was performed using SuperScript^{TM} III First-Strand Synthesis System following the manufacturer's protocol. Briefly, 5 µg of total RNA was mixed with 1 µl oligo$(dT)_{20}$ (50 µM) and 1 µl of 10 mM dNTPs (2.5 mM each) in a final volume of 10 µl. The mixture was then incubated at 65℃ for 5 minutes and then placed on ice for 1 minute. Next, 10 µl of cDNA reaction mix [2 µl of 10×RT buffer, 4 µl of 25 mM $MgCl_2$, 2 µl of 0.1 M DTT, 1 µl of RNaseOUT^{TM} (40 U/µl) and 1 µl of SuperScript^{TM} III RT (200 U/µl)] was added to the mixture and incubated for 50 minutes at 50℃. The reaction was terminated at 85℃ for 5 minutes. Finally, 1 µl of RNase H was added to the reaction and incubated at 37℃ for 20 minutes to remove the RNA template.

2.3.2 Polymerase Chain Reaction (PCR)

Materials:

1) *i-pfu* DNA polymerase (Intron Biotechnology, Seonggnam, Korea).

Methods:

All PCRs in this study were performed on an ABI PCR 2720 thermocycler (Applied Biosystem, USA) using gene specific primers as listed in Table 2.1.

Table 2.1: Genes-specific primers used in PCR.

Name	Sequences 5' - 3'	GC%	bases	Basic Tm (℃)
LMP1-F	***CACC**ATGGAACACGACCTTGAGAGG	56	25	61
LMP1-RS	****TTA**GTCATAGTAGCTTAGCTGAACTGGGCC	46.67	30	62
LMP1-R	GTCATAGTAGCTTAGCTGAACTGGGCC	51.85	27	61
LMP2A-F	***CACC**ATGGGGTCCCTAGAAATG	54.55	22	57
LMP2A-RS	****TTA**TACAGTGTTGCGATATGGGGTCG	46.15	26	58
LMP2A-R	TACAGTGTTGCGATATGGGGTC	50	22	55
LMP2B-F	***CACC**ATGAATCCAGTATGCCTGCCTG	53.85	26	61
LMP2B-RS	****TTA**TACAGTGTTGCGATATGGGGTCGGTGG	50	30	63
LMP2B-R	TACAGTGTTGCGATATGGGGTCGGTG	53.85	26	61

*The bold and underlined residues represent the specific sequences require for directional cloning.

**The bold and underlined residues represent the stop codon.

The reactions were carried out in a total volume of 15 µl each reaction under optimized parameter as in Table 2.2.

Table 2.2: Components of PCR mixture and amplification conditions.

Components	Concentration of stock	Working concentration	One PCR reaction (µl)	Amplification Conditions
Ultra pure water (deionized water)	-	-	9.4	Denaturation (1×) 94℃ – 2 minutes Denaturation (40×) 94℃ – 20 seconds Annealing Tm℃ – 15 seconds Extension 72℃ – 30 seconds Final extension (1×) 72℃ – 5 minutes
10X *i*-pfu Buffer (Intron, Korea)	10X	1X	1.5	
dNTPs (Intron, Korea)	10mM	0.8mM	1.2	
Forward primer	10µM	0.5µM	0.75	
Reverse primer	10µM	0.5µM	0.75	
i-pfu DNA polymerase (Intron, Korea)	2.5U/µl	1U/15µl	0.4	
Template cDNA	-	-	1	
Total Volume			**15**	

2.3.3 Gene insertion to pcDNA3.1 vector

Materials:

1) pcDNA3.1 Directional TOPO cloning Kit (Invitrogen, USA)
2) One Shot TOP10 chemically competent *E.coli* (Invitrogen, USA)
3) LB agar: 1% (w/v) tryptone (BD, USA), 0.5% (w/v) yeast extract (BD, USA), 1% (w/v) NaCl (Merck, Germany), 1% (w/v) Bacto Agar powder (BD, USA)
4) SOC medium: 2% (w/v) tryptone, 0.5% (w/v) yeast extract, 0.05% (w/v) NaCl, 0.5% $MgSO_4.7H_2O$ and 20 mM glucose
5) Ampicillin (Bioshop, Canada)

Methods:

The *LMP-1*, *LMP-2A* and *LMP-2B* amplified products were cloned into pcDNA™3.1 Directional TOPO® vector. The pcDNA3.1 Directional TOPO expression vector is a linearized vector which provides greater than 90% efficiency of directional cloning of blunt-end PCR products into vector. In this system, PCR products are directionally cloned by adding CACC to the forward primer. The overhang with GTGG from the vector will invade to the double stranded PCR products and displacing the GTGG from the PCR products (Figure 2.1). This vector employs topoisomerase I

activity to efficiently clone the PCR product into vector (Shuman, 1991 and Shuman, 1994) thus forming a circular plasmid. The cloning site is located downstream of CMV promoter. There is no specific antibody for LMP-2B therefore for tagging of LMP-2B protein with V5 epitope and polyhistidine region, the termination codon was removed from the PCR primer.

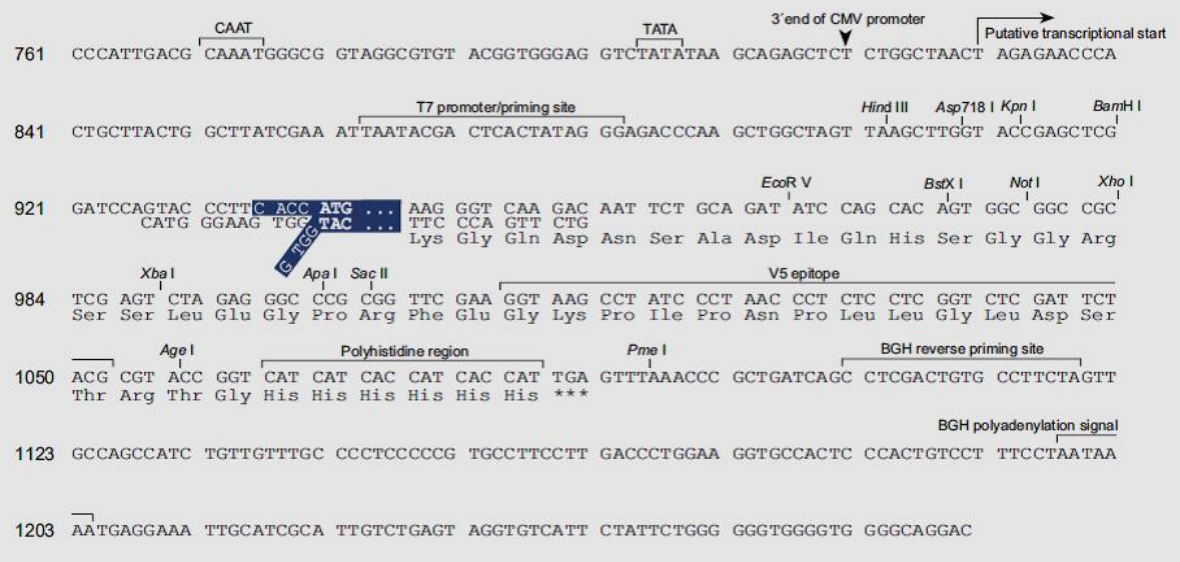

Figure 2.1: Multiple-cloning site (MCS) and Insertion site of pcDNA3.1 directional cloning vector.

Briefly, 4 µl of PCR product was mixed with 1 µl of salt solution (0.2 M NaCl and 0.01 M $MgCl_2$) and 1 µl of vector (0.5 µg/µl). The cloning reaction mixture was left to incubate at room temperature for 5 minutes. Next, 2 µl of the mixture was added to the TOP10 chemically competent *E. coli*. Then, it underwent heat shock treatment at 42℃ for 30 seconds to transform the vector which carried the desired gene fragment into competent cells. Immediately after that, 250 µl of SOC medium was added to the transformants and incubated at 37℃ for 1 hour. The heat shock method was described elsewhere (Sambrook *et al.*, 1989). The *E.coli* tranformants were spread onto ampicillin selection plate (LB agar + 100 µg/ml ampicillin) and incubated at 37℃ for 16 hours.

2.3.4 Boiling PCR for transformants

Materials:

1) *Taq* DNA polymerase (Finnzymes, Finland)
2) Glycerol (Unilab, Australia)

Methods:

Single colony was selected for boiling PCR assessments to verify insertion of the gene and orientation of inserted gene in the vector. A single colony was picked and added into the PCR master mix containing the components as mentioned in Section 2.3.2 except for the DNA polymerase which was replaced with *Taq* DNA polymerase. In order to determine the correct insert and orientation of the genes, amplification was performed with the T7 forward primer (5' TAATACGACTCACTATAGGG 3') and insert-specific reverse primer (Table 2.1). The expected size of the amplicon will be 76-bp longer when T7 forward primer was used. The colony with correct insert and orientation was cultured overnight and glycerol stocks were prepared (LB + 15% (v/v) glycerol) then stored at -80℃.

2.3.5 Plasmid Extraction

Materials:

1) QIAprep® Spin Miniprep Kit (Qiagen, Germany)

Methods:

Plasmid from the *E.coli* transformants was extracted using QIAprep® Spin Miniprep Kit according to manufacturer's protocol. Briefly, overnight 5 ml bacterial culture was harvested by centrifugation at 4000 rpm (Sigma 3-16PK) for 10 minutes. The bacterial pellet was resuspended with 250 µl of Buffer P1 and lysed with 250 µl of Buffer P2. The debris was precipitated with 350 µl of Buffer N3 and pelleted via centrifugation at 13000 rpm for 10 minutes. The supernatant was transferred into a spin column followed by centrifugation at 13000 rpm for 1 minute and the flow-through was discarded. The column was washed with 750 µl of Buffer PE and plasmid was eluted with 50 µl of Milli-Q water. Finally, the concentration and purity of plasmid DNA was checked through spectrophotometer and agarose gel electrophoresis.

2.3.6 Sequencing of Inserted Genes

The pcDNA3.1 recombinant vectors with correct insertions were sent to Advance Interactive Technologies (AIT) Pte. Ltd., Singapore for sequencing using T7 and gene internal primers (Table 2.3). Then, the sequences were compared and aligned with Epstein-Barr virus (EBV) genome, strain B95-8 from NCBI (Gene Bank Acc. No.: V01555) using Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0 and Megalign (DNASTAR).

Table 2.3: List of sequencing primers.

Name	Sequences (5'-3')	EBV coordinate	bases	Basic Tm (℃)
T7	TAATACGACTCACTATAGGG	-	20	48
S2-LMP1	TGCTGTTCATCTTCGGGTGC	169068-169049	20	54
S3-LMP1	CTCTGCTCTCAAAACCTAGG	168612-168593	20	52
S2-LMP2A	ACATATACGAAGAAGCGGGC	166889-166908	20	52
S3-LMP2A	GACGCTGTTTTGCAGCTGAG	647-666	20	54
S4-LMP2A	CTGCATGTTATTACTGATTGTCGC	1287-1310	24	54
S2-LMP2B	TGCTCCTGATACTAGCGTAC	555-574	20	52
S3-LMP2B	ACTTCAAGAGTTTAAGCAGCAC	1161-1182	22	51
BGH reverse	TAGAAGGCACAGTCGAGG	-	18	50

2.4 Expression of LMP-1, LMP-2A and LMP-2B

Materials:

1) Fugene® HD Transfection Reagent (Roche, Switzerland)
2) Opti-MEM® (GIBCO/BRL, Grand Island, NY)
3) pECFP-N1 (Clontech, USA)
4) DMEM-10% FBS medium (as mentioned in Section 2.1)

Methods:

LMP-1, LMP-2A and LMP-2B expressing cells were established by transfecting the cells with pcDNA3.1-LMP-1, pcDNA3.1-LMP-2A and pcDNA3.1-LMP-2B-V5 constructs. The fluorescence protein expression vector, pcDNA3.1-CFP, was used to monitor the transfection efficiencies. Transfection of the epithelial cells was performed by Fugene® HD Transfection Reagent following manufacturer's protocol. Briefly, 1×10^6 cells were seeded in T-25 cell culture flask in DMEM-10 with no antibiotic and incubated for 24 hours at 37℃ with 5% CO_2. For transfection, 6 µg of plasmid (3 µg of pcDNA3.1-empty vector + 3 µg of pcDNA3.1-LMP-1 or pcDNA3.1-LMP-2A or pcDNA3.1-LMP-2B for the respective LMPs expression; 3 µg of pcDNA3.1-LMP-1 + 3 µg of pcDNA3.1-LMP-2A or pcDNA3.1-LMP-2B-V5 for co-expression of LMPs) was added into 600 µl of opti-MEM® medium. Subsequently, the diluted plasmid was added with 30 µl of Fugene® HD Transfection Reagent (Roche, Switzerland) in a ratio of 1:5 and mixed well. The mixture was incubated for 15 minutes at room temperature for transfection-complex formation. Next, the transfection-complex was added into the seeded cells in drop-wise manner. The cells were incubated for 48 hours at 37℃ with 5% CO_2. After 48 hours incubation, the cells were harvested for downstream analysis.

2.5 Analysis of genes expressions in LMP-1, LMP-2A and LMP-2B expressing cells

2.5.1 Analysis of RNA expression

2.5.1.1 RNA extraction

(As mentioned in Section 2.2)

2.5.1.2 cDNA synthesis

(As mentioned in Section 2.3.1)

2.5.1.3 PCR

Procedures were as mentioned in Section 2.3.2 but the forward primer was changed to S3-LMP1 for LMP-1 gene expression (453bp). For both LMP-2A and LMP-2B, the same forward primer (S4-LMP-2A) was used to amplify the common sequences (316bp).

2.5.1.4 Semi-quantitative RT-PCR

The synthesized cDNAs were quantitated with spectrophotometer and diluted to have the same concentration. The cDNA (700 ng) was subjected to semi-quantitative RT-PCR for comparison of the expressional levels of genes among different samples. The PCR composition and condition was described in Table 2.4.

Table 2.4: Components and conditions for semi-quantitative RT-PCR.

Components	Concentration of stock	Working concentration	One PCR reaction (µl)	Amplification Conditions
Ultra pure water (deionized water)	-	-	15.0	Denaturation (1×) 94℃ – 2 minutes Denaturation 94℃ – 30 seconds Annealing Tm℃ – 30 seconds Extension 72℃ – 30 seconds Final extension (1×) 72℃ – 5 minutes
10× PCR Buffer (15mM $MgCl_2$)	10X	1X	2.0	
dNTPs	10mM	0.25mM	0.5	
Forward primer	10µM	0.25µM	0.5	
Reverse primer	10µM	0.25µM	0.5	
Taq DNA polymerase	2U/µl	1U	0.5	
Template cDNA	-	-	1	
Total Volume			**20**	

The number of PCR cycles for each gene was optimized to ensure the amplification fall in the linear phase. Five µl of the PCR aliquots were removed and visualized after 15 cycles, 5 additional cycles will be carried out if it is not in linear phase. For each pair of genes specific primers (Table 2.5), three independent RT-PCRs were performed using three different set of RNA samples as template. RT-PCR for GAPDH (house-keeping gene) expression was used to normalize the amount of cDNA used. PCRs for GAPDH were performed as independent reactions. The PCR products were visualized in 2% (w/v) agarose gel and the intensity of the bands was quantified using Image J software.

Table 2.5: List of Primers used for semi-quantitative RT-PCR.

Gene	Primer Sequence (5'-3')	Tm (°C)	Product size (bp)	Ref.
14-3-3σ	GGCCATGGACATCAGCAAGAA CGAAAGTGGTCTTGGCCAGAG	58	137	Liu *et al.*, 2006
p53	TGTCCCTTCCCAGAAAACCTACC CCACTCGGATAAGATGCTGAGGAG	58	305	Wang and El-Deiry, 2006
p21	GTGGACCTGTCACTGTCTTGTAC CTTCCTCTTGGAGAAGATCAGC	56	151	Hata, 2005
p27	AACGTGCGAGTGTCTAACGG CCCTCTAGGGGTTTGTGATTCT	51	597	Weinmann *et al.*, 2008
Reprimo	GCAATCTGCTCATCAAGTCCGAG CCCCGCATTCCAAGTAAGTAGC	58	384	Takahashi *et al.*, 2005

2.5.1.5 Real Time Quantitative PCR

Materials:

1) SYBR Green PCR Master Mix (Applied Biosystem, USA)

Methods:

Real time quantitative PCR was performed in a volume of 15 µl reaction which contained 100 ng of cDNA template, 1×SYBR Green PCR Master mix and 0.1 mM forward and reverse gene specific primers using ABI 7000 Real time PCR machine (Applied Biosystem, USA). Each PCR reaction was optimized to eliminate the presence of primer dimer. For each pair of genes specific primers (Table 2.5), three independent RT-PCRs were performed using three different sets of RNA samples as template. The PCR conditions were as follows:

1 cycle	:	95℃ 10 mins
40 cycles	:	Step 1 95℃, 15s
		Step 2 60℃, 1min

The fluorescent product was determined at amplification step of each cycle. After amplification, melting curve was carried out by heating the PCR product to 94℃ for 5 mins and cooling at 0.5℃/10s to 55℃. The fluorescence data was collected at 0.5℃ intervals.

The relative fold change of the gene of interest was determined by comparing its expression level to GAPDH or β-actin internal control as follows: The relative fold change = $2^{-\Delta\Delta Cq}$, where $\Delta\Delta C_q = \left[[C_{qTarget}\ (\text{sample}) - C_{qGAPDH}\ (\text{sample})] - [C_{qTarget}\ (\text{Empty vector}) - C_{qGAPDH}\ (\text{empty vector})] + [C_{qTarget}\ (\text{sample}) - C_{q\beta\text{-actin}}\ (\text{sample})] - [C_{qTarget}\ (\text{Empty vector}) - C_{q\beta\text{-actin}}\ (\text{empty vector})]\right]/2$. C_q is quantification cycle according to Bustin *et al.*, (2009). Primers for *GAPDH* (Forward-5' CCA CCC ATG

GCA AAT TCC 3' and Reverse-5' CAG CAT CGC CCC ACT TG 3'), *β-actin* (Forward-5' AAA AGC CAC CCC ACT TCT CTC T 3' and Reverse-5' AAT GCT ATC ACC TCC CCT GTG T 3'), *14-3-3σ* (Forward-5' TGT CCA GTA TTG AGC AGA AAA GCA 3' and Reverse-5' CAC GCC CTG GAG CTC AGT 3') and *Reprimo* (Forward-5' CTG GCC CTG GGA CAA AGA C 3' and Reverse-5' TCA AAA CGG TGT CAC GGA TGT 3'), were designed using Primer express 3.0.

2.5.2 Analysis of protein by Western blotting

2.5.2.1 Protein extraction

Materials:

1) Radio-immuno-precipitation assay (RIPA) buffer: 50 mM Tris-Cl (Sigma, USA), 150 mM NaCl (Merck, Germany), 0.5% (w/v) Na-deoxycholate (Sigma, USA), 0.1% (w/v) Sodium dodecyl sulphate (SDS) (Amresco, Ohio), 1% (v/v) NP-40 (Sigma, USA).
2) Protease inhibitor cocktail set I (Calbiochem, La Jolla, CA)
3) Phosphatase inhibitor cocktail set V, 50× (Calbiochem, La Jolla, CA)

Methods:

The transfected cells in T-25 plate were washed with ice-cold PBS and harvested with cell scraper. After centrifugation at 800 rpm for 5 minutes, the supernatant was discarded. Then, 194 µl of RIPA buffer was added to the pellet with 2 µl of protease inhibitor cocktail and 4 µl of phosphatase inhibitor then transferred to a 1.5 ml Eppendorf tube. The cell lysate was incubated for 30 minutes on ice and mixed thoroughly every 5 minutes. After the incubation, the cell lysate was centrifuged at 13000 rpm for 30 minutes at 4℃. The supernatant was collected and stored at -20℃ before use.

2.5.2.2 Bradford assay

Materials:

1) Bradford Protein Assay (Bio-Rad, USA)
2) Bovine Serum Albumin (BSA) (Bio-Rad, USA)

Methods:

Cell lysate was quantified with Bradford's method with improved dye reagent from Bio-Rad. Six concentrations of BSA (0, 0.1, 0.2, 0.3, 0.4, 0.5 mg/ml) were prepared in 10µl each in duplicate in microtiter plate to act as a standard for protein concentration measurement. The sample was prepared in 10× dilution and 20× dilution. A 200 µl of Bradford dye reagent was added to each well and mixed. The plate was incubated for 5 minutes in room temperature to allow colour development. The optical density at 590 nm for each sample was collected from MRX microplate reader (Dynex, Chantilly, VA). The protein concentration for each sample was determined from the standard curve.

2.5.2.3 SDS-PAGE Electrophoresis

Materials:

1) Monomer solution (30.8% T 2.7%C_{Bis}): For 200 ml solution; 60 g acrylamide (Sigma, USA), 1.6 g N,N'-methylenebisacrylamide (Sigma, USA).
2) Running gel buffer (4×): 1.5 M Tris-Cl, pH 8.8
3) Stacking gel buffer (4×): 0.5 M Tris-Cl, pH 6.8
4) SDS (10% w/v)
5) Ammonium Persulphate (APS) (10% w/v) (Fluka, Switzerland)
6) Tetramethylethylenediamine (TEMED) (Fluka, Switzerland)
7) Sample treatment buffer (6×): 7 ml 0.5M Tris-Cl (pH 6.8), 2.6 ml Glycerol, 1 g 1,4-Dithio-DL-threitol (DTT), 60 µl of 10% (w/v) Bromophenol Blue, 400 ul 10% (w/v) SDS
8) Gel Running Buffer (10×): 0.25 M TRis-HCl, 1.9 M Glycine (Sigma, USA), 1% (w/v) SDS, pH 8.6
9) PageRuler™ Prestained Protein ladder (Fermentas, Canada)

Methods:

The extracted proteins were mixed with 6× sample treatment buffer and heat denatured at 94℃ for 5 minutes (at 70℃ for 5 minutes for LMP2-A and LMP-2B). Fifty µg of the protein was loaded on a 12% SDS polyacrylamide gel for separation (Table 2.6). Five µl of PageRuler™ Prestained Protein ladder was used as molecular weight marker.

Table 2.6: Components of the running gel and stacking gel.

Reagents	12% running gel	10% running gel	4% stacking gel
Monomer solution	3.2 ml	2.67 ml	665µl
Gel buffer	2.00 ml	2.00 ml	1.25 ml
10% SDS	80 µl	80 µl	50 µl
Mili-Q	2.8 ml	3.33 ml	3.0 ml
10% APS	120 µl	120 µl	50 µl
TEMED	8 µl	8 µl	5 µl

The gel percentage was dependent on the molecular weight of the interested protein. The protein separation was performed in a Mini-PROTEAN® 3 cell (Bio-rad) with 1× gel running buffer. Electrophoresis was achieved with 100V for 140 minutes at 4℃.

2.5.2.4 Immunoblotting

Materials:

1) Polyvinylidene Flouride (PVDF) membrane (PALL Corporation, Pensacola)
2) Methanol (R&M marketing, Essex, United Kingdom)
3) Tween-20 (Sigma®, USA)
4) Blocking solution: milk diluents (KPL, Inc., Gaithersburg, MD)
5) 5-bromo-4chloro-3indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) Phophatase Substrate System (KPL, Inc., Gaithersburg, MD)
6) Western Lightning® Plus–ECL (Perkin Elmer, USA)
7) 1× Tris Buffered Saline (TBS): 5 mM Tris-HCI, pH7.5, 150 mM NaCl

Methods:

After separating proteins using SDS-PAGE, the proteins were transferred electrophoretically to a PVDF membrane using Trans-Blot® SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad) for 25 minutes at 12V. The transfer efficiency was determined by observing the prestained ladder. After a complete transfer, the membrane was washed with TBST buffer (0.1% tween-20 in TBS) for 10 minutes and blocked with blocking solution (0.2% non-fat milk in TBST) for 1 hour at room temperature. Then, it was probed with primary antibody at room temperature for one hour. After washing for 3 times with TBST for 5 minutes, the membrane was probed with secondary antibody for 1 hour. After washing 3 times with TBST for 5 minutes, the protein signal was detected with BCIP/NBT phosphate substrate system at room temperature for 10 minutes for AP-conjugated secondary antibody. For HRP-conjugated secondary antibody, protein signal was detected by Western Lightning® Plus–ECL detection system according to manufacturer's protocol. The list of antibodies used and dilution for each antibody are described in Table 2.7.

Table 2.7: List of Antibodies used in Western blotting.

Antibodies	Dilution (in 0.1% non-fat milk)	Manufacturer
CS1-4 LMP1	1:1000	Dako, Denmark
B14-7 LMP-2A	1:1000	Serotec, UK
V5 epitope	1:1000	Invitrogen, USA
Alpha-Tubulin	1:1000	Abnova, Taiwan
14-3-3 sigma	1:1000	Abnova, Taiwan
Reprimo	1:1000	Genetex, USA
HRP-anti mouse/ rat	1:2000	Dako, Denmark
AP-anti mouse	1:5000	Sigma, USA
HRP-anti rabbit	1:1000	Abnova, Taiwan
HRP-anti Goat	1:1000	Abnova, Taiwan

2.6 Flow cytometry for cell cycle Analysis

Materials:

1) Propidium iodide (Sigma, USA)
2) RNase A solution (Novagen, EMD Biosciences, Inc, Madison, WI)

Methods:

Forty-eight hours after transfection, the cells were harvested prior to propidium iodide staining. The cells were trypsinized and washed twice with PBS, then collected in 15ml round bottom tube (Becto Dickinson, San Jose, CA). Next, the cells were fixed in ice-cold absolute ethanol at 4℃ for 1 hour and then collected by centrifugation. The pellet was washed once with PBS and incubated for 5 minutes on ice in 5ml of PBS with 0.25% Triton-X 100. The cells were then collected and incubated in PBS containing 10 µg/ml propidium iodide and 10 µg/ml RNaseA at 4℃ for 20 minutes. The cells were kept in 4℃ prior to FACS analysis using FACS Calibur flow cytometer (Becto Dickinson San Jose, CA). The histograms from FACS were generated with Winmbi and further analyzed with Cylchred cell cycle analysis software.

2.7 Data Analysis

All collected data were evaluated with Graphpad PRISM 5.0. Results for semiquantitative PCR and qPCR were evaluated with student's *t*-test and results from flow cytometry were evaluated with ANOVA. All statistical tests with P-values < 0.05 were considered statistically significant.

3.1 Cloning and sequence analysis of LMP-1, 2A and 2B derived from B95.8 cells

Overexpression of LMP-1 may contribute to cytostatic effect to the expressing cells. Therefore, to evaluate the effects of LMPs on cell cycle regulation, we first constructed the expression vector (pcDNA3.1) that allowed expression of LMPs under the control of CMV promoter.

LMP-1, 2A and *2B* genes for cloning were derived from B95.8 cell line. The total RNA (Figure 3.1A) was first subjected to cDNA synthesis and followed by PCR to amplify the genes (Figure 3.1B) prior to cloning into pcDNA3.1 vector (Appendix A).

LMP-1, 2A and *2B* PCR products were ligated into pcDNA3.1 vector with topoisomerase activity and transformed into *E.coli*. The recombinant plasmids were purified from the positive transformants which were first verified using colony PCR to confirm the presence of inserts with correct sizes (Figure 3.2). The sequences of the inserted genes in the extracted plasmids were confirmed by sequencing reactions. Subsequently, the sequences were compared to Epstein-Barr virus (EBV) genome, strain B95.8 in NCBI database (GenBank Acc. No.: V01555). The sequences of the inserted genes perfectly matched with the sequences of LMP-1, LMP-2A and LMP-2B in EBV B95.8 strain (Appendix B).

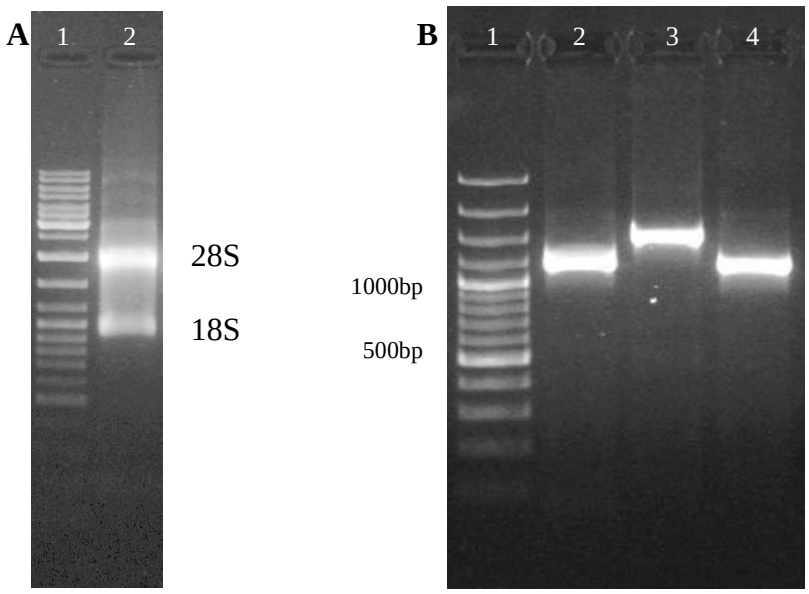

Figure 3.1: RT-PCR and cloning of LMP-1, 2A and 2B derived from B95.8 cell line. (**A**) Gel electrophoresis of total RNA isolated from B95.8 cell line with GeneRuler™ DNA Ladder Mix (Fermentas) as a size indicator in lane 1. (**B**) RT-PCR amplification of LMP-1, 1158bp (Lane 2), LMP-2A, 1491bp (Lane 3) and LMP-2B, 1134bp (Lane 4) with GeneRuler™ 100 bp plus DNA Ladder (Fermentas) as a size indicator in lane 1.

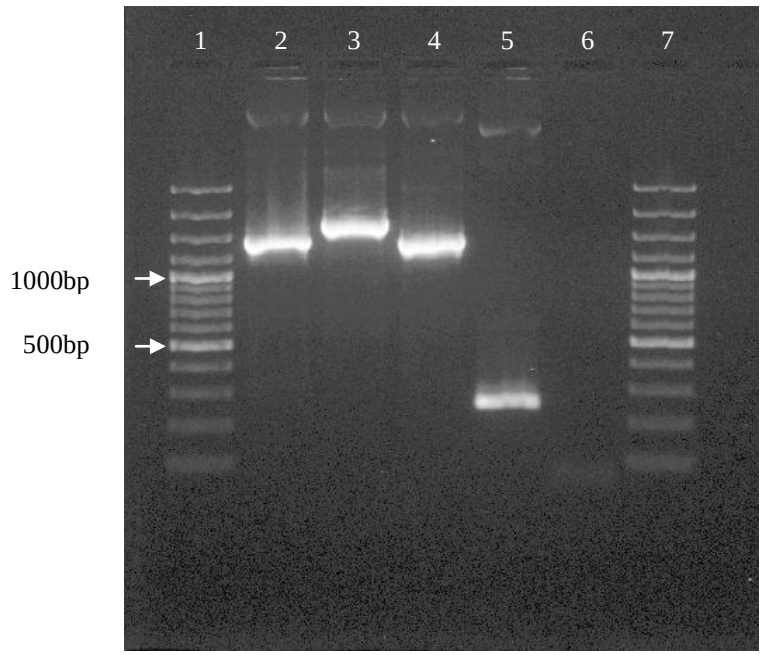

Figure 3.2: Verification of gene insertion into pcDNA3.1 with T7 and BGH primers. Lane 1: Fermentas GeneRuler™ 100 bp Plus DNA Ladder, Lane 2: pcDNA3.1-LMP1 (1414bp), Lane 3: pcDNA3.1-LMP2A (1747bp), Lane 4: pcDNA3.1-LMP2B (1387bp), Lane 5: pcDNA3.1-empty vector (249bp), Lane 6: Negative control (PCR reaction without template), Lane 7: Fermentas GeneRuler™ 100 bp Plus DNA Ladder.

3.2 Transfection optimization

HEK293T is widely used to express and study LMPs properties. In order to study the LMPs cytostatic effect, these LMPs must be able to achieve their optimal expression levels. Before transfection of LMPs constructs, the efficiency of pcDNA3.1 gene delivery system was optimized using cyan fluorescent protein (CFP). The cyan fluorescent gene from fluorescent CFP-expressing plasmid, pECFP-N1 (Clontech,USA), was subcloned into pcDNA3.1 vector and subsequently transfected into HEK293T cells for determining the optimum ratio of plasmid DNA to Fugene® HD transfection reagent. The transfection efficiency of about 70% was obtained by using

the ratio of 1:5 (1µg DNA to 5µl of Fugene® HD) in which about 70% of the cells expressed the CFP at 24 hours after transfection (compare Fig 3.3A and B).

A **B**

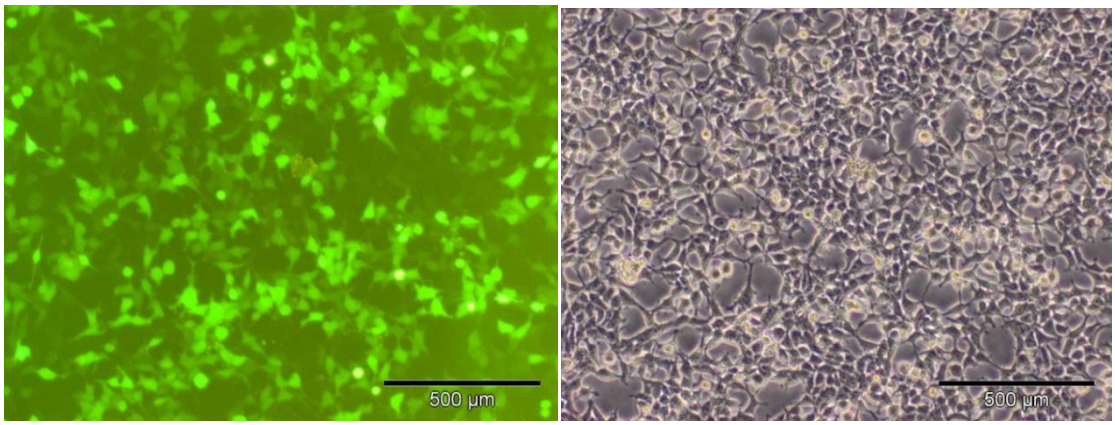

Figure 3.3: Transfection of HEK293T cells with the amount of pcDNA3.1-CFP (µg**) to Fugene HD (**µl**) at the ratio of 1:5.** 100× magnification revealing intracellular CFP expression. (A) Cells transfected with pcDNA3.1-CFP. (B) Bright field picture of transfected cells.

3.3 Expression of LMP-1, LMP-2A, LMP-2B in HEK293T cell line

After optimization of the gene delivery system, the recombinant plasmids were transfected into HEK293T cells to express the LMPs. The expression levels of these LMPs were verified by RT-PCR and western blotting.

By RT-PCR, the expressions of LMP-1, LMP-2A and LMP-2B mRNA were detected in HEK293T cells (Figure 3.4) after transient transfection. Forty-eight hours post-transfection, the cDNAs from transfected cells were subjected to RT-PCR using gene specific primers and the products were analyzed via agarose gel eletrophoresis. As shown in Figure 3.4, all the transfected cells expressed LMPs at a detectable level except cells transfected with pcDNA3.1-empty vector.

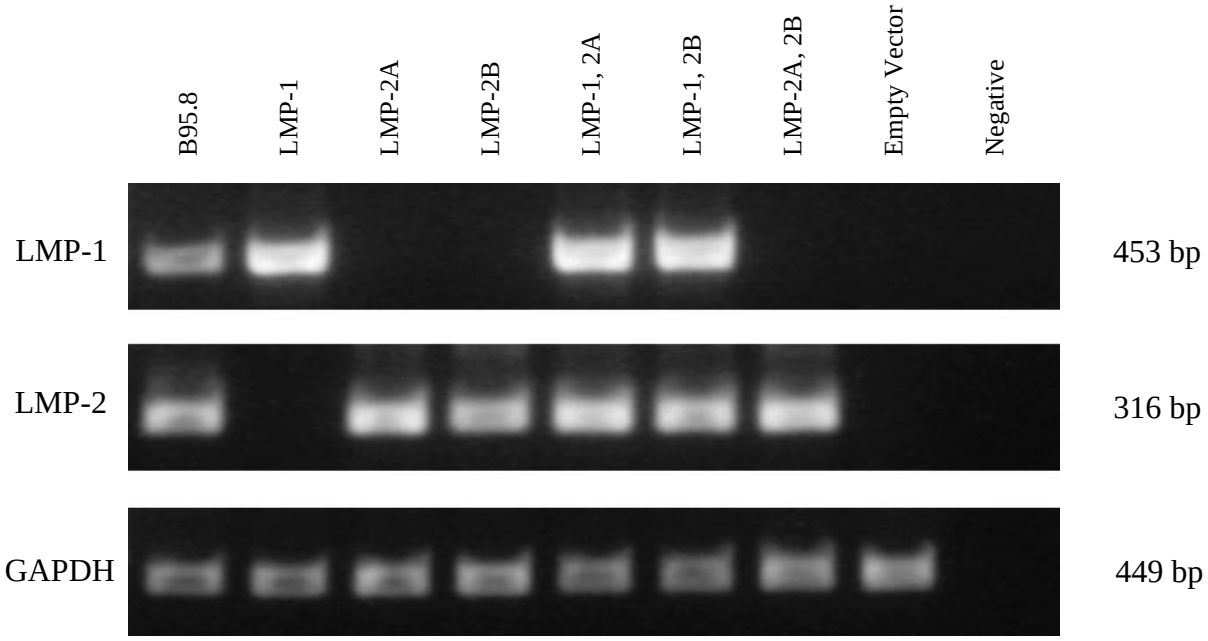

Figure 3.4: Transcription of LMP-1 and LMP-2 in HEK293T. Both LMP-2A and LMP-2B share a common sequence therefore the same primer pair was used to verify the expression of LMP-2. Same amount of cDNAs were subjected to semi-quantitative PCR and the amplified products were analyzed using 1% agarose gel electrophoresis. The cDNA from B95.8 cells was used as a positive control for LMPs gene expression. GAPDH was used as an internal control for cDNA abundance normalization.

The expression of LMPs proteins in the transfected cells was further confirmed using western blot assay at 48 hours after transfection (Figure 3.5). Western blot analysis revealed that LMPs with the molecular weights of 63kDa, 53kDa and 45kDa were expressed in cells transfected with pcDNA3.1-LMP-1, pcDNA3.1-LMP-2A and pcDNA3.1-LMP-2B-V5 constructs respectively. These observations indicated that the expression vectors were successfully transfected into cells and expressed LMPs at detectable levels.

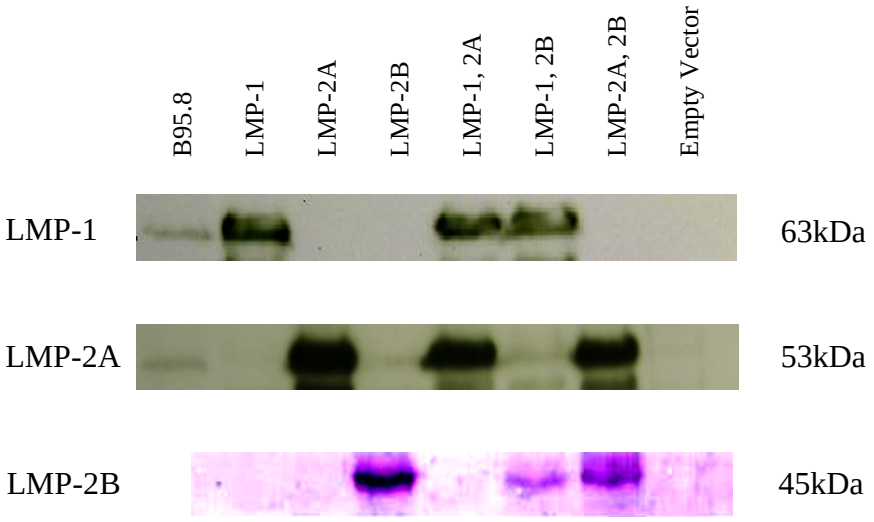

Figure 3.5: Detection of LMPs protein expression in HEK293T after transfection with various pcDNA3.1 constructs. Fifty µg of total protein lysates were separated in 10% SDS-PAGE and transferred to PVDF membranes for blotting with anti-LMP1 (CS1-4), anti-LMP2A (14B7) and anti-V5 antibodies to detect LMP1, LMP2A andLMP-2B proteins respectively. B95.8 cell lysate was used as a positive control for LMP-1 and LMP-2A proteins.

3.4 Reduction in cell density in all LMP-1 expressing cells

LMP-1 is toxic to cells and causes cytostasis when expressed in high levels (Hammerschmidt *et al.*, 1989; Floettmann *et al.*, 1996). Indeed, forty-eight hours post-transfection, cells were examined microscopically for effects of LMPs expression on cell viability. We observed reduction in cell density in all LMP-1 expressing cells (Figure 3.6) 48 hours post-transfection. There were less or no obvious phenotypical changes observed in other cells compared to their control cells. The control cells (pcDNA3.1-empty vector) showed a typical appearance of epithelial cells with flattened cell morphology and exhibited clear cell-to-cell contact and adhesion.

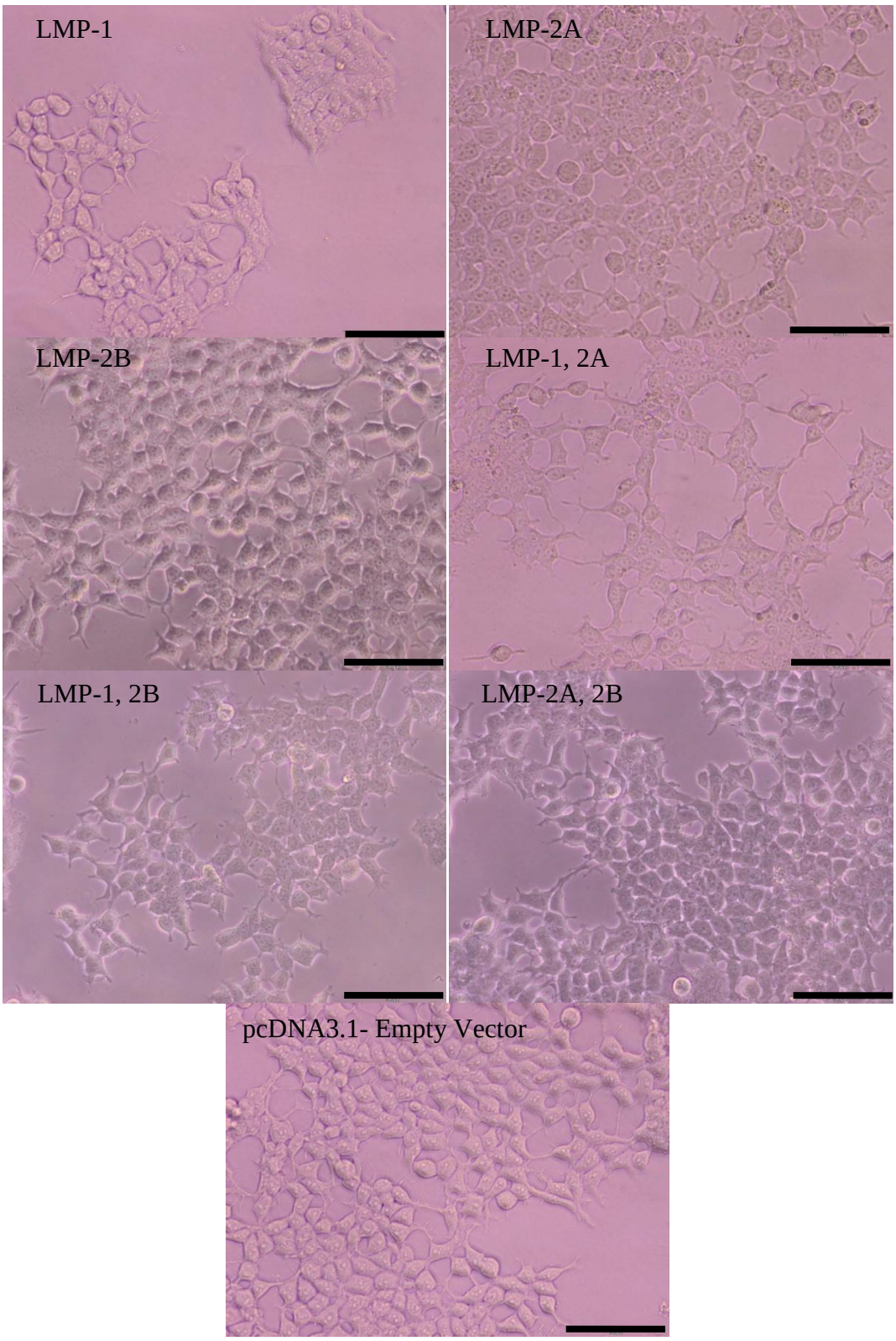

Figure 3.6: Effects of LMPs expression on cell viability and morphology. The LMP-1 expressing cells contain lesser cells compared to the cells transfected with empty vector. (Scale bar, 100μm).

3.5 Upregulation of *14-3-3σ* and *Reprimo* in LMP-1 expressing cells from semi-quantitative RT-PCR analysis

Due to the observation of a reduced cell density in all LMP-1 expressing cells, we further explored the effect of LMP-1 on cell cycle progression. The mRNA levels of several cell cycle regulator genes were analyzed in LMPs expressing cells by semi-quantitative RT-PCR. The *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) gene was used as an internal control. There were no significant changes in the transcription of *p53*, *p27* and *p21* among all the transfected cells (Figure 3.7A). However, *14-3-3σ* and *Reprimo* which are mediators for G_2 cell cycle arrest (Hermeking *et al.*, 1997; Ohki *et al.*, 2000) showed significant upregulation in all LMP-1 expressing cells with about 2 folds, compared to negative control (pcDNA3.1-empty vector) (Figure 3.7). There are no significant changes of these gene expression levels in LMP-2A, LMP-2B and LMP-2A, 2B expressing cells. The fold change of each gene is summarized in Figure 3.7B. Expression levels of *14-3-3σ* and *Reprimo* were further analyzed with real-time quantitative PCR.

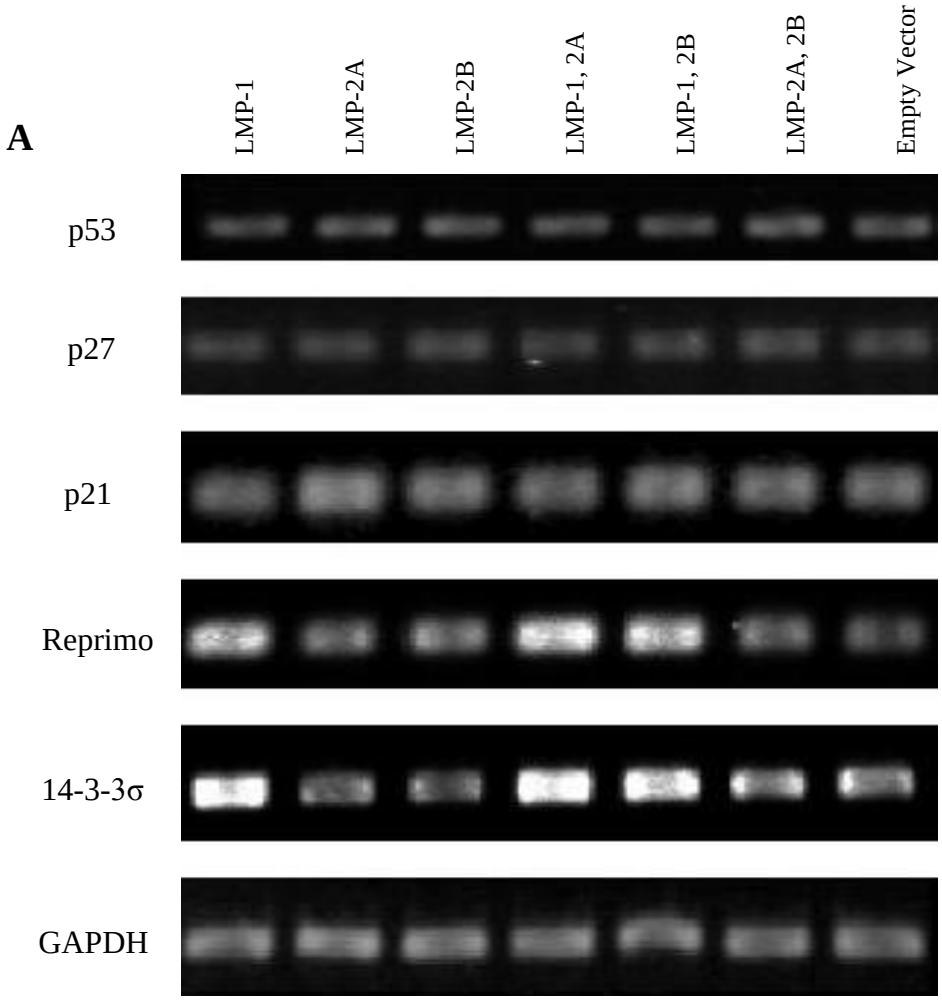

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Figure 3.7, continued.

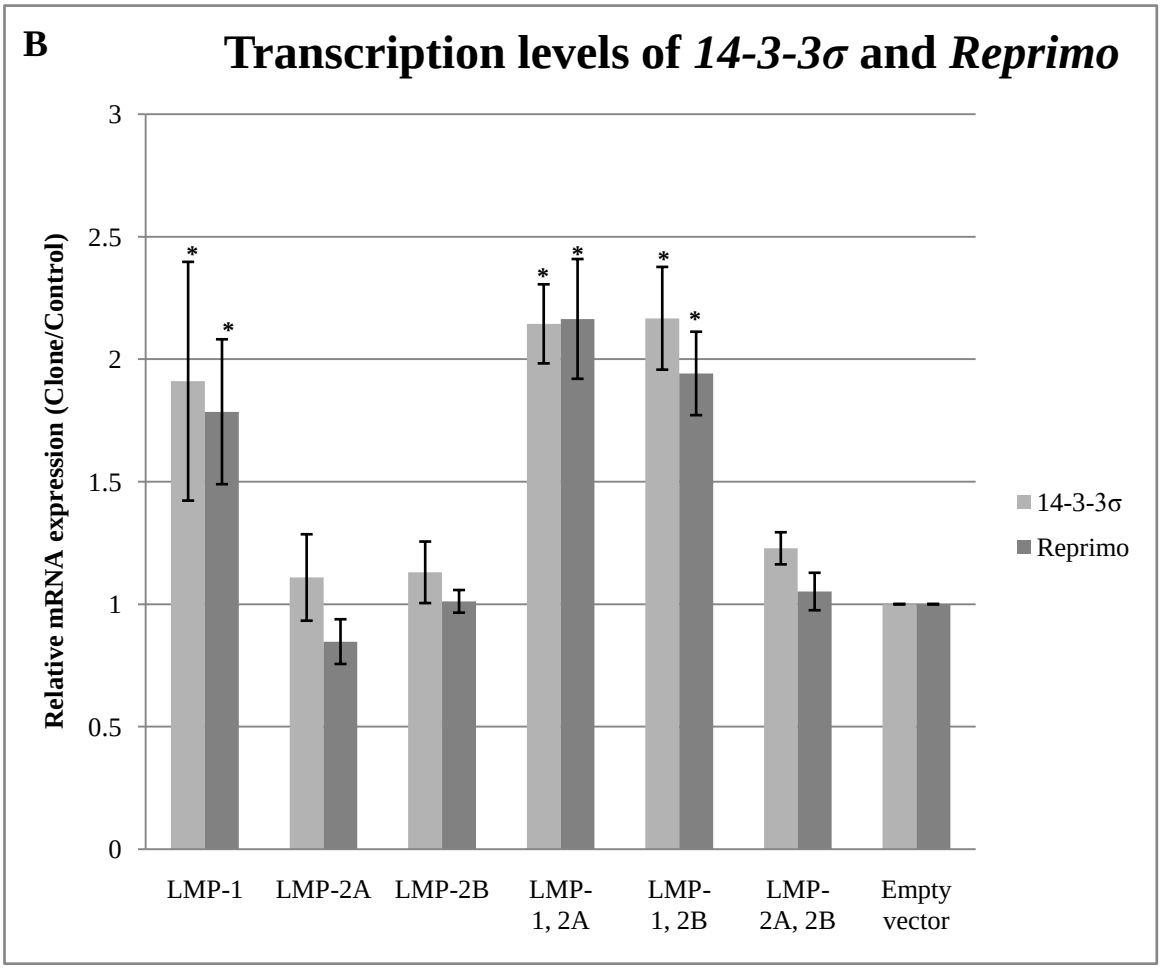
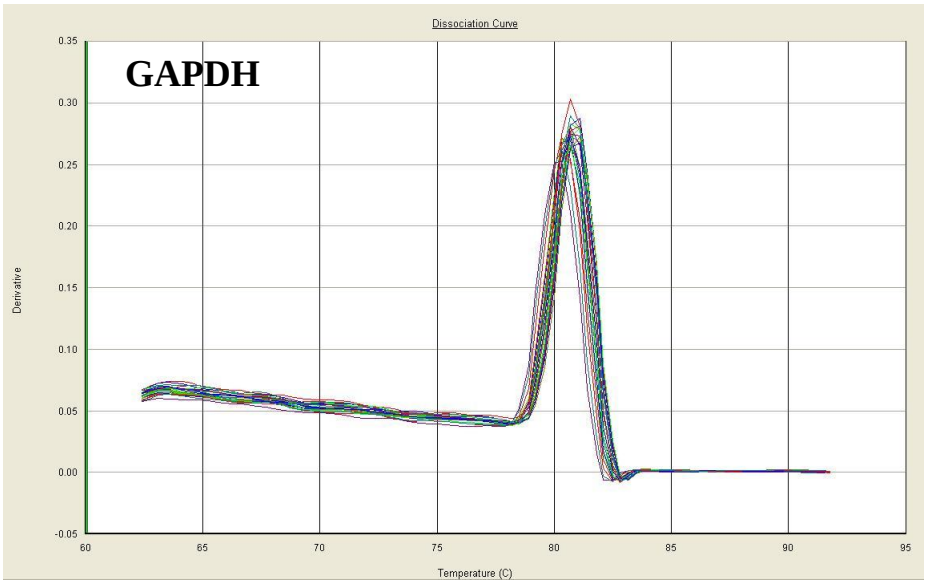


Figure 3.7: Semi-quantitative comparison of mRNA expression levels of *p53, p27, p21, Reprimo* and *14-3-3σ* in LMPs expressing cells.(**A**) Gel electrophoresis of the RT-PCR products. (**B**) Relative transcripts levels for all the transfected cells. Total RNA was reverse-transcribed and cDNAs were subjected to semi-quantitative PCR and the amplified products were analyzed using 1.5% agarose gel electrophoresis. GAPDH was used as internal control for cDNA abundance normalization. The densitometric value of each amplified products was normalized to negative control (pcDNA3.1-empty vector). Data shown are the mean of three independent experiments with standard error as error bars. *Indicates where value is significant in comparison to negative control (pcDNA3.1-empty vector) (student's *t*-test, *=$p<0.05$). Data are representative of three or more independent experiments.

3.6 Upregulation of *14-3-3σ* and *Reprimo* in LMP-1 expressing cells from real-time quantitative PCR assay

The upregulation of *14-3-3σ* and *Reprimo* was further analyzed with real-time PCR assay. *GAPDH* and *β-actin* were used as endogenous controls. Values for Relative Quantitative (RQ) were determined from the C_q of each reaction. In order to determine specific amplification, melting curves of the PCR products were studied and only single peak for all amplifications were observed (Figure 3.8). Upregulation of these genes were confirmed in all the LMP-1 expressing cells (Figure 3.9). The gene expression of *14-3-3σ* was upregulated in LMP-1, LMP-1, 2A and LMP-1, 2B expressing cells by 3.49 fold ($p<0.05$), 2.78 fold ($p<0.05$) and 2.39 fold ($p<0.05$), respectively when compared to the empty vector. *Reprimo* was upregulated by 2.08 fold ($p<0.05$), 2.27 fold ($p<0.05$) and 1.76 fold ($p<0.05$), respectively when compared to the empty vector. Once again, these upregulation of genes were not observed in LMP-2A, LMP-2B and LMP-2A, 2B expressing cells.



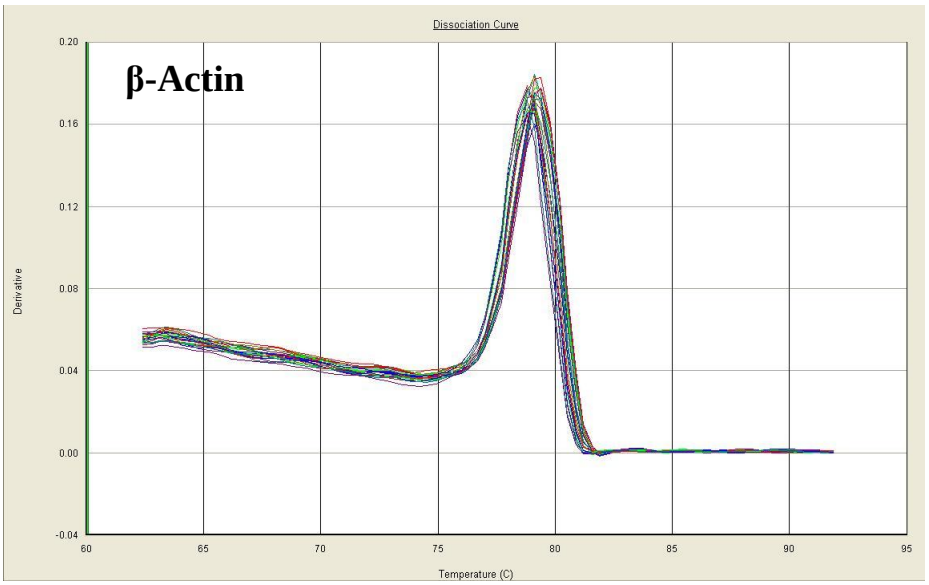


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Figure 3.8, continued.

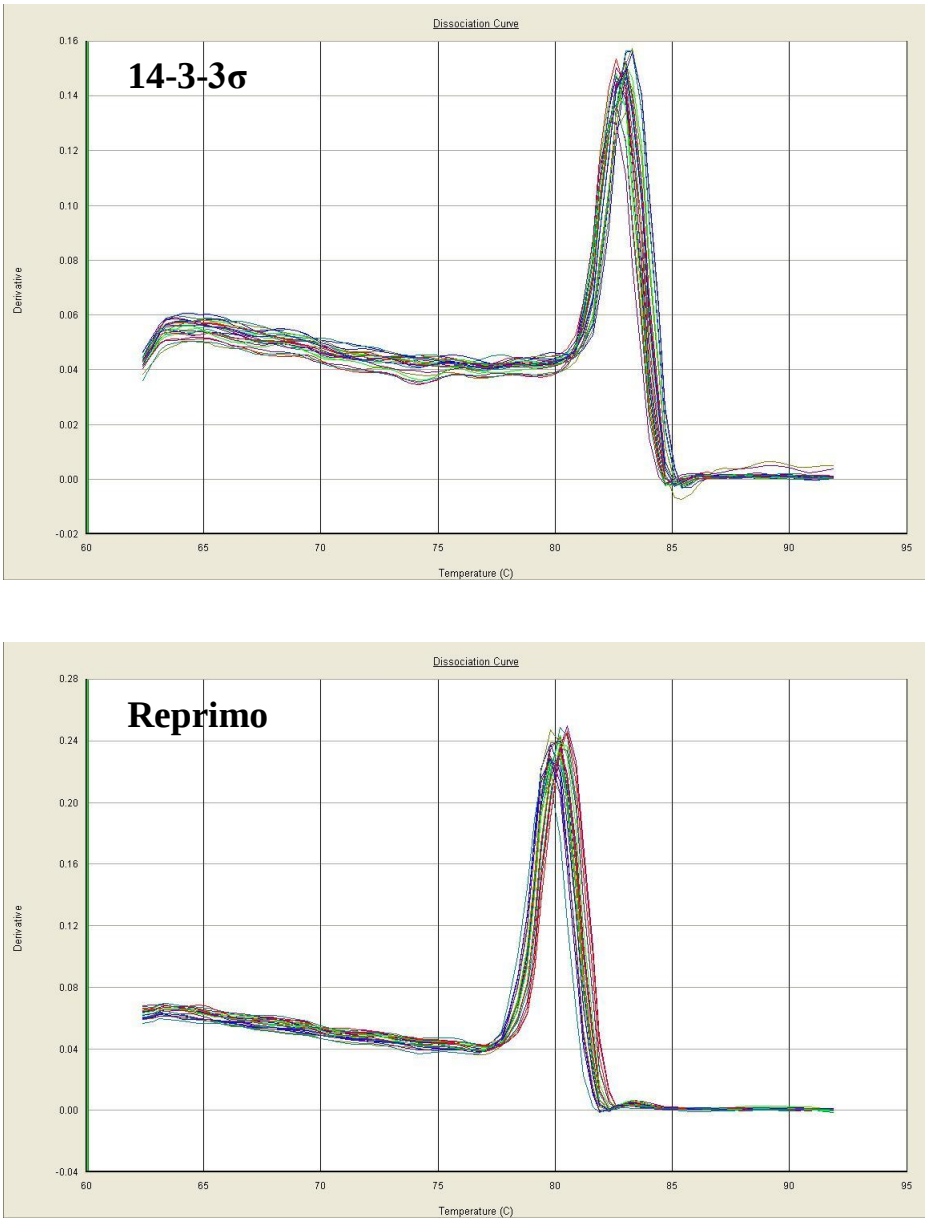

Figure 3.8: Melting curve profiles for PCR amplifications of *GAPDH*, *β-actin*, *14-3-3σ* and *Reprimo*. Only single peak was observed in all amplification reactions.

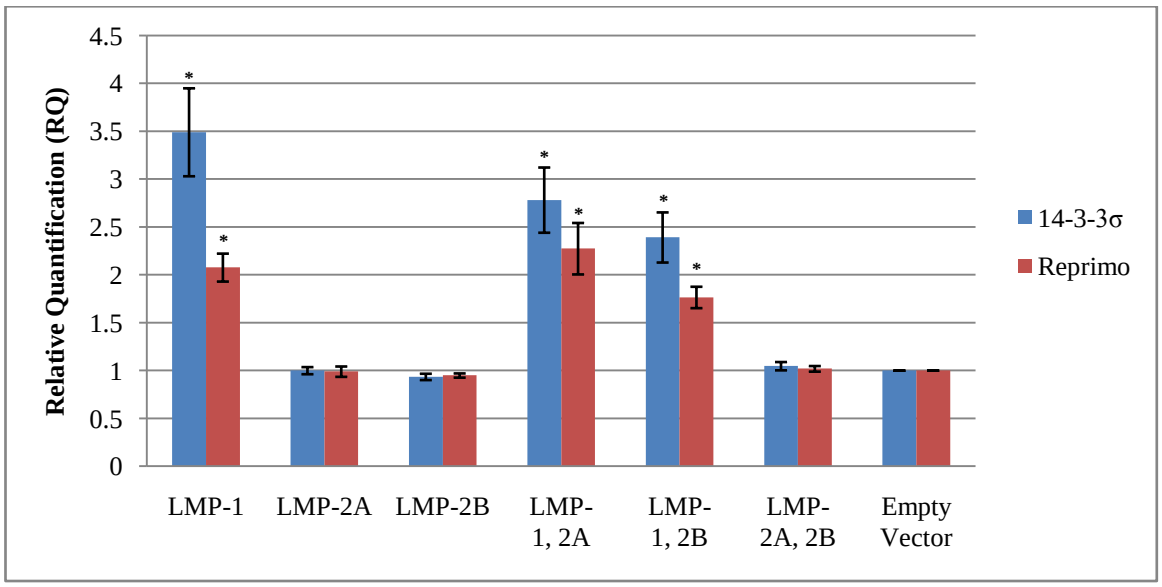

Figure 3.9: The relative mRNAs expressions of *14-3-3σ* and *Reprimo* in LMPs expressing cells. Relative gene expression levels for all the transfected cells were determined using real-time quantitative analysis. Data shown are the mean of three independent experiments with standard error as error bars. *Indicates where value is significant in comparison to negative control (pcDNA3.1-empty vector) (Student's *t*-test, *=$p<0.05$). Data are representative of three or more independent experiments.

3.7 14-3-3σ and Reprimo proteins were upregulated in all the LMP-1 expressing cells

In this study, the protein expression levels of 14-3-3σ and Reprimo were further examined with specific antibodies via western blotting analysis. As shown in Figure 3.10, protein levels of 14-3-3σ and Reprimo were upregulated in all the LMP-1 expressing cells.

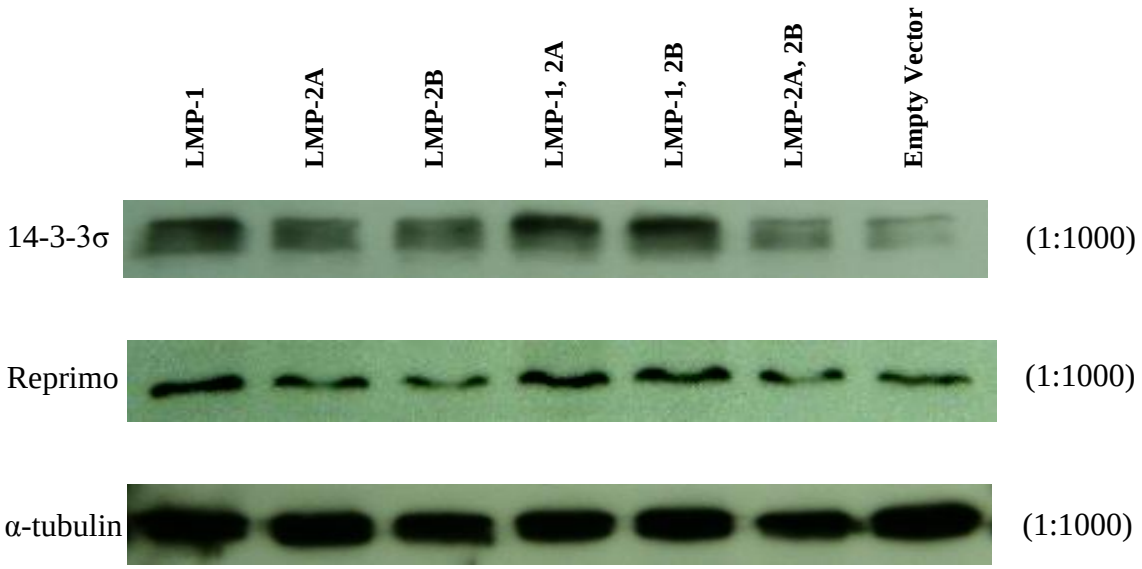

Figure 3.10: Western blotting analysis of 14-3-3σ and Reprimo in all the transfected cells. Cells transfected with empty vector were used as a negative control. Fifty µg of total protein lysates were separated in 12% SDS-PAGE and transferred to PVDF membranes for blotting with specific antibodies. Protein levels for 14-3-3σ and Reprimo in the various transfected cells were shown and α- tubulin protein level was detected as loading control.

3.8 Overexpression of LMP-1 arrests cells in G_2/M phase

In order to understand the effects of LMPs expression on cell cycle progression, transfected cells were subjected to flow cytometry analysis based on DNA content in nuclei stained by PI. Forty-eight hours post-transfection, cell cycle analysis revealed an increase in G_2/M phase in all the LMP-1 expressing cells compared to the control cells (Figure 3.11). Our data has shown that about 25.13% ± 3.93% ($p<0.05$) of the cells expressing only LMP-1 accumulated in G_2/M phase 48 hours after transfection, in contrast to only about 16.73% ± 0.26% of negative control cells (pcDNA3.1-empty vector) in the same phase. When LMP-1 was coexpressed with LMP-2A or LMP-2B, the proportion of cells accumulated in G_2/M phase were 25.93% ± 1.07% ($p<0.01$) and 24.97% ± 3.88% ($p<0.05$), respectively (Figure 3.11B and Table 3.1). The results revealed that the overexpression of LMP-1 capable to arrest the cells in G_2/M phase. Interestingly, there are some increments of cells in sub G_1 phase of LMP-1 expressing cells that maybe due to the cytotoxic effect of LMP-1 (Hammerschmidt *et al.*, 1989). In contrast, LMP-2A and LMP-2B had no effects on cell cycle progression when express alone or coexpress with each other and LMP-1. Details of cell cycle distributions for transfected cells and HEK293T cells are summarized in Table 3.1.

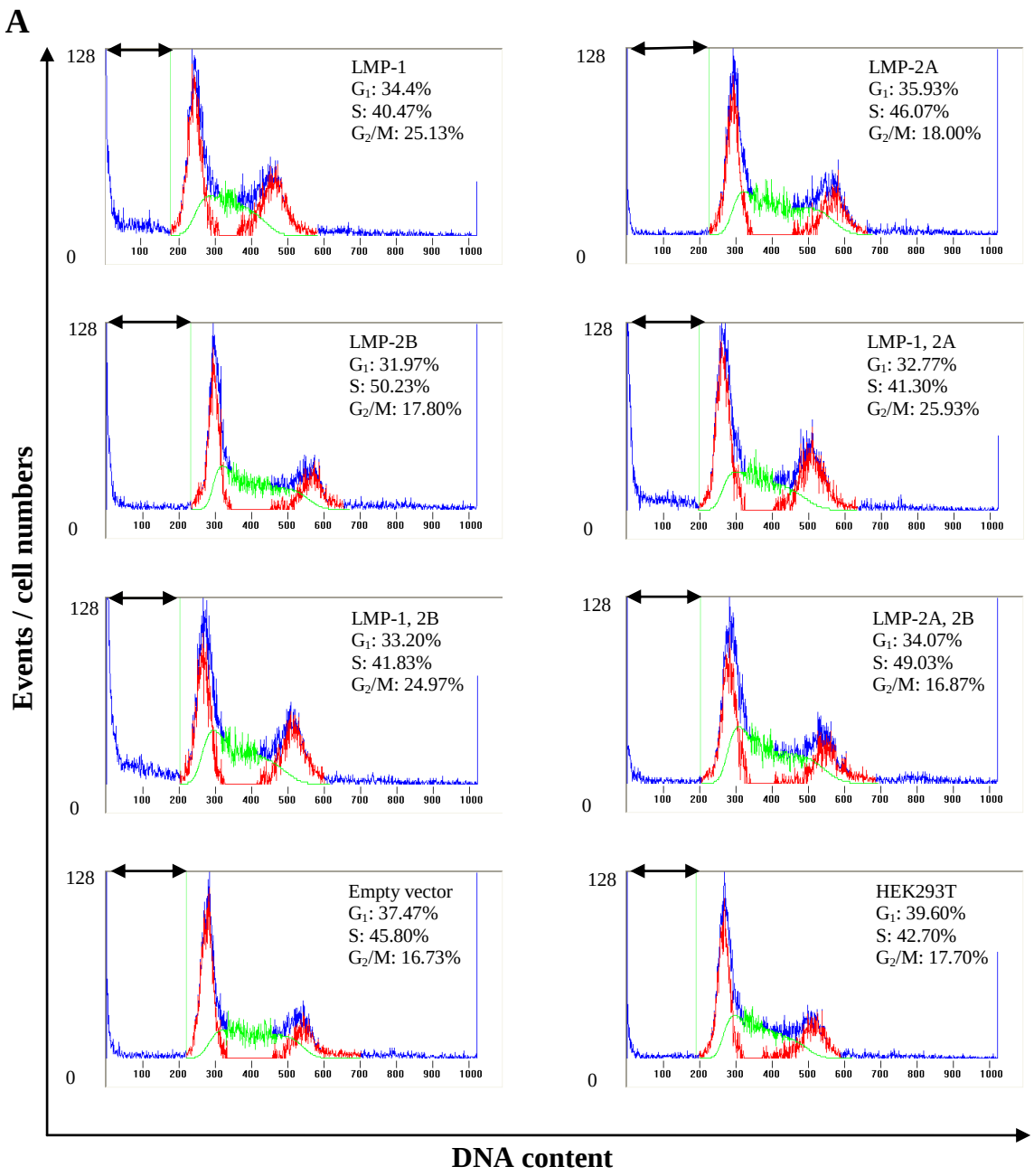

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Figure 3.11, continued.

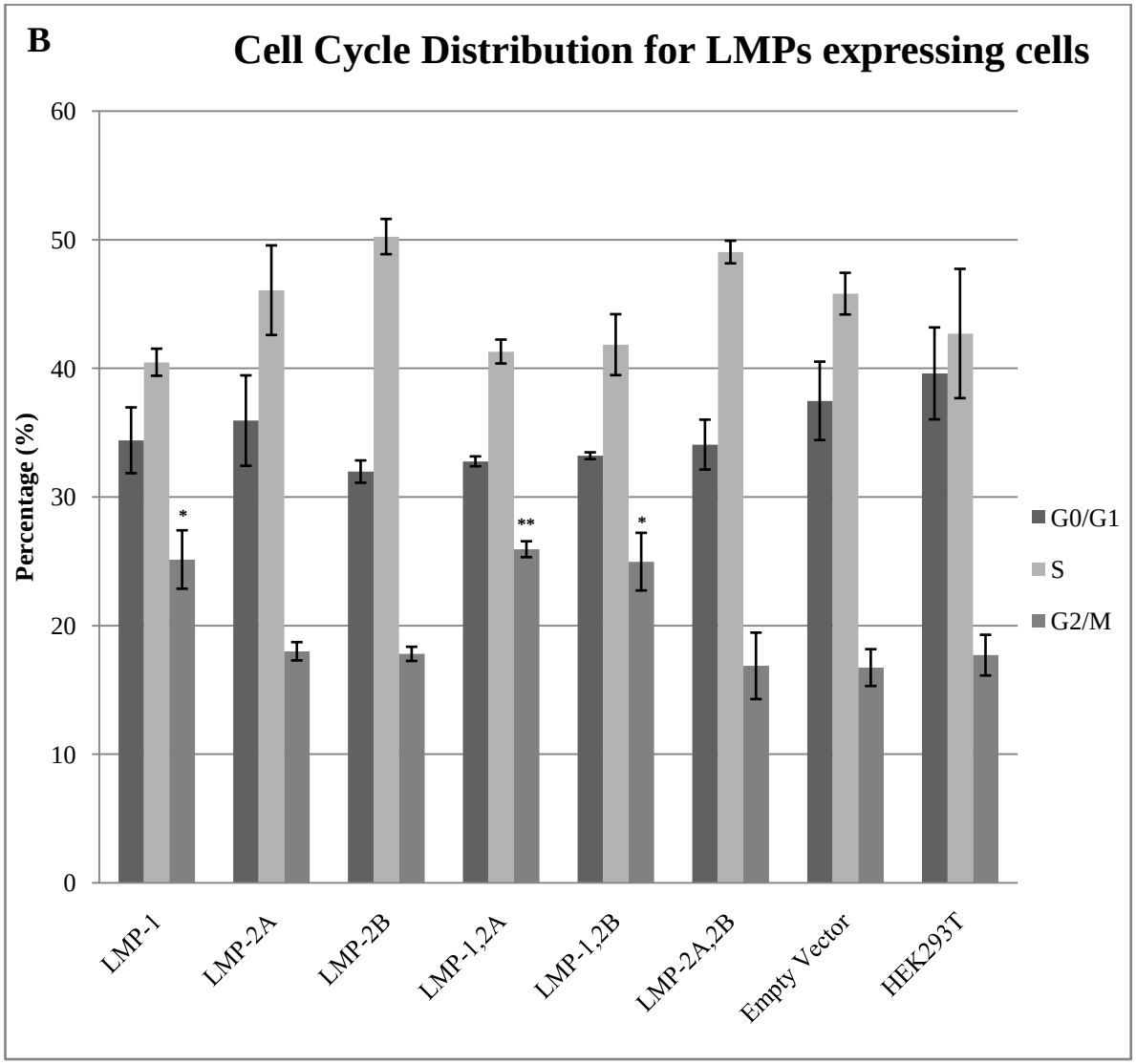

Figure 3.11: Flow cytometry analysis of HEK293T cells. (**A**) Histograms represent cell cycle distributions of HEK293T cells transfected with LMPs constructs and non-transfected parental cells. DNA profiles were analyzed using Cylchred software. G_1 and G_2/M phases are shown in red, and S phase is shown in green. The arrow indicates the apoptotic-sub G_1fraction of the cells. (**B**) Cell cycle phase distribution was analysed. Transfected cells were compared to cells transfected with pcDNA3.1 empty vector (mock). Data shown are the mean of three independent experiments with standard error as error bars. *Indicates where value is significant in comparison to negative control

(pcDNA3.1-empty vector) (ANOVA,**=p<0.01; *=p<0.05). Data are representative of three or more independent experiments.

Table 3.1: Cell Cycle distribution of transfected cells and HEK293T cells.

Clones	G_0/G_1(%)	SD (%)	S (%)	SD (%)	G_2/M (%)	SD (%)	Total
LMP-1	34.40	4.43	40.47	1.82	25.13*	3.93	100.00
LMP-2A	35.93	6.09	46.07	6.03	18.00	1.23	100.00
LMP-2B	31.97	1.50	50.23	2.37	17.80	0.95	100.00
LMP-1,2A	32.77	0.67	41.30	1.61	25.93**	1.07	100.00
LMP-1,2B	33.20	0.46	41.83	4.11	24.97*	3.88	100.00
LMP-2A,2B	34.07	3.36	49.03	1.52	16.87	4.47	100.00
Empty Vector	37.47	5.48	45.80	5.51	16.73	0.26	100.00
HEK293T	39.60	6.19	42.70	8.70	17.70	2.74	100.00

*Indicates where value is significant in comparison to control (ANOVA,**=p<0.01 *=p<0.05).

4.1 Expression of LMPs

There are 2 sub-types of EBV, Type I and Type II. Type I is represented by the prototype B95.8 strain while Type 2 was firstly dicovered to be associated with some cases of Burkitt's lymphomas in central Africa and New Guinea. The LMPs genes for our study were derived from B95.8 EBV strain. For LMP-1 itself, there are many sequence variations derived from different patients nowaday. In 1991, Hu and co-workers discovered a different sequence of LMP-1 from the Chinese NPC patients that is different in the loss of *XhoI* restriction site in the N-terminal and the 30-bp deletion adjacent to the CTAR2 domain compared to prototype B95.8. This variant is also known as CAOLMP-1. To date, there are many NPC-derived LMP-1 variants have been isolated from Asia and Europe. One of these LMP-1 variants is NLMP-1 that derived from NPC patient from Taiwan. NLMP-1 was reported to be more potent in transforming rodent fibroblast and able to induce tumor in nude mice as compared to B95.8 prototype (Chen *et al.*, 1992). In another experiment, NLMP-1 expressing Balb/c 3T3 cells was shown to produce lower level of nitric oxide that eventually can lead to cellular apoptosis as compared to wild type LMP-1. This ability of NLMP-1 may be an important contribution for cell survival (Yoshizaki *et al.,* 2001). Other NPC LMP-1 variants such as C15 was isolated from Mediterranean Caucasian (Gilligan *et al.,* 1990); LMP-1 Groups A-D European NPC (Sandvej *et al.*,1997); 2117-LMP-1 isolated from Hong Kong (Huang *et al.,* 1989), DV-Arg335 and DV-Gly335 also isolated from Hong Kong (Cheung *et al.,* 1998) and NPC-7 which isolated from China (Midgley *et al.,* 2000). In Malaysia, only limited LMP1 sequences isolated from patients with nasopharyngeal carcinoma has been reported (Tan *et al.,* 2007). Different clinical variants are likely to have variation in the functions of the LMP-1 genes. The selection of LMP-1 strains or variants for study should be under careful consideration. For our

study, the wild-type LMPs sequences from EBV B95.8 were selected with the consideration of the integrity of the genome as a major prototype of EBV strain.

Next, LMP-1 contains both oncogenic and toxic properties; therefore, there was a need to develop appropriate method to express LMP-1. In order to induce cytostatic effect which caused by LMP-1 and study LMPs interaction in cell cycle arrest, it must be expressed efficiently in the cells. Therefore, a few considerations were taken when carried out the study. The pcDNA3.1 mammalian expression vector was selected due to its human cytomegalovirus (CMV) immediate early enhancer/promoter which is capable for high-level constitutive expression of the gene of interest in a wide range of mammalian cells lines (Anderson, *et al.*, 1989; Boshart *et al.*, 1985; Nelson *et al.*, 1987). The HEK293T is a derivative of HEK293 line transfected with SV40 T-antigen which is important for rapidly ampliflying plasmid containing SV40 origin of replication such as pcDNA3.1 (Gluzman, 1981). SV40 plasmids undergo multiple rounds of duplication within one cell generation, usually accumulating to over 10,000 plasmid copies per cell in just 48 h after transfection (Chittenden *et al.*, 1991). Due to these criteria, LMPs were successfully expressed at high level compared to its endogenous expression level in B95.8 (Figure 3.5).

4.2 Overexpression of LMP-1 Induces Cell cycle Arrest

LMP-1 is a typical oncogene that is capable to transform rodent fibroblast (Wang *et al.*, 1985). However, LMP-1 is also able to inhibit cell proliferation or induce cytostatic effect when expressed in high level (Cuomo *et al.*, 1992, Floettmann *et al.*, 1996; Hammerschmidt *et al.*, 1989; Kaykas and Sugden, 2000). When LMP-1 was over-expressed in one to four times higher than its endogenous expression level in EBV-positive B cells, cytostasis in HEK293 cells for at least 6 days was induced (Kaykas and Sugden, 2000). Floettmann *et al.* (1996) also showed that LMP-1 induced cytostatic effect in BJAB, DG75 and Akata cells but some of the cells resumed proliferation after 4 days. The recovered cells may be part of the cells with lower expression level of LMP-1. Hence, LMP-1 may have growth advantage in those cells with lower expression levels.

In our present study, we demonstrated that LMP-1 expressing HEK293T cells exhibited cytostatic effect when expressed in higher level than B95.8 cells. This was evident from the observation of a reduction in cell density in the LMP-1 expressing cells (Figure 3.6). Moreover, in agreement with Floettmann *et al.* (1996) and Deng *et al.* (2003), our flow cytometry analysis (Figure 3.11) further revealed that LMP-1 is able to influence cell cycle progression and arrested cells at the G_2/M phase of the cell cycle. The cytostatic effect of cellular components or drugs can cause the cell to stop growing and it will usually followed by either cytotoxicity or escape from the stasis. From our observation (Figure 3.11), LMP-1 induced some cytotoxicity that caused the cells to undergo cell death and that may be the consequences of the cytostatic effect.

In constrast, LMP-2A and LMP-2B are unable to induce any changes in cell cycle progression. This suggested that LMP-2A and LMP-2B may not have any effect on cell cycle progression. Furthermore, LMP-2A and LMP-2B also unable to induce any significant changes to the cytostatic effect of LMP-1 and this can be further suggested that LMP-2 may not be an interaction partner for LMP-1 to activate cytostasis.

There are possible consequences of this LMP-1 induce cytostasis. Upon infection of primary B-cells, EBV DNA is replicated rapidly and this amplification is thought to be vital in maintaining an optimal level of viral gene expression. Eventually, the number of DNA copies stabilizes. The stabilization may be caused by LMP-1's role in growth inhibition whereby a higher LMP-1 expression level restricts cell proliferation (Kaykas and Sugden, 2000). In Burkitt's lymphoma, one of the fastest proliferating human tumors, the expression of LMP-1 is lost (Rowe *et al*., 1987). The rapid proliferation of this tumor may be contributed by the loss of LMP-1's growth inhibitory properties. In addition, the susceptibility of EBV infected cells to exterior cytotoxic signals such as chemotherapy drug (Cisplatin, Doxorubicin and Fluorouracil) that may be limited by the system which LMP-1 employs to induce cytostasis (Kaykas and Sugden, 2000).

Besides LMP-1, multiple oncogenes have been proved to induce both growth-promoting and growth-inhibitory effects. This paradox effects are observed in cellular oncoprotein Ras, c-Myc and Bcl-2 and the viral oncoproteins E1A and E7. These oncoproteins have significant role in promoting cell proliferation or survival. However, under certain circumstances, these oncoproteins are able to suppress cell proliferation either by cell cycle arrest or induction of p53-dependent and -independent apoptosis (Evan *et al.*, 1992; Debbas and White, 1993; Howes *et al.*, 1994; O'Reilly *et al.*, 1996; Serrano *et al.*, 1997; Evan and Littlewood, 1998). Moreover, these oncoproteins are able

to suppress its oncogenic properties via specific mechanism (Evan and Littlewood, 1998).

4.3 LMP-1 may have Induced Cell Cycle Arrest via Upregulation of 14-3-3α and Reprimo

To investigate the candidate genes that involved in LMP-1 induced cell cycle arrest, we evaluated the expression level of different cell cycle arrest genes in LMPs expression cells. Based on our observations, LMP-1 may induce cell cycle arrest at G_2/M phase of cell cycle via upregulation of 14-3-3σ and Reprimo expression (Figure 3.7, Figure 3.9 and Figure 3.11).

Recently, researches in 14-3-3 proteins have achieved increasing interest in many fields of biology. 14-3-3σ protein belongs to a highly conserved acidic protein family which has seven isoforms in mammals. Most of the 14-3-3 family members are involved in regulating signaling pathway, apoptosis, adhesion, cellular proliferation, diferentiation and survival (Mhawech, 2005). Of all the seven isoforms, only 14-3-3σ seemed to be involved directly to human cancer (Hermeking, 2003; Hermeking, 2006; Robert *et al.*, 2002). 14-3-3σ is a p53 regulated inhibitor of G_2/M progression (Hermeking *et al.*, 1997; Mhawech, 2005). Ectopic expression of 14-3-3σ protein results in G_2 arrest via inhibition of cyclin dependent kinase activities in many breast cancer cell lines (Laronga *et al.*, 2000).

Reprimo, a highly gylcosylated protein and p53 regulated G_2/M progression regulator, is a candidate gene that is involved in G_2/M cell cycle arrest. Ectopic expression of p53 protein induced its mRNA expression and subsequently induced G_2/M cell cycle arrest. In arrested cells, Reprimo is able to inhibit the activity of cdc2 and nuclear translocation of cyclin B1. Therefore, Reprimo is considered to be another p53-induced cell cycle regulator (Ohki *et al.*, 2000). Recently, Reprimo is identified as a

potential biomarker for early detection of gastric cancer (Bernal *et al.*, 2008). Besides, loss of Reprimo expression due to aberrant methylation was identified in various human malignancies and lung cancer (Suzuki *et al.*, 2005; Takahashi *et al.*, 2005).

In our study, we identified another two downstream targets of p53 which are 14-3-3σ and Reprimo that may be involved in G_2/M arrest in LMP-1 expressing cells. However, p53 protein is disabled in 293T cells by E1B and T large antigen; therefore we suggest that 14-3-3σ and Reprimo may be upregulated via p53-independent pathway. According to Zhang et al. (2004), 14-3-3σ is found to be stimulated by PI3K signaling pathway when in response to Insulin-like growth factor 1 (IGF-I). They have shown that IGF-I-induced expression of 14-3-3σ consistently even when p53 expression was knockdown with siRNA in MCF-7 cells. Therefore, LMP-1 is probably bypass p53 to induce 14-3-3σ expression in HEK293T cells since LMP-1 involved in many signaling pathways. However, the exact mechanism still needs further investigation. On the other hand, Reprimo expression in p53-independent manner is not well studied; consequently this occurrence may need additional study. Both 14-3-3σ and Reprimo are involved in regulation of cdc2 and cyclin B1 pathway (Ohki *et al.*, 2000; Taylor and Stark, 2001; Stark and Taylor, 2006). G_2/M arrest is important checkpoint to ensure normal DNA replication and abnormality of DNA replication is closely associated with genomic instability, tumorigenesis and treatment of tumor (Hoeijmakers, 2001; Khanna and Jackson, 2001; Nigg, 2001; O'Connell *et al.*, 2000; Zhou and Elledge, 2000).

On the other hand, since LMPs are commonly co-expressed and co-localized to the same compartment (Longnecker and Kieff, 1990; Lynch *et al.*, 2002; Rovedo and Longnecker, 2007), they may probably interact with each other in tumorigenesis. However, in our present observation, LMP-2A and 2B were found to have no significant changes on LMP-1 induced 14-3-3σ and Reprimo expression as well as G_2/M arrest. This phenomenon showed that LMP-1 may be a stronger inducer protein

that can overcome the effect of LMP-2 or LMP-2A and 2B have no direct interaction with LMP-1 in controlling cell cycle progression. This observation still needs further investigation to unveil the consequences and functions of LMPs interaction.

4.4 Limitations

There are several limitations in our current study that need to be addressed and improved for future work. Firstly, different transfection efficiency in between the plates might affect the expression of the LMPs and thus the interpretation of the results. Cell sorter may be useful for making sure that the cells studied contained high level of LMPs. Secondly, in order to study LMPs cytostasis, LMP-1 must be expressed in high levels. In our study, HEK293T cells were selected due to its SV40 T large antigen that can increase the expression level of LMPs by replicating plasmid that contains SV40 origin. However, not all the cells can achieve high and similar expression level therefore a better expression method such as inducible system with doxycycline or tetracyclin from stable clone will be able to improve and confirm the results. In addition, more than one cell line should be used to increase the persuasive of our study. Lastly, additional functional study (or proteins in selected pathways) may be added to our study to reveal a better picture of this mechanism.

Result obtained in this study was only part of the puzzle from LMP-1 induced cell cycle arrest therefore it may not sufficient for us to derive conclusive evidences on the exact mechanism of LMP-1 induced cell cycle arrest. Therefore, this phenomenon may be deserved to further study using different approach such as RNA interference or gene knockout techniques. Besides, elucidating the mechanism of LMP-1 induced cell cycle arrest in different cell types is important for understanding the growth regulation in particular tumor type. Perhaps, we may see a better picture of LMP-1 function in cell cycle with investigating the location of LMP-1 in the cell. In that respect, further study

can therefore be implemented to study the precise machinery of LMP-1 induced cell cycle arrest for understanding this distinct mechanism in EBV associated malignancies.

5.1 Conclusion

In conclusion, LMP-1, 2A and 2B were successfully cloned into pcDNA3.1 expression vector. Moreover, these LMPs were efficiently expressed in HEK293T cells. LMP-1 was able to upregulate the expression of 14-3-3σ and Reprimo. We have shown that LMP-1 may induce cell cycle arrest in G_2/M phase through the upregulation of 14-3-3σ and Reprimo. In addition, LMP-2 may not interfere with the function of LMP-1 in inducing cell cycle arrest. According to our study, LMP-1 induced the expression of 14-3-3σ and Reprimo and this mechanism may play some roles in EBV-associated human malignancies. Further studies will be required to determine the precise machinery by which LMP-1 induces cell cycle arrest for better understanding of this distinct mechanism.

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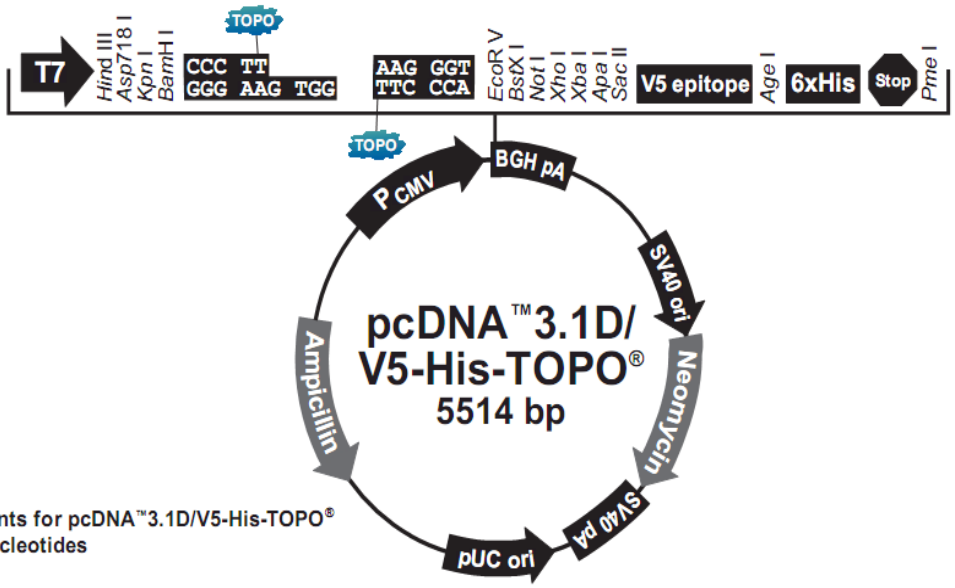
APPENDIX A: pcDNA3.1 vector



Comments for pcDNA™3.1D/V5-His-TOPO®
5514 nucleotides

CMV promoter: bases 232-819
T7 promoter/priming site: bases 863-882
TOPO® recognition site 1: bases 930-934
Overhang sequence (complementary strand): bases 935-938
TOPO® recognition site 2: bases 939-943
V5 epitope: bases 1011-1052
Polyhistidine (6xHis) tag: bases 1062-1079
BGH reverse priming site: bases 1102-1119
BGH polyadenylation signal: bases 1108-1332
SV40 early promoter and origin: bases 1833-2142
Neomycin resistance gene: bases 2217-3011
SV40 early polyadenylation signal: bases 3189-3319
pUC origin: bases 3700-4373 (complementary strand)
Ampicillin (*bla*) resistance gene: bases 4518-5378 (complementary strand)
bla promoter: bases 5379-5477 (complementary strand)

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APPENDIX B: Sequences of LMP-1, LMP-2A and LMP-2B in EBV B95.8 strain

Epstein-Barr virus (EBV) genome, strain B95-8 GenBank: V01555.2

LMP-1

ATGGAACACGACCTTGAGAGGGGCCCACCGGGCCCGCGACGGCCCCCTCGAGGACCCCCC 60
CTCTCCTCTTCCCTAGGCCTTGCTCTCCTTCTCCTCCTCTTGGCGCTACTGTTTTGGCTG 120
TACATCGTTATGAGTGACTGGACTGGAGGAGCCCTCCTTGTCCTCTATTCCTTTGCTCTC 180
ATGCTTATAATTATAATTTTGATCATCTTTATCTTCAGAAGAGACCTTCTCTGTCCACTT 240
GGAGCCCTTTGTATACTCCTACTGATGATCACCCTCCTGCTCATCGCTCTCTGGAATTTG 300
CACGGACAGGCATTGTTCCTTGGAATTGTGCTGTTCATCTTCGGGTGCTTACTTGTCTTA 360
GGTATCTGGATCTACTTATTGGAGATGCTCTGGCGACTTGGTGCCACCATCTGGCAGCTT 420
TTGGCCTTCTTCCTAGCCTTCTTCCTAGACCTCATCCTGCTCATTATTGCTCTCTATCTA 480
CAACAAAACTGGTGGACTCTATTGGTTGATCTCCTTTGGCTCCTCCTGTTTCTGGCGATT 540
TTAATCTGGATGTATTACCATGGACAACGACACAGTGATGAACACCACCACGATGACTCC 600
CTCCCGCACCCTCAACAAGCTACCGATGATTCTGGCCATGAATCTGACTCTAACTCCAAC 660
GAGGGCAGACACCACCTGCTCGTGAGTGGAGCCGGCGACGGACCCCCACTCTGCTCTCAA 720
AACCTAGGCGCACCTGGAGGTGGTCCTGACAATGGCCCACAGGACCCTGACAACACTGAT 780
GACAATGGCCCACAGGACCCTGACAACACTGATGACAATGGCCCACATGACCCGCTGCCT 840
CAGGACCCTGACAACACTGATGACAATGGCCCACAGGACCCTGACAACACTGATGACAAT 900
GGCCCACATGACCCGCTGCCTCATAGCCCTAGCGACTCTGCTGGAAATGATGGAGGCCCT 960
CCACAATTGACGGAAGAGGTTGAAAACAAAGGAGGTGACCAGGGCCCGCCTTTGATGACA1020
GACGGAGGCGGCGGTCATAGTCATGATTCCGGCCATGGCGGCGGTGATCCACACCTTCCT 1080
ACGCTGCTTTTGGGTTCTTCTGGTTCCGGTGGAGATGATGACGACCCCCACGGCCCAGTT 1140
CAGCTAAGCTACTATGACTAA 1161

LMP-2A

ATGGGGTCCCTAGAAATGGTGCCAATGGGCGCGGGTCCCCCTAGCCCCGGCGGGGATCCG 60
GATGGGTACGATGGCGGAAACAACTCCCAATATCCATCTGCTTCTGGCTCTTCTGGGAAC 120
ACCCCCACCCCACCGAACGATGAGGAACGTGAATCTAATGAAGAGCCCCCACCGCCTTAT 180
GAGGACCCATATTGGGGCAATGGCGACCGTCACTCGGACTATCAACCACTAGGAACCCAA 240
GATCAAAGTCTGTACTTGGGATTGCAACACGACGGGAATGACGGGCTCCCTCCCCCTCCC 300
TACTCTCCACGGGATGACTCATCTCAACACATATACGAAGAAGCGGGCAGAGGAAGTATG 360
AATCCAGTATGCCTGCCTGTAATTGTTGCGCCCTACCTCTTTTGGCTGGCGGCTATTGCC 420
GCCTCGTGTTTCACGGCCTCAGTTAGTACCGTTGTGACCGCCACCGGCTTGGCCCTCTCA 480
CTTCTACTCTTGGCAGCAGTGGCCAGCTCATATGCCGCTGCACAAAGGAAACTGCTGACA 540
CCGGTGACAGTGCTTACTGCGGTTGTCACTTTCTTTGCAATTTGCCTAACATGGAGGATT 600
GAGGACCCACCTTTTAATTCTCTTCTGTTTGCATTGCTGGCCGCAGCTGGCGGACTACAA 660
GGCATTTACGTTCTGGTGATGCTTGTGCTCCTGATACTAGCGTACAGAAGGAGATGGCGC 720
CGTTTGACTGTTTGTGGCGGCATCATGTTTTTGGCATGTGTACTTGTCCTCATCGTCGAC 780
GCTGTTTTGCAGCTGAGTCCCCTCCTTGGAGCTGTAACTGTGGTTTCCATGACGCTGCTG 840
CTACTGGCTTTCGTCCTCTGGCTCTCTTCGCCAGGGGGCCTAGGTACTCTTGGTGCAGCC 900
CTTTTAACATTGGCAGCAGCTCTGGCACTGCTAGCGTCACTGATTTTGGGCACACTTAAC 960
TTGACTACAATGTTCCTTCTCATGCTCCTATGGACACTTGTGGTTCTCCTGATTTGCTCT 1020
TCGTGCTCTTCATGTCCACTGAGCAAGATCCTTCTGGCACGACTGTTCCTATATGCTCTC 1080
GCACTCTTGTTGCTAGCCTCCGCGCTAATCGCTGGTGGCAGTATTTTGCAAACAAACTTC 1140
AAGAGTTTAAGCAGCACTGAATTTATACCCAATTTGTTCTGCATGTTATTACTGATTGTC 1200
GCTGGCATACTCTTCATTCTTGCTATCCTGACCGAATGGGGCAGTGGAAATAGAACATAC 1260
GGTCCAGTTTTTATGTGCCTCGGTGGCCTGCTCACCATGGTAGCCGGCGCTGTGTGGCTG 1320
ACGGTGATGTCTAACACGCTTTTGTCTGCCTGGATTCTTACAGCAGGATTCCTGATTTTC 1380
CTCATTGGCTTTGCCCTCTTTGGGGTCATTAGATGCTGCCGCTACTGCTGCTACTACTGC 1440
CTTACACTGGAAAGTGAGGAGCGCCCACCGACCCCATATCGCAACACTGTATAA 1494

LMP-2B

ATGAATCCAGTATGCCTGCCTGTAATTGTTGCGCCCTACCTCTTTTGGCTGGCGGCTATT 60
GCCGCCTCGTGTTTCACGGCCTCAGTTAGTACCGTTGTGACCGCCACCGGCTTGGCCCTC 120
TCACTTCTACTCTTGGCAGCAGTGGCCAGCTCATATGCCGCTGCACAAAGGAAACTGCTG 180
ACACCGGTGACAGTGCTTACTGCGGTTGTCACTTTCTTTGCAATTTGCCTAACATGGAGG 240
ATTGAGGACCCACCTTTTAATTCTCTTCTGTTTGCATTGCTGGCCGCAGCTGGCGGACTA 300
CAAGGCATTTACGTTCTGGTGATGCTTGTGCTCCTGATACTAGCGTACAGAAGGAGATGG360
CGCCGTTTGACTGTTTGTGGCGGCATCATGTTTTTGGCATGTGTACTTGTCCTCATCGTC 420
GACGCTGTTTTGCAGCTGAGTCCCCTCCTTGGAGCTGTAACTGTGGTTTCCATGACGCTG 480
CTGCTACTGGCTTTCGTCCTCTGGCTCTCTTCGCCAGGGGGCCTAGGTACTCTTGGTGCA 540
GCCCTTTTAACATTGGCAGCAGCTCTGGCACTGCTAGCGTCACTGATTTTGGGCACACTT 600
AACTTGACTACAATGTTCCTTCTCATGCTCCTATGGACACTTGTGGTTCTCCTGATTTGC 660
TCTTCGTGCTCTTCATGTCCACTGAGCAAGATCCTTCTGGCACGACTGTTCCTATATGCT 720
CTCGCACTCTTGTTGCTAGCCTCCGCGCTAATCGCTGGTGGCAGTATTTTGCAAACAAAC 780
TTCAAGAGTTTAAGCAGCACTGAATTTATACCCAATTTGTTCTGCATGTTATTACTGATT 840
GTCGCTGGCATACTCTTCATTCTTGCTATCCTGACCGAATGGGGCAGTGGAAATAGAACA 900
TACGGTCCAGTTTTTATGTGCCTCGGTGGCCTGCTCACCATGGTAGCCGGCGCTGTGTGG 960
CTGACGGTGATGTCTAACACGCTTTTGTCTGCCTGGATTCTTACAGCAGGATTCCTGATT 1020
TTCCTCATTGGCTTTGCCCTCTTTGGGGTCATTAGATGCTGCCGCTACTGCTGCTACTAC 1080
TGCCTTACACTGGAAAGTGAGGAGCGCCCACCGACCCCATATCGCAACACTGTATAA 1137