行政院國家科學委員會專題研究計畫　成果報告

探討 EB 病毒蛋白 BALF1 在其感染細胞中的功能

計畫類別：個別型計畫
計畫編號：NSC94-2320-B-040-024-
執行期間：94 年 08 月 01 日至 95 年 07 月 31 日
執行單位：中山醫學大學醫學系微生物及免疫學科

計畫主持人：梁熾龍

報告類型：精簡報告
報告附件：出席國際會議研究心得報告及發表論文
處理方式：本計畫涉及專利或其他智慧財產權，2 年後可公開查詢

中　華　民　國 95 年 10 月 30 日

中文摘要

EB病毒是人類疱疹病毒 (herpesvirus) 的一員。它能夠持續存活於B淋巴細胞，並透過其潛伏性膜蛋白1 (LMP1)活化細胞中的Bcl-2蛋白質，導致B細胞轉形 (transformation) 及癌化 (tumorigenesis)。換言之，Bcl-2蛋白在其中扮演非常重要的角色。近來在EB病毒基因研究中，發現有一種會出現在潛伏期及溶裂期的早期溶裂型蛋白質(an early lytic protein)，其結構與Bcl-2相似，我們稱它為BALF1。雖有相似的結構，但是我們對其在病毒感染細胞中的角色與功能至今仍不清楚。根據初步研究結果，BALF1能夠抑制EB病毒的 Zta protein 的 transcript，但對 EBNA1的 transcript 則無明顯影響。合理推論，BALF1可能調控EB病毒的生活史。此外，BALF1 不僅活化EB病毒腫瘤基因 LMP1的轉錄活性，亦增加細胞內兩個與細胞周期(cell cycle)有關的基因表現。其分別為 c-Myc 與 E2F1。在另一方面，BALF1能夠抑制細胞DNA修復活性 (cell DNA repair activity) 及某些細胞凋亡 (apoptosis) 的進行。他意味著BALF1會改變細胞的生長並推測其可能會採用Bcl-2相同方式達到細胞癌化的現象。其中包括抑制細胞修復活性及細胞凋亡的進行。然而是否如推測所云，至今證據仍嫌不足，因此我們必須進一步追蹤探討 BALF1 在感染細胞中的功能。EB病毒是屬於DNA腫瘤病毒，它與許多癌症疾病有關。本次研究計劃，我們希望從中獲得 BALF1 基因對其宿主及病毒本身調控機制的資訊，並透過此資訊找出宿主與病毒間互動的關係。藉由深入了解彼此關係，期望發展出抑制EB病毒的藥物及因病毒所產生疾病的基因治療方法。

關鍵詞: EB病毒, DNA修復活性

英文摘要

Epstein-Barr virus (EBV) is a human herpesvirus, which is persistent in B lymphoid cells and triggers transformation and tumorigenesis of latently infected B cells *in vitro* via LMP-induced cellular survival protein, Bcl-2. In other words, Bcl-2 protein in EBV-infected B cells plays an important role in prolonging life span of cells. Recently, an early lytic gene, BALF1, which expressed in both latent and lytic stage and was structurally homologous to Bcl-2 protein, was characterized in EBV infected cells. However the role and function of BALF1 in EBV infected cells still remain to be investigated. According to the present studies, we have known that the result of RT-PCR showed BALF1 had no effect of the expression of EBNA1 but suppressed Zta transcripts. It seems reasonable that BALF1 may be involved in EBV reactivation. In addition, BALF1 induced not only the transcription activity of EBV oncogene LMP1 but also the expression of cell-cycle promoting proteins, c-Myc and E2F1. On the other hand, BALF1 was involved in suppressing the DNA repair activity of cells and the ceramide or α-Fas/INF-γ-mediated cell apoptosis. That means BALF1 seems change cell growth. It is notably implicated BALF1 possibly contributed to trumorigenesis by mimicking the same pathway of Bcl-2, such as suppression of DNA repair and apoptosis. However, these evidences are not enough to determine completely the role of BAlF1 in cells, we still need to accumulate more evidences to clearly address the function of BALF1. EBV is a DNA tumor virus, which is associated with many tumors. In this study, the determination of the function of BALF1 in infected cells will further provide us to realize the relationship of EBV and its associated tumors. Moreover, this information will be applied to the development of drugs for inhibition of its intrinsic activities and non-immunogenic vectors for use in human gene therapy.

Keywords: Epstein-Barr virus, cell DNA repair activity

Introduction

Epstein-Barr virus (EBV) is a human herpesvirus, which establishes a latent, growth-transforming infection in primary human B-lymphocytes. In the transformed B-lymphocytes, EBV expresses only night latent proteins (six EBNAs, three LMPs) and two abundant small RNAs (EBER1, 2) (reviewed by Kieff and Rickinson, 2001). Upon reactivation by inducing agents, several early and late genes of EBV are activated and induced to enter lytic infection. EBV is also associated with many malignant diseases, including Burkitt's lymphoma (BL) (de-The *et al*., 1978), Hodgkin's disease, T-cell lymphoma and nasopharyngeal carcinoma (NPC) (Henle *et al.,* 1970; Henle *et al.,* 1976; Chang *et al.,* 1990).

Apoptosis is important in the elimination of malignant or virally infected cells through a genetic program of enzymatic and morphologic events (Cheng *et al*., 1996, Fadeel *et al*., 1999, Kawanishi 1997, Chau *et al*, 2000). In organism, a famous superfamily, Bcl-2 family, controls cell homeostasis and programmed cell death (apoptosis) (Kawanishi 1997). Bcl-2, which is a prototype of Bcl-2 family, is first identified as a proto-oncogene in follicular lymphoma (Kirsch *et al*., 1999). It is known to cooperate with its family members to maintain lymphoid cells by heterodimers or homodimers. Cell transfomation or apoptosis may be dependent on this interaction of Bcl-2 family members in which the caspase cascade is trigged or inhibited. Therefore, Bcl-2 family members are pivotal in determining the destiny of cells and central to lymphoid homeostasis. Previous studies have shown that EBV encoded a special gene, which was homologous to Bcl-2. This protein was named as BHRF1. Due to the similarity of Bcl-2, scientists are highly interested in the function of BHRF1 and speculated that it may be involved in inhibiting cell apoptosis induced by a various stimuli (Pearson *et al*., 1987, Henderson *et al*., 1993, Foghsgaard and Jaattela, 1997, Fanidi *et al*., 1998). Recently other scientists discovered another Bcl-2-like EBV gene, which was thought to be the second v-Bcl-2 of EBV. It is named as BALF1, which is 0.7 kb in size and shows a predicted 220-amino acid protein in a region of early EBV transcripts, indicating that it inhibits apoptosis through the association of Bax and Bak (Marshall *et al*., 1999). Analysis of BALF1 in amino acid sequences reveals the structure features in functionally important BH domains, BH1 to BH4 are similar to that of Bcl-2 (Kroemer, 1997). The predicted amino acid sequence of BALF1 shows three unique features for a v-Bcl-2. First, a glycine within BH1 domain of BALF1 is replaced

by a serine where in virtually all Bcl-2 family members there is a critical glycine (Yin *et al*., 1994). Mutation of this glycine to alanine in BH1 domain abolishes anti-apoptotic function. Secondly, all other gamma-herpesvirus Bcl-2-like members possess hydrophobic C-termini capable of integrated into organellar membranes. However, EBV BALF1 lacks the C-termini of hydrophobic domain, as do EB1 (Lakshmi *et al*., 1992, Brun *et al*., 1996, Cheng *et al*., 1997). Finally, in contrast to the divergence in the BH4 domain of other Bcl-2 members compared to Bcl-2, BALF1 is very similar to Bcl-2 and Bcl-xl, which region is conserved. Thus the feature unique to BALF1 probably provides its distinct functions compared to those cellular Bcl-2 family members in some respects (Lakshmi *et al*., 1992, Brun *et al*., 1996, Cheng *et al*., 1997, Nava *et al*., 1997, Sarid *et al*., 1997). Interestingly, there is closer similarity between BALF1 and Bcl-2 than between BHRF1 and Bcl-2. It seems to mean that BALF1 could be more potential than BHRF1 in EBV growth transformation of primary B cells and EBV-mediated tumor diseases. However Bellows group provided the opposite results in the function of BALF1. They found that EBV BALF1 lacks anti-apoptotic function of Bcl-2 and impairs the ability of BHRF1 to inhibit apoptosis (Bellows *et al*., 2002). This phenomenon is just like cellular counterpart where anti-apoptotic Bcl-2 proteins modulate the function of pro-apoptotic Bcl-2 members. On the other hand, recent report showed that BALF1 was not only expressed during the very early lytic infection but also expressed during latently infected tumor cells (Marshall *et al*., 1999, Cabras *et al*., 2005). It also proved that BALF1 could render cells to serum independent (Cabras *et al*., 2005). All these results allow us to raise the possibility that BALF1 may be playing an additional role in latently infected tumor cells by modulating the cell transformation and apoptosis. Taken together, those data leave many questions and allow us to be confused and controversial. In other words, it needs to provide more substantial evidences on the study of BALF1 function to unravel the unknown role of BALF1 in infected cells. To date, two latent genes, LMP1 and EBNA1 are thought to be two potential oncogenes for EBV induced transformation (Kilger *et al*., 1998, Leight and Sugden, 2000). However, whether BALF1 is potentially to be the third EBV oncogene needs to further investigated. Therefore**, the purpose of this project is to clarify the function of BALF1 on the EBV infected cells.**

In this study, we first generated a Myc-tagged BALF1 expression plasmid, which encoded a BALF1 protein with a predicted molecular weight of 27 kDa. Overexpression

of BALF1 induced the promoters of LMP1 and Zta, but less effect on EBNA1 promoter. Further examination revealed that BALF1 induced some cell-cycle associated regulators, such as E2F and c-Myc proteins. In addition, the expression of BALF1 suppressed not only ceramide- or anti-Fas/INF-γ-mediated apoptosis but also DNA repair activity. The results showed us that this suppression by BALF1 could be by means of activation of those cell-cycle factors, such as E2F1 and c-Myc or by LMP1-mediated Bcl-2 activation. From the preliminary results, it is implicated that the life cycle of EBV can be regulated by BALF1 via modulating cell transformation-associated proteins during EBV infection.

Results

A approximate 27 kDa of BALF1 protein expressed in cells

To test the function of BALF1 on the EBV genes and cellular genes, we first generated a Myc-tagged BALF1 expression plasmid and examine whether BALF1 expressed after transfection of BALF1 expression plasmid into either 293 or P3HR-1 cells. The result indicated that the molecular mass of the BALF1 protein detected by western blotting was in agreement with the predicted molecular mass of 27kDa (Fig.1).

Expression of BALF1 in EBV positive cells suppresses Zta transcript expression, but has no effect on EBNA1

To further illustrate the effect of BALF1 on EBV genes, EBNA1 and Zta, we introduced BALF1 into P3HR-1 cells (an EBV positive Burkitt's lymphoma cell line) by electroporation and detected the gene expression of EBNA1 and Zta by RT-PCR. The result of RT-PCR showed that overexpression of BALF1 in P3HR1 cells has a less effect on the expression of EBNA1, but obviously suppresses transcript expression of Zta gene 24hr after transfection (Fig.2). That means that BALF1may be involved in the EBV reactivation during its life cycle.

Expression of BALF1 results in activation LMP1 promoter, but less effect on EBNA1 promoter

EBNA1 and LMP1 are the EBV latent genes, which are associated with cell transformation and maintenance of episomal form. It will be interesting for us to test whether BALF1 regulate and determine the fate of EBV life cycle. In an attempt to test possibility, we cotransfected BALF1 into 293 cells with either LMP1 promoter reporter gene (p12-Luc) or EBNA1 promoter reporter plasmid (Qp125) for 24hr. The promoter activity of the transfected cells was measured by luciferase assay system (Promega)

24hr post transfection. As shown in Fig. 3, the expression of BALF1 in cells clearly activated about 2-3 fold of LMP1 promoter activity (panel B), but had less effect on EBNA1 promoter activity (panel A). The results indicated the role of BALF1 in EBV-infected cells seems to contribute to cell transformation.

BALF1 activates the expression of c-Myc and E2F1 proteins in 293 cells

It has previously been proposed that the inhibition of EBV reactivation by cell cycle- promoting factors, c-Myc and E2F1 occurs through inhibition of the transactivation function of Zta protein (Young *et al*., 2000; DeGregori, 2002; Lin *et al*., 2004). That means that c-Myc or E2F1 proteins may play an important role in regulation of EBV reactivation and promote cell proliferation. Our current data indicated that BALF1 inhibited Zta transcripts and activated LMP1 promoter activity. It will be very interesting to see for us whether BALF1 affects on the EBV gene expression is due to the regulation of both c-Myc and E2F1 protein. To prove this possibility, the 293 cells or 293 cells were transfected with the BALF1expression plasmid (pc-Myc-BALF1) or vector control (pc-Myc). 24hr after transfection, the transfected cells were used to perform Western Blotting. The results revealed that the expression of BALF1 in 293 cells obviously induced the expression of both c-Myc and E2F1 protein (Fig. 4).

BALF1 suppresses DNA repair activity of 293 cells

c-Myc is a proto-oncogene that normally regulates cellular growth and proliferation (Evan *et al*., 1992). Overexpression of c-Myc is thought to contribute to tumorigenesis by inducing genomic instability, such as suppression of DNA repair activity (Li and Dang, 1999). Because BALF1 activated the expression of c-Myc protein in 293 cells (Fig 4), we further address whether BALF1 induces genomic instability by means of induction of c-Myc protein. To prove this possibility, we used HCR assay to determine the DNA repair activity. In this assay, the plasmids with either BALF1 or empty vector, and either a UV-treated or un-treated pCMV-Luc luciferase reporter were cotransfected into 293 cells. The fold of HCR assay was determined as described in Material and Methods. As shown in Fig. 5, the cells with BALF1 have a higher fold of HCR than the cells with an empty vector do. It means that BALF1 suppresses the DNA repair activity of 293 cells.

BALF1 inhibits apoptotic DNA fragmentation induced by α-Fas/INF-γ

Members of E2F1 family, especially E2F1 have important roles in regulating cell

proliferation and apoptosis. As shown in Fig. 5, BALF1 induced E2F1 expression in 293 cells. That means whether BALF1 could trigger cell proliferation and apoptosis through induction of E2F1 protein. To address the possibility, the Hela cells were first transfected with BALF1 expression plasmid or the empty vector. 24hr post transfection, the genomic DNA of the transfected cells was used to perform apoptotic DNA fragmentation assay. The result indicated that BALF1 expression inhibits not only transfection-induced apoptosis but also α-Fas/INF-γ-induced apoptosis (Fig. 6).

Expression of BALF1 in EBV positive cells results in inhibition of ceramide-induced apoptosis

Bcl-2 is important for inhibition of cell apoptosis and maintenance of homeostasis. Owing to the similarity of BALF1 to Bcl-2 in structure, we wanted to see whether the function of BALF1 was similar to that of Bcl-2, which inhibiting the cell apoptosis. To prove the possibility, the P3HR-1 cells with or without the ectopic BALF1 were treated with ceramide (an apoptosis-inducing agent) for various time-course, such as 0, 6, 12 and 24 hr. As shown in Fig.7, the BALF1 protein obviously had the ability to suppress ceramide-mediated apoptosis 24 hr post-treatment. The result indicated that BALF1 indeed interfered with signal pathway of ceramide-mediated apoptosis via unknown mechanism.

Discussion

EBV is thought to be a DNA tumor virus because of its association with several human cancers, such as B cell lymphoma, Hodgkin's disease, and nasopharyngeal carcinoma (Kieff and Rickinson, 2001). Despite studying on EBV for many years, the precise mechanism by which the EBV drives primary B cells from quiescence into the activation state and develops into the EBV-associated tumorigenesis are still partially understood. On the studies of the recombinant EBV, it have been demonstrated that only six EBV latent genes, EBNA1, 2, 3A, 3C, LP, and LMP1 are crucial to promote cell transformation (Dirmeier *et al.*, 2003; Humme *et al.*, 2003). In addition to these six latent genes, two Bcl-2-like EBV proteins, BHRF1 and BALF1 can be still the possible candidates to cause EBV-mediated tumors in fact. BHRF1 has an anti-apoptotic activity, but is rarely expressed in nasopharyngeal carcinoma. As for BALF1, it is expressed not only in lytic infection, but also in latently infected tumors such as BL and NPC (Gabes *et al*, 2005). Therefore, BALF1 is considered to a most possible candidate for EBV mediated cell transformation. However the possible significance of BALF1 in the

latently infected tumors still remains to be characterized so far.

DNA repair are essential for the maintenance of genomic integrity. Improperly repaired chromosomal damage results in tumorigenesis. Recent report showed Bcl-2 suppressed DNA repair by enhancing c-Myc transcriptional activity (Tsujimoto *et al*.,1985; McDonnell and Korsmeyer, 1991). In preliminary study, we have obtained that a BALF1 protein with a predicted molecular weight of 27 kDa had a less effect on the transcript expression of EBV EBNA1 but suppressed the transcripts of Zta gene in P3HR1. This result allows us to speculate that BALF1 could be involved in EBV reactivation. Meanwhile, the data also showed that BALF1 induced the expression of cell-cycle associated regulators, c-Myc and E2F1 and suppressed the DNA repair activity. According to the preliminary results, we logically speculated the suppression of cell DNA repair activity by BALF1 could be due to activation of these two cell-cycle regulators, c-Myc and E2F1. On the other hand, the RT-PCR result showed that the BALF1 notably activated the transcription activity of EBV oncogene LMP1 in the latently infected cells. As we know, LMP1 is a 63 kD membrane protein, which engaged the TRAF proteins to promote cell transformation and mediated DNA repair, which resulted in accumulation of un-repaired DNA and consequent genomic instability, to lead to tumorigenesis (Liu *et al*., 2004). It is therefore speculated that BALF1 could contribute cell oncogenesis based on activation of LMP1 function. Currently, several reports indicated that Bcl-2 contribute to the development of carcinogenesis by not only attenuating DNA repair but also inhibiting apoptosis (Linete *et al*., 1995; Strasser *et al*., 1993). Intriguingly, we also observed that BALF1 possessed the ability to suppress cell apoptosis mediated by ceramide- or α-Fas/INF-α in the apoptotic assay. It seems logical that BALF1may develop carcinogenesis by mimicking the same way of Bcl-2 developed carcinogenesis. Therefore, if we further explore the relationship of BALF1 and more other cell-cycle regulators or observe the formation of tumors by BALF1 is due to change which pathways of apoptosis or genomic instability. That will be helpful to understand the role of BALF1 in infected cells. Though EBV induced tumorigenesis is still not clear so far, it is believed that the results of this project will help us to find the right way to understanding of how EBV cause a tumor.

Materials and Methods

Cell culture

EBV-positive Burkitt's lymphoma (BL) cell lines, Rael (type I) and P3HR-1 or EBV-negative burkitt's lymphoma cells, CA46 and Akata, were maintained in RPMI 1640 (GIBCO, USA) supplemented with 15% (v/v) fetal bovine serum (FBS). Hela cells and 293 cells were cultured in Delbecco's modified Eagle medium (DMEM) with 10% FBS.

Plasmid construction

The DNA fragment covering the full-length BALF1are generated by polymerase chain reaction (PCR) with genomic DNA isolated from Rael cells and one set of primers. The BALF1 fragment synthesized by PCR was inserted into EcoRI site of pCMV-Myc expression vector (Clontech), which consisted of the CMV promoter and BALF1 open reading fragment named as pCMV-Myc-BALF1.

RNA extraction and RT-PCR

RNA was extracted from the cells according to the manufacturer's instructions (Viogen) and then the extracted RNA was used to carry out RT-PCR. Briefly, the five μg of RNA was added into the 20ul of reaction, which contained 1X reverse transcriptase (RT) buffer and 1U of RT to generate those cDNA fragments. One hr after incubation at 42^{o}C, the one fourth of cDNA reaction was used to perform the PCR analysis with three sets of the specific primers (GAPDH, EBNA1 and Zta). Finally the products of PCR were subject to be fractionated by using 2% agarose gel electrophoresis and analyzed under UV after staining.

DNA transfection and Luciferase assays

The indicated amount of expression plasmids were introduced into the suspension cells (Rael, LCL) by electroporation at 960 μF and 0.22 kV using the Gene Pulser (Bio-Rad, USA) and the transfected cells were incubated for 24 hr. The cells were then harvested and lysed in 50 μl of lysis buffer (Promega, USA). The protein concentration of the cell lysate was measured by using a Bio-Rad protein assay reagent (Bio-Rad, USA). A 50μg- sample of protein extract was used to measure the luciferase activity in an illuminometer (Berthold autolumat model LB953, Germany). The epithelial cells were transfected with the indicated plasmids through Lipofectamine 2000 (Invitrogen). 48hr after incubation, the steps of luciferase assay in epithelial cells were similar to that in the suspension cells as described above.

Western blotting

Western blot analysis was carried out by incubation of cell lysates with the indicated antibody. Briefly, the cells were lysed for 30 min. on ice in 0.5% NP-40 lysis buffer containing 50mM HEPES (pH 7.4) 250mM NaCl, 10% glycerol, 2mM EDTA, 1mM PMSF, 2μg / ml aprotinin, 2μg / ml pepstatin and 2μg / ml leupeptin. Cell debris was removed by centrifugation at 10,000Xg for 10 min at 4^{o}C. The protein concentration of cell lysate was measured by Bradford method (Bio-Red, Richmond CA). The lysate was applied to the proper percentage of SDS-PAGE and then transferred to the nitrocellulose membrane. The membrane was incubated with polyclonal antibodies of anti-Myc (Santa Cruz Biotechnology Inc.), -E2F1 or -actin and reacted with the horseradish peroxidase-conjugated anti-rabbit antibodies as secondary antibody. After ECL treatment, the membrane was exposed to Kodak BioMax film.

DNA frgmentation assay

$5x10^{6}$ cells were collected in a 1.5ml tube and washed with 1xPBS and then incubated in 500ul of lysis buffer at 4^{0}C for 20 min. After 12,000rpm centrifugation at 4^{0}C, the cell extract was transferred into a new tube and incubated with 200u/ml proteinase K at 50^{0}C for 8hr. After proteinase digestion, RNase was added into the mixture for incubation at 37^{0}C for 6hr. The genomic DNA was purified from the treated cell extract by using equal volume of phenol/cholorform (1:1). After purification, the genomic DNA was precipitated by using an equal volume of isopropanol and an one-tenth of sodium acetate at -20^{0}C for overnight. The genomic DNA was pelletted down by centrifugation at 4^{0}C at 12,000rpm for 30min. Finally the genomic DNA was dissolved in 8ul of TE buffer and analyzed apoptosis by 2% agarose gel.

HCR activity assay

HCR activity assay was previously described by using a luciferase reporter plasmid (Tran *et al*., 2002; Liu *et al*., 2004). Briefly, the luciferase plasmid (pCMV-Luc) either UV-treated or un-treated; together with renilla plasmid as internal control are cotransfected into the cells with BALF1 expression plasmid or empty vector by using Lipofectamine 2000 (Invitrogen). 24 hr post transfection, the cell lysates from different treatments of cells were prepared to detect the activity of luciferase by using Dual-Glo Luciferase Assay System according to manufacturer's stand procedure (Promega USA). Fold of HCR were determined as follows (Liu *et al*., 2004): First, the repair conversion was calculated through dividing the normalized luciferase activity from cells transfected

with UV-treated pCMV-Luc by non-treated pCMV-Luc tranfectants. The fold of HCR was calculated through dividing the repair conversion of effector tansfectants by that of vector tansfectants.

Detection of apoptotic cells by flow cytometry

The presence of apoptotic cells was detected by propidium iodide staining followed by flow cytometric analysis of hypodiploid cells. Cells (1 x 10^6) were fixed in 2 ml of 70% ethanol/PBS and placed at 4°C for at least 30 min. After centrifugation, cells were resuspended in 800 μl of PBS containing 40 μg/ml of propidium iodide (Sigma-Aldrich) and 100 μg/ml of RNase (Calbiochem), and incubated at room temperature for 30 min. The DNA content of cells was analyzed on a FACScan (Becton Dickinson, Mountain View, CA) with excitation set at 488 nm.

Reference

Bellows, D. S., Howell, M., Pearson, C., Hazlewood, S. A., and Hardwick, J. M. (2002). Epstein-Barr virus BALF1 is a BCL-2-like antagonist of the herpesvirus antiapoptotic BCL-2 proteins. **J Virol** *76*, 2469-2479.

Bissonnette, R. P., Echeverri, F., Mahboubi, A., and Green, D. R. (1992). Apoptotic cell death induced by c-myc is inhibited by bcl-2. **Nature** *359*, 552-554.

Brummelkamp, T. R., Bernards, R., and Agami, R. (2002). A system for stable expression of short interfering RNAs in mammalian cells. **Science** *296*, 550-553.

Brun, A., Rivas, C., Esteban, M., Escribano, J. M., and Alonso, C. (1996). African swine fever virus gene A179L, a viral homologue of bcl-2, protects cells from programmed cell death. **Virology** *225*, 227-230.

Cabras, G., Decaussin, G., Zeng, Y., Djennaoui, D., Melouli, H., Broully, P., Bouguermouh, A. M., and Ooka, T. (2005). Epstein-Barr virus encoded BALF1 gene is transcribed in Burkitt's lymphoma cell lines and in nasopharyngeal carcinoma's biopsies. **J Clin Virol** *34*, 26-34.

Chang, Y. S., Shih, L. Y., Peng, C. L., Chen, S. H., and Liu, S. T. (1990). Characterization of two newly established EBV-containing lymphoblastoid cell lines from patients with myeloid leukemias. **Leuk Res** *14*, 309-320.

Chau, B. N., Cheng, E. H., Kerr, D. A., and Hardwick, J. M. (2000). Aven, a novel inhibitor of caspase activation, binds Bcl-xL and Apaf-1. **Mol Cell** *6*, 31-40.

Cheng, E. H., Levine, B., Boise, L. H., Thompson, C. B., and Hardwick, J. M. (1996). Bax-independent inhibition of apoptosis by Bcl-XL. **Nature** *379*, 554-556.

Cheng, E. H., Nicholas, J., Bellows, D. S., Hayward, G. S., Guo, H. G., Reitz, M. S., and Hardwick, J. M. (1997). A Bcl-2 homolog encoded by Kaposi sarcoma-associated virus, human herpesvirus 8, inhibits apoptosis but does not heterodimerize with Bax or Bak. **Proc Natl Acad Sci U S A** *94*, 690-694.

de-The, G., Geser, A., Day, N. E., Tukei, P. M., Williams, E. H., Beri, D. P., Smith, P. G., Dean, A. G., Bronkamm, G. W., Feorino, P., and Henle, W. (1978). Epidemiological evidence for causal relationship between Epstein-Barr virus and Burkitt's lymphoma from Ugandan prospective study. **Nature** *274*, 756-761.

DeGregori, J. (2002). The genetics of the E2F family of transcription factors: shared functions and unique roles. **Biochim Biophys Acta** *1602*, 131-150.

Dirmeier, U., Neuhierl, B., Kilger, E., Reisbach, G., Sandberg, M. L., and Hammerschmidt, W. (2003). Latent membrane protein 1 is critical for efficient growth transformation of human B cells by epstein-barr virus. **Cancer Res** *63*, 2982-2989.

Evan, G. I., Wyllie, A. H., Gilbert, C. S., Littlewood, T. D., Land, H., Brooks, M., Waters, C. M., Penn, L. Z., and Hancock, D. C. (1992). Induction of apoptosis in fibroblasts by c-myc protein. **Cell** *69*, 119-128.

Fadeel, B., Zhivotovsky, B., and Orrenius, S. (1999). All along the watchtower: on the regulation of apoptosis regulators. **Faseb J** *13*, 1647-1657.

Fanidi, A., Hancock, D. C., and Littlewood, T. D. (1998). Suppression of c-Myc-induced apoptosis by the Epstein-Barr virus gene product BHRF1. **J Virol** *72*, 8392-8395.

Felsher, D. W., and Bishop, J. M. (1999). Transient excess of MYC activity can elicit genomic instability and tumorigenesis. **Proc Natl Acad Sci U S A** *96*, 3940-3944.

Foghsgaard, L., and Jaattela, M. (1997). The ability of BHRF1 to inhibit apoptosis is dependent on stimulus and cell type. **J Virol** *71*, 7509-7517.

Gratama, J. W., Oosterveer, M. A., Zwaan, F. E., Lepoutre, J., Klein, G., and Ernberg, I. (1988). Eradication of Epstein-Barr virus by allogeneic bone

marrow transplantation: implications for sites of viral latency. **Proc Natl Acad Sci U S A** *85*, 8693-8696.

Hannon, G. J., Chubb, A., Maroney, P. A., Hannon, G., Altman, S., and Nilsen, T. W. (1991). Multiple cis-acting elements are required for RNA polymerase III transcription of the gene encoding H1 RNA, the RNA component of human RNase P. **J Biol Chem** *266*, 22796-22799.

Henderson, S., Huen, D., Rowe, M., Dawson, C., Johnson, G., and Rickinson, A. (1993). Epstein-Barr virus-coded BHRF1 protein, a viral homologue of Bcl-2, protects human B cells from programmed cell death. **Proc Natl Acad Sci U S A** *90*, 8479-8483.

Henderson, S., Rowe, M., Gregory, C., Croom-Carter, D., Wang, F., Longnecker, R., Kieff, E., and Rickinson, A. (1991). Induction of bcl-2 expression by Epstein-Barr virus latent membrane protein 1 protects infected B cells from programmed cell death. **Cell** *65*, 1107-1115.

Henle, G., and Henle, W. (1976). Epstein-Barr virus-specific IgA serum antibodies as an outstanding feature of nasopharyngeal carcinoma. **Int J Cancer** *17*, 1-7.

Henle, W., Henle, G., Ho, H. C., Burtin, P., Cachin, Y., Clifford, P., de Schryver, A., de-The, G., Diehl, V., and Klein, G. (1970). Antibodies to Epstein-Barr virus in nasopharyngeal carcinoma, other head and neck neoplasms, and control groups. **J Natl Cancer Inst** *44*, 225-231.

Humme, S., Reisbach, G., Feederle, R., Delecluse, H. J., Bousset, K., Hammerschmidt, W., and Schepers, A. (2003). The EBV nuclear antigen 1 (EBNA1) enhances B cell immortalization several thousandfold. **Proc Natl Acad Sci U S A** *100*, 10989-10994.

Kawanishi, M. (1997). Epstein-Barr virus BHRF1 protein protects intestine 407 epithelial cells from apoptosis induced by tumor necrosis factor alpha and anti-Fas antibody. **J Virol** *71*, 3319-3322.

Kilger, E., Kieser, A., Baumann, M., and Hammerschmidt, W. (1998). Epstein-Barr virus-mediated B-cell proliferation is dependent upon latent membrane protein 1, which simulates an activated CD40 receptor. **Embo J** *17*, 1700-1709.

Kirsch, D. G., Doseff, A., Chau, B. N., Lim, D. S., de Souza-Pinto, N. C., Hansford, R., Kastan, M. B., Lazebnik, Y. A., and Hardwick, J. M. (1999).

Caspase-3-dependent cleavage of Bcl-2 promotes release of cytochrome c. **J Biol Chem** *274*, 21155-21161.

Kolesnick, R. N., Goni, F. M., and Alonso, A. (2000). Compartmentalization of ceramide signaling: physical foundations and biological effects. **J Cell Physiol** *184*, 285-300.

Kroemer, G. (1997). The proto-oncogene Bcl-2 and its role in regulating apoptosis. **Nat Med** *3*, 614-620.

Leight, E. R., and Sugden, B. (2000). EBNA-1: a protein pivotal to latent infection by Epstein-Barr virus. **Rev Med Virol** *10*, 83-100.

Li, Q., and Dang, C. V. (1999). c-Myc overexpression uncouples DNA replication from mitosis. **Mol Cell Biol** *19*, 5339-5351.

Lin, Z., Yin, Q., and Flemington, E. (2004). Identification of a negative regulatory element in the Epstein-Barr virus Zta transactivation domain that is regulated by the cell cycle control factors c-Myc and E2F1. **J Virol** *78*, 11962-11971.

Linette, G. P., Hess, J. L., Sentman, C. L., and Korsmeyer, S. J. (1995). Peripheral T-cell lymphoma in lckpr-bcl-2 transgenic mice. **Blood** *86*, 1255-1260.

Marshall, W. L., Yim, C., Gustafson, E., Graf, T., Sage, D. R., Hanify, K., Williams, L., Fingeroth, J., and Finberg, R. W. (1999). Epstein-Barr virus encodes a novel homolog of the bcl-2 oncogene that inhibits apoptosis and associates with Bax and Bak. **J Virol** *73*, 5181-5185.

McDonnell, T. J., and Korsmeyer, S. J. (1991). Progression from lymphoid hyperplasia to high-grade malignant lymphoma in mice transgenic for the t(14; 18). **Nature** *349*, 254-256.

Nava, V. E., Cheng, E. H., Veliuona, M., Zou, S., Clem, R. J., Mayer, M. L., and Hardwick, J. M. (1997). Herpesvirus saimiri encodes a functional homolog of the human bcl-2 oncogene. **J Virol** *71*, 4118-4122.

Nicoletti, I., Migliorati, G., Pagliacci, M. C., Grignani, F., and Riccardi, C. (1991). A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. **J Immunol Methods** *139*, 271-279.

Pearson, G. R., Luka, J., Petti, L., Sample, J., Birkenbach, M., Braun, D., and Kieff, E. (1987). Identification of an Epstein-Barr virus early gene encoding a second component of the restricted early antigen complex. **Virology** *160*, 151-161.

Rao, L., Debbas, M., Sabbatini, P., Hockenbery, D., Korsmeyer, S., and White, E. (1992). The adenovirus E1A proteins induce apoptosis, which is inhibited by the E1B 19-kDa and Bcl-2 proteins. **Proc Natl Acad Sci U S A** *89*, 7742-7746.

Ruvolo, P. P. (2003). Intracellular signal transduction pathways activated by ceramide and its metabolites. **Pharmacol Res** *47*, 383-392.

Sarid, R., Sato, T., Bohenzky, R. A., Russo, J. J., and Chang, Y. (1997). Kaposi's sarcoma-associated herpesvirus encodes a functional bcl-2 homologue. **Nat Med** *3*, 293-298.

Strasser, A., Harris, A. W., and Cory, S. (1993). E mu-bcl-2 transgene facilitates spontaneous transformation of early pre-B and immunoglobulin-secreting cells but not T cells. **Oncogene** *8*, 1-9.

Tsujimoto, Y., Gorham, J., Cossman, J., Jaffe, E., and Croce, C. M. (1985). The t(14;18) chromosome translocations involved in B-cell neoplasms result from mistakes in VDJ joining. **Science** *229*, 1390-1393.

Verheij, M., Bose, R., Lin, X. H., Yao, B., Jarvis, W. D., Grant, S., Birrer, M. J., Szabo, E., Zon, L. I., Kyriakis, J. M., *et al.* (1996). Requirement for ceramide-initiated SAPK/JNK signalling in stress-induced apoptosis. **Nature** *380*, 75-79.

Yin, X. M., Oltvai, Z. N., and Korsmeyer, S. J. (1994). BH1 and BH2 domains of Bcl-2 are required for inhibition of apoptosis and heterodimerization with Bax. **Nature** *369*, 321-323.

Young, L. S., Dawson, C. W., and Eliopoulos, A. G. (2000). The expression and function of Epstein-Barr virus encoded latent genes. **Mol Pathol** *53*, 238-247.

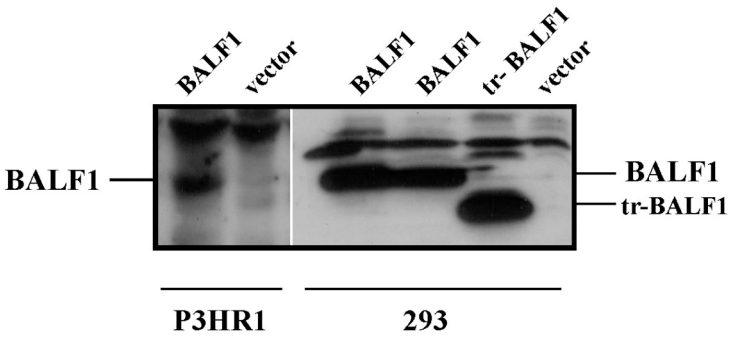

Fig. 1 The expression of BALF1 in P3HR1 and 293 cells. The cells (P3HR1 or 293) were transfected with full-length-BALF1 plasmid(BALF1), truncated BALF1(tr-BALF1) or pc-Myc(vector) by using electroporation or lipofectamine. 24hr post transfection, the expression of BALF1 protein was analyzed by western blotting with anti-Myc antibody.

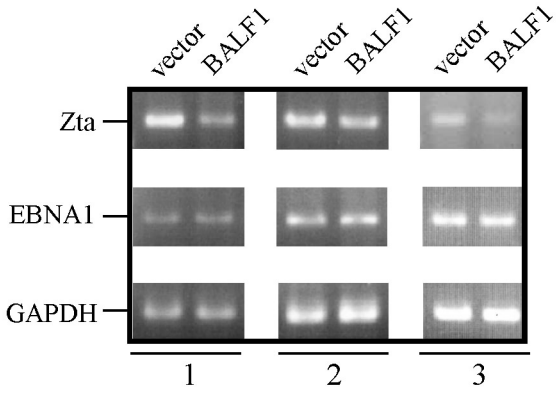

Fig. 2 The expression of EBV EBNA1 and Zta transcripts regulated by BALF1. The P3HR1 cells were transfected with either pc-Myc-BALF1 expression plasmid (BALF1) or pc-Myc (vector) by electroporation. 24 hr after transfection, the transfected cells were used to detect the transcripts of EBNA1 and Zta by RT-PCR. The number of 1, 2 and 3 represents three independent experiments.

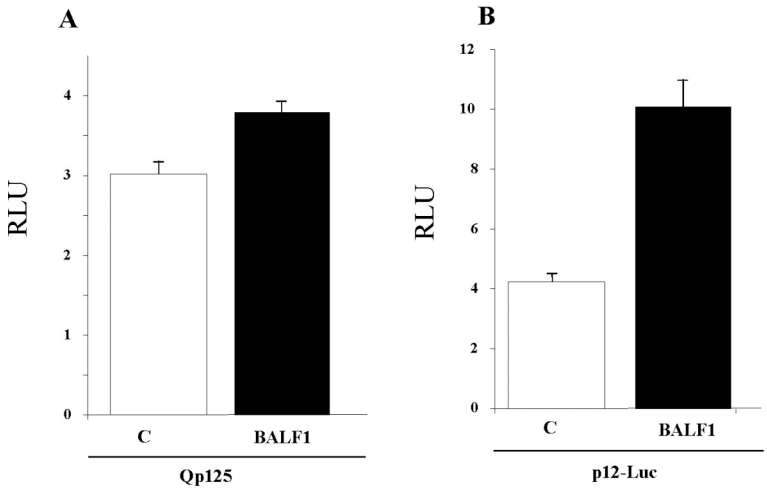

Fig. 3 The effect of BALF1 on the EBV Q, LMP 1 and Z promoters The BALF1 expression plasmid was transfected into 293 cells with Q promoter, Qp125 (panel A), or LMP1 promoter, p12-Luc (panel B). 24hr post tansfection, the promoter activity was determined by luciferase assay as described in Materials and Methods. C serves as empty vector control. the number of Y axis is the relative luciferase unit (RLU) normalized by internal control (Renilla). The data shown in panel A and C are representative results of two independent experiments.

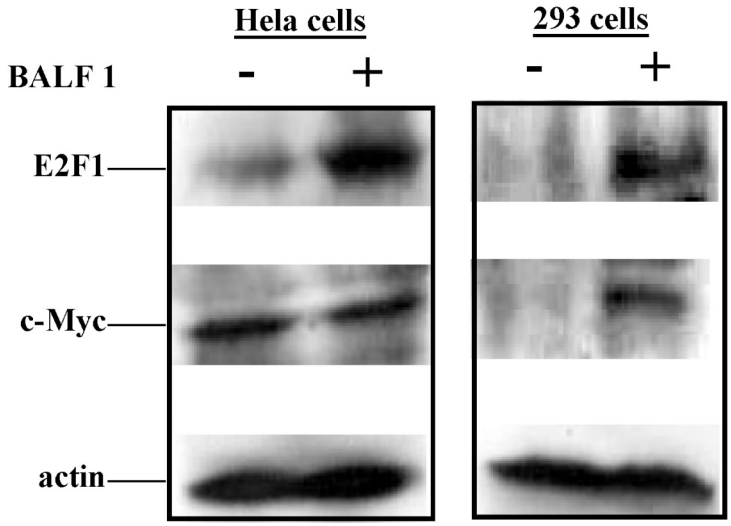

Fig. 4 BALF 1 regulates the expression of c-Myc and E2F1 proteins. The cells were tansfected with pc-Myc-BALF1 expression plasmid or pc-Myc vector. 24hr after transfection, the cell lysates from the transfected cells were used to perform the western blot analysis with anti-c-Myc, E2F1 and actin antibodies. the actin acts as internal control.

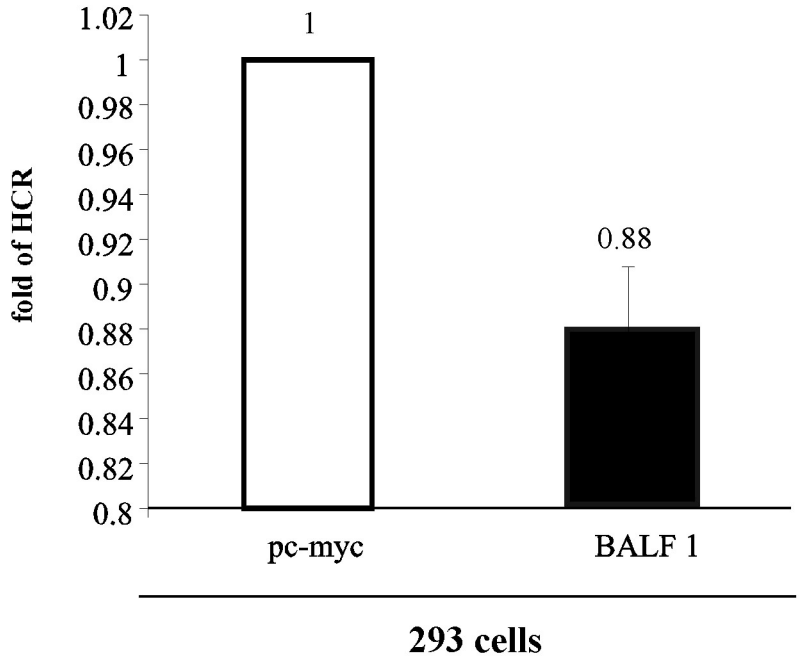

Fig. 5 BALF1 suppresses the DNA repair activity of 293 cells. The 293 cells were cotransfected with 3 μg of the pc-Myc-BALF1 expression plasmid (BALF1) or 3 μg of empty vector (pc-Myc), together with an UV-treated (0.1J/cm^2) pCMV-Luc reporter plasmid (500ng) or the un-treated plasmid and a renilla plasmid (50ng), which serves as a internal control for normalizing the luciferase activity. 24hr post transfection, luciferase activity were determined . Fold of HCR represents DNA repair activity, which was calculated as described in Materials and Methods. The data of HCR represents the average of at least three independent experiments.

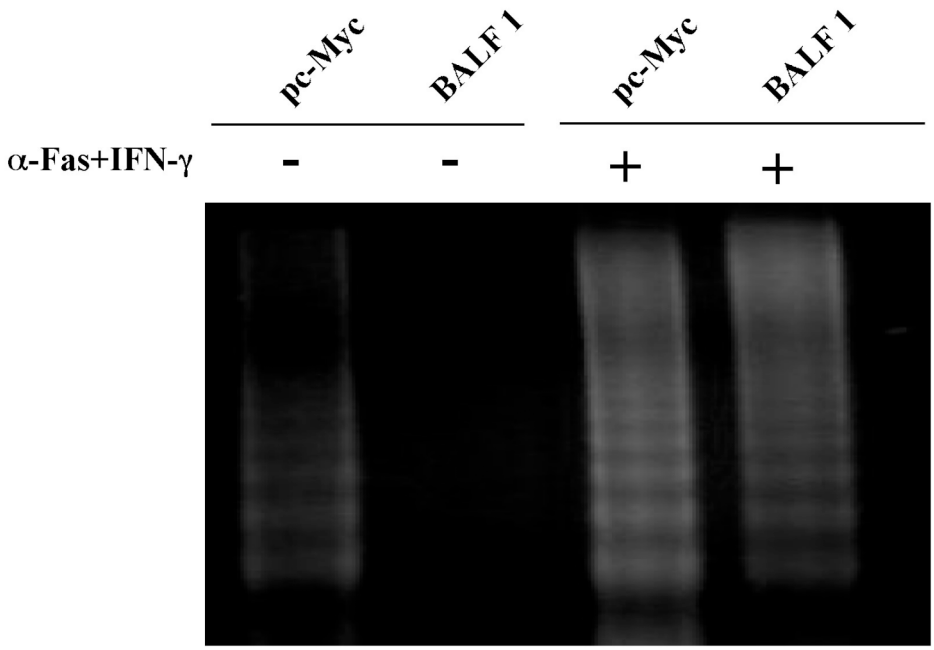

Fig. 6 The BALF 1 inhibits the apoptotic DNA fragmentation of cells. The Hela cells were transfected with a BALF 1 expression plasmid (BALF1) or an empty plasmid (pc-Myc). 24hr after transfection, the genomic DNA of the transfected cells was purified by an equal volume of phenol/chlorofrom solution. The DNA frgmentation ladder was fractionated on 2% agarose gel electophoresis as described in Materials and Methods.

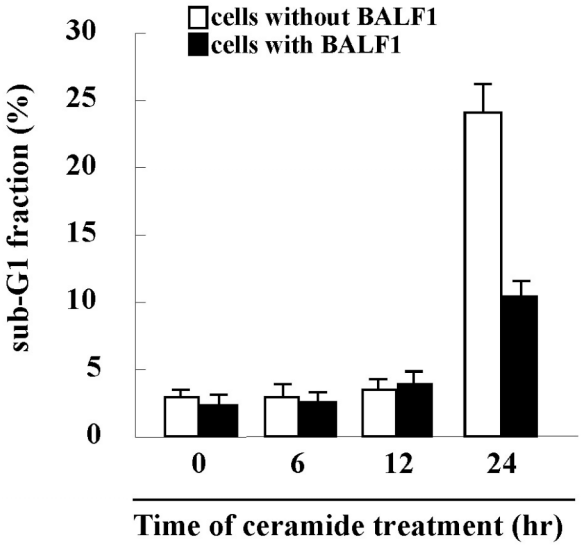

Fig. 7 Flow cytometry analysis of sub-G1 fraction in P3HR-1 cells transfected with BALF1. The EBV positive P3HR1 cells were transfected with pcMyc-BALF1 or empty vector. 24hr post transfection, the cells were collected at indicated time points to analyze the sub-G1 cell percentage by flow cytometry with propidium iodide staining. The data were representative results of two independent experiments.

成果自評

1. To research for he mechanism of EBV transformation in primary B cells is always our major goal in the future work. In this project, our purpose is to demonstrate the role of BALF1 in infected cells. The content and experimental design of this project very similar to that of the proposed original project. This project is only completed to about 50 percent, because of the lack of long-term support for human and financial resources. If NSC continues to support more human and financial resources, this project must be finished soon.
2. There are some experiments, which need to be improved in this project, such as the systems of ChIP and cell apoptosis. If the defects of these experiments can be corrected, it is believed that we will succeed in finishing the proposed project.
3. As this project is completely finished, we will not only publish our project on the science Journal but also establish a set of new therapeutic methods for EBV induced diseases. In addition, BALF1 can be a new diagnostic marker of EBV mediated tumors.