

## Original Article

# Dual expression of Epstein-Barr virus, latent membrane protein-1 and human papillomavirus-16 E6 transform primary mouse embryonic fibroblasts through NF- $\kappa$ B signaling

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**Abstract:** The prevalence of Epstein-Barr virus (EBV) and high-risk human papilloma virus (HPV) infections in patients with oral cancer in Okinawa, southwest islands of Japan, has led to the hypothesis that carcinogenesis is related to EBV and HPV co-infection. To explore the mechanisms of transformation induced by EBV and HPV co-infection, we analyzed the transformation of primary mouse embryonic fibroblasts (MEFs) expressing EBV and HPV-16 genes, alone or in combination. Expression of EBV latent membrane protein-1 (LMP-1) alone or in combination with HPV-16 E6 increased cell proliferation and decreased apoptosis, whereas single expression of EBV nuclear antigen-1 (EBNA-1), or HPV-16 E6 did not. Co-expression of LMP-1 and E6 induced anchorage-independent growth and tumor formation in nude mice, whereas expression of LMP-1 alone did not. Although the singular expression of these viral genes showed increased DNA damage and DNA damage response (DDR), co-expression of LMP-1 and E6 did not induce DDR, which is frequently seen in cancer cells. Furthermore, co-expression of LMP-1 with E6 increased NF- $\kappa$ B signaling, and the knockdown of LMP-1 or E6 in co-expressing cells decreased cell proliferation, anchorage independent growth, and NF- $\kappa$ B activation. These data suggested that expression of individual viral genes is insufficient for inducing transformation and that co-expression of LMP-1 and E6, which is associated with suppression of DDR and increased NF- $\kappa$ B activity, lead to transformation. Our findings demonstrate the synergistic effect by the interaction of oncogenes from different viruses on the transformation of primary MEFs.

**Keywords:** LMP-1, E6, co-expression, transformation, primary mouse embryonic fibroblast, NF- $\kappa$ B

## Introduction

Tumor viruses transform cells by expression of virally-encoded oncogenes and are associated with a variety of malignancies. Among them, Epstein-Barr virus (EBV) and Human papillomavirus (HPV) are the causative agents in multiple malignancies [1, 2]. EBV latent genes are differentially expressed in these malignancies [1]. Primary human B lymphocytes are immortalized by EBV infection *in vitro* into lymphoblastoid cell lines (LCLs) because of latent genes expression. Infection of EBV with mutation of single latent gene such as EBV nuclear antigen 2 (EBNA-2), EBNA-3A, EBNA-3C, or LMP-1, show loss of induction of LCLs demonstrating that immortalization of primary lymphocytes require

synergism of these latent genes [3-5]. However LCLs have high telomerase activity and genomic instability, tumorigenesis by LCLs requires additional genetic alterations in the host [6].

HPV-encoded genes, particularly E6 and E7 from high-risk HPV strains are essential for transformation [7, 8]. Although these genes expressions immortalize primary rodent cells [9], E6 or E7 expression alone did not induce transformation [10]. In addition, co-expression of E6 and E7, with activated ras is required for inducing transformation in primary cells [11].

The mechanistic association between dual infection with two types of virus and carcinogenesis is not well understood. Very few reports

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**Table 1.** Primers used for PCR amplification of EBV and HPV genes

Viral gene	Primer sequence
EBV LMP-1	Forward: GGAATTCCTTCTCAACTGCCTTGCT
	Reverse: AACCGGTTGCAAGCCTATGACATGGTAATG
EBV EBNA-1	Forward: GGAATTCCTTGATCTCTTTTAGTGGAATCATGTC
	Reverse: CCGCTCGAGCGGGGCGTCTCCTAACAGTTACA
HPV16 E6	Forward: GAAGATCTCAAGCAGACATTTTATGCACC
	Reverse: CCGCTCGAGCGTTACAGCTGGGTTTC

directly demonstrate transformation induced by synergistic effect of viral co-infection. EBV-infected Human herpes virus type 8 (HHV-8)-positive primary effusion lymphoma cells have increased tumorigenesis in SCID mice, indicating viral co-operation in cancer development [12]. Although Al Moustafa et al suggested a possible association between HPV and EBV infections and human oral carcinogenesis [13], possible associations between HPV and EBV dual infection and cancer remain to be clarified.

Tshako et al reported higher HPV infection rates in oral squamous cell carcinoma patients in Okinawa, southwest islands in Japan [14, 15] and they also demonstrated many positive signals of HPV DNA integration into the nuclei of oral squamous cell carcinoma in Okinawa. Both high prevalence of and a high integration rate of HPV suggests that HPV is related to oral squamous cell carcinoma in Okinawa. Also, > 70% of oral cancer in Okinawa were positive for EBV DNA and expression of LMP-1 and EBNA-2 [15-17]. Similarly, 82.5% of oral cancers in Taiwan where locate close to Okinawa exhibit EBV infection and express latent genes and some structural proteins [18].

Furthermore, 47% of nasopharyngeal carcinomas in Taiwan and 60% (36/60) of oral cancers in Okinawa were co-infected with EBV and HPV [15, 19]. Interestingly, only 7.3% (3/41) of oral cancers in Sapporo, northern Japan, were co-infected [15]. The rates of co-infection reflect the rates of single viral infection with either EBV or HPV: ~75% for both viruses in Okinawa versus only 40.5% and 26.2%, respectively, in Sapporo.

Based on these molecular epidemiological data, we hypothesized that malignant transformation of oral cancers in Okinawa are caused

by EBV and HPV dual infection. We showed that mouse embryonic fibroblast (MEF) cell lines were oncogenically transformed by co-expression of EBV LMP-1 and HPV-16 E6, whereas expression of each gene individually was not sufficient. This transformation occurred through suppression of DNA damage response (DDR) and activation of NF- $\kappa$ B. Knock down of LMP-1 or E6 in the cells with co-expressing

these genes reversed the increase in cell proliferation and anchorage-independent growth and reduced NF- $\kappa$ B activation. Our findings provide insights into the molecular mechanism of transformation caused by synergistic expression of HPV and EBV genes.

### Materials and methods

#### Cell cultures

CF-1 MEFs were purchased from ATCC (Manassas, VA) and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 15% fetal bovine serum (FBS). EBV transformed lymphocyte cell line B95-8 was maintained in RPMI 1640 with 10% FBS.

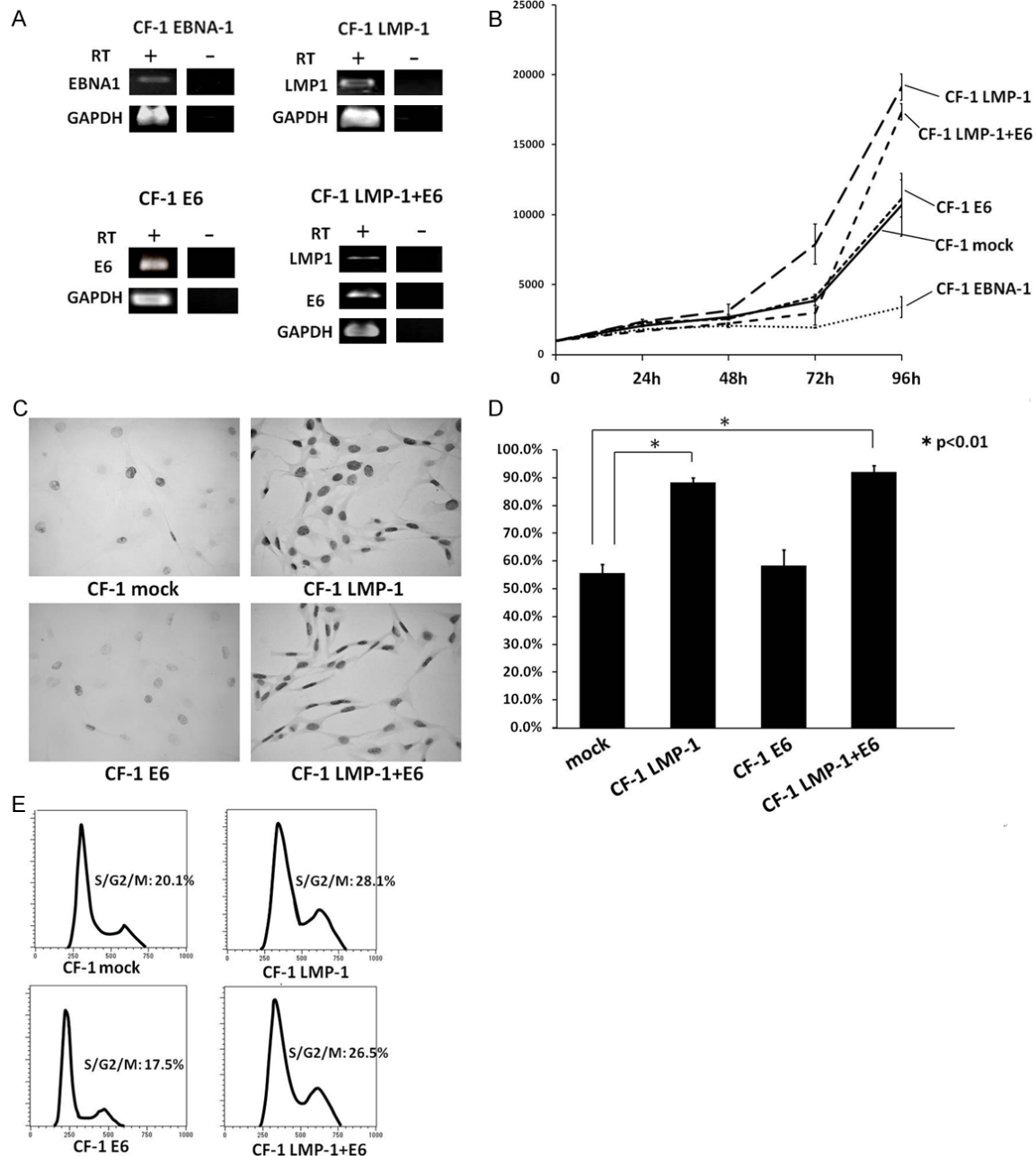
#### Plasmids

The HPV-16 whole genome in pBR322 was a kind gift from the Japanese Cancer Research Resource Bank (JCRB, Ibaraki, Osaka) by permission of Dr. Zur Hausen. PCR primers are listed in **Table 1**. HPV-16 E6 was amplified by PCR using primers (shown in **Table 1**) containing restriction enzyme recognition sites. The PCR product was digested with endonuclease and subcloned into the retrovirus vector plasmid pMSCV-hygro (Clontech, Mountain View, CA) to yield pMSCV-hygro-E6. EBV LMP-1 and EBV EBNA-1 were amplified by PCR from B95-8 genomic DNA, digested with restriction enzymes and inserted into pMSCV-neo and pMSCV-puro (Clontech) to yield pMSCV-neo-LMP-1 and pMSCV-puro-EBNA-1, respectively. The NF- $\kappa$ B-luc (Stratagene, La Jolla, CA) reporter plasmid and pGL4.74 (Promega, Madison, WI) were used for luciferase assay.

#### Small interfering RNA (siRNA) transfection

For LMP-1 and E6 knockdown, siRNAs were designed and synthesized (Invitrogen, Carlsbad,

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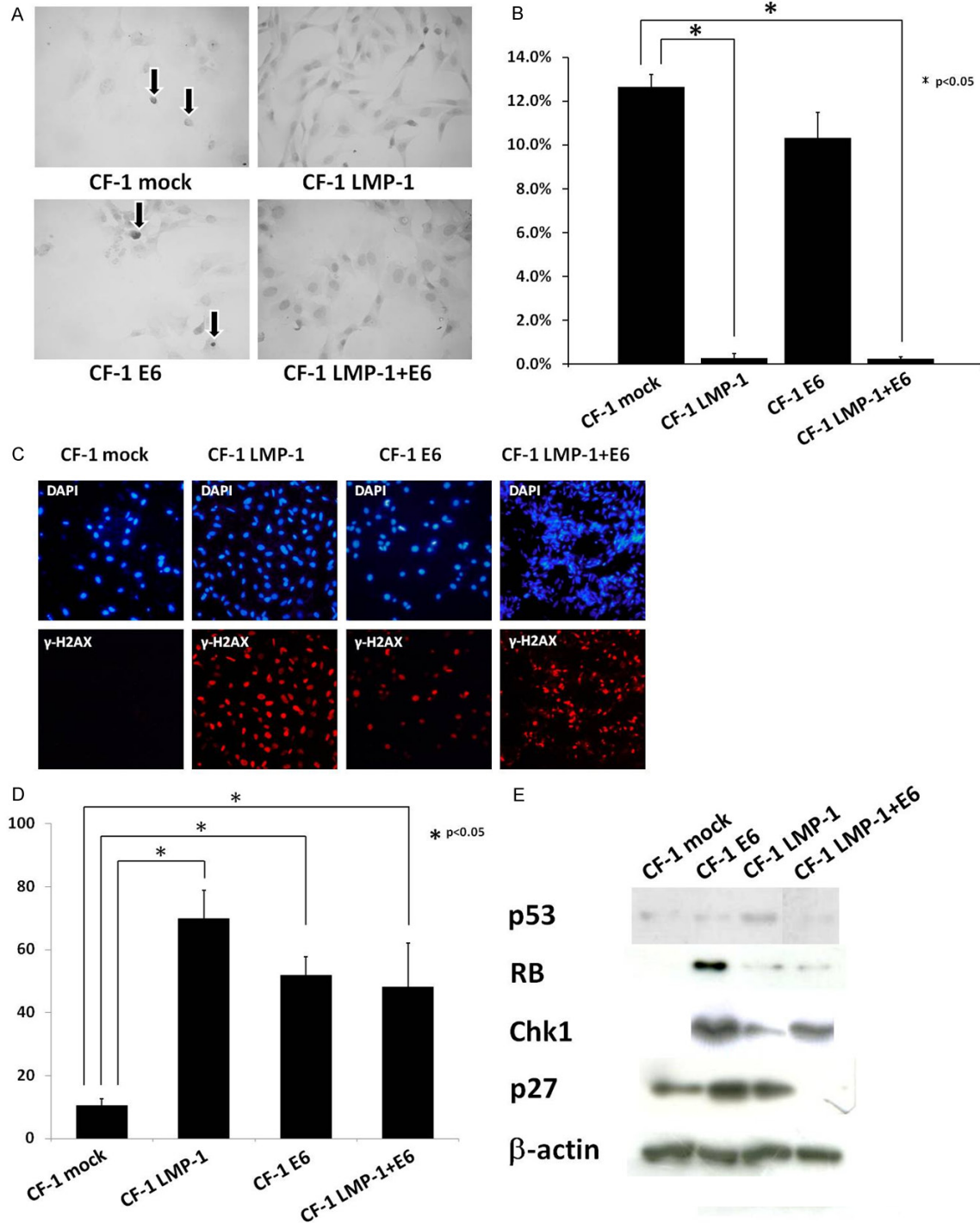


**Figure 1.** LMP-1, EBNA-1, and E6 expression in mouse embryonic fibroblasts (MEFs) and their effects on cell proliferation. (A) RT-PCR analysis of MEFs infected with retrovirus expressing EBV LMP-1 or EBNA-1 and/or HPV16 E6. Expression of GAPDH was used as a positive control. RT+ indicates that DNase treated RNA was reacted with reverse transcriptase for cDNA synthesis, and RT- indicates that DNase-treated RNA was not reacted with reverse transcriptase to check for genomic DNA contamination. (B) Cell proliferation assays with MEFs expressing EBV LMP-1, HPV16 E6, LMP-1+E6, and MEFs transduced with the empty vector (mock). Cell proliferation was assayed using CCK-8, a modified method of the MTT assay. Data are presented as mean  $\pm$  SDs from three independent experiments. (C, D) PCNA immunostaining in MEFs expressing EBV LMP-1, HPV16 E6, LMP-1+E6, and MEFs transduced with the empty vector (mock). At least 200 cells were counted in each assay. Data are mean  $\pm$  SDs from three independent experiments and are presented as PCNA-positive cells/total cells in (D). \* $P < 0.05$ . (E) Flow cytometry analysis of DNA content in MEFs expressing EBV LMP-1, HPV16 E6, LMP-1+E6, and MEFs transduced with the empty vector (mock). The percentages of cells in S or G2/M phases are indicated.

CA). The sequences of siRNA duplexes for LMP-1 were 5'-AUCAGUAGGAGUAUACAAAGG-

GCUCTT-3' and 5'-GAGCCCUUUGUAUACUCCUACUGAUTT-3', and those for E6 were 5'-CACA-

## Transformation by LMP-1 and E6 co-expression



**Figure 2.** Apoptosis, DNA damage, and induction of DDR due to expression of EBV LMP-1, HPV16 E6, and LMP-1+E6. (A, B) MEFs expressing LMP-1, E6, or LMP-1+E6, or mock were cultured under serum-free conditions, and TUNEL was used to detect apoptotic cells. At least 200 cells were counted in each experiment. The arrows in (A) indicate apoptotic cells. Data in (B) are mean  $\pm$  SDs from three independent experiments and are presented as TUNEL-positive cells/total cells. \* $P < 0.05$ . (C, D)  $\gamma$ H2AX immunofluorescence in MEFs expressing LMP-1, E6, or LMP-1+E6, or mock.  $\gamma$ H2AX positivity is an indicator of DNA damage. At least 200 cells were counted in each experiment. Data in (D) are mean  $\pm$  SDs from three independent experiments and are presented as  $\gamma$ H2AX-positive cells/total cells. \* $P < 0.05$ . (E) Analysis by Western blotting of p53, RB, Chk1, and p27 in MEFs expressing LMP-1, E6, or LMP-1+E6, or mock.  $\beta$ -actin was used as a loading control.

## Transformation by LMP-1 and E6 co-expression

GAGCUG CAAACAACUAUACAUTT -3' and 5'-AUG-UAUAGUUGUUUGCAGCUCUGUGTT-3'. These sequences of non-specific siRNA controls were 5'-GCCCUAAAGAUGGCCAGCCAUCUUUTT-3' and 5'-AAAGAUGGCUGGCCAUCUUUAGGGCTT-3'.

One day prior to siRNA transfection, MEFs with or without viral gene expression were plated at  $5 \times 10^4$  cells/well on 6-well plates. LMP-1 siRNA, E6 siRNA, or control (non-specific) siRNA were transfected into the cells using Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions and analyzed 48 h after transfection.

### *Retrovirus production and construction of viral gene-expressing MEFs*

The plasmids pMSCV-hygro-E6, pMSCV-neo-LMP-1, and pMSCV-puro-EBNA-1 (10  $\mu$ g each) were transfected into the packaging cell line, PT-67 (Clontech) using Lipofectamine 2000 (Invitrogen). In brief, 1 day before transfection, PT-67 cells were plated on 60-mm plates and grown until 80% confluence. The culture media were collected 24, 48, and 72 h after transfection and filtered through a 0.45- $\mu$ m cellulose acetate membrane. The viral titers of the supernatants determined by serial dilution ranged between  $4 \times 10^4$  and  $1 \times 10^6$  cfu/ml. MEFs were infected for 24 h with MSCV retrovirus encoding EBV or HPV genes and placed under drug selection (neomycin for LMP-1, puromycin for EBNA-1, and hygromycin for E6) to obtain CF-1 LMP-1, CF-1 EBNA-1, and CF-1 E6. The MEF lines co-expressing viral proteins were produced by re-infection of the individual viral cell lines with the appropriate supernatant and drug selection to yield CF-1 LMP-1+E6. Viral gene expression in retrovirus-infected cells was confirmed by reverse transcription PCR (**Figure 1A**). As a negative control, CF-1 cells were infected with empty retrovirus to yield CF-1 mock.

### *Cell proliferation assays*

MEFs expressing EBV and/or HPV genes were plated on 96-well plates at 1000 cells/well. Cell proliferation assays were performed every 24 h using Cell Counting Kit-8 (CCK-8: Dojindo, Kumamoto, Japan), which utilizes [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium

salt], a modification of the MTT colorimetric assay.

### *RT-PCR*

Total RNA was extracted using RNeasy (Qiagen, Valencia, CA) and treated with recombinant DNase I (Takara, Shiga, Japan) at 37°C for 30 min. After adding EDTA and incubating at 80°C to inactivate DNase, cDNA synthesis was performed using SuperScript III (Invitrogen) at 42°C for 50 min. cDNA synthesis was confirmed by PCR amplification of GAPDH.

### *Immunocytochemistry*

MEFs were fixed in 4% paraformaldehyde (PFA) for 15 min, and washed with phosphate-buffered saline (PBS). Fixed cells were permeabilized with 0.25% Triton X-100 for 3 min, washed with PBS, and treated with 0.3% hydrogen peroxide to inactivate endogenous peroxidase. After blocking with 10% bovine serum albumin (BSA) for 2 h, cells were incubated at room temperature for 1 h with primary antibodies that recognize proliferating cell nuclear antigen (PCNA: Abcam, Cambridge, MA),  $\gamma$ -H2AX (Gene Tex, Irvine, CA), epithelial membrane antigen (EMA: Dako, Glostrup, Denmark) or keratin (AE1/AE3: Dako). PCNA, EMA, and AE1/AE3 immunostaining were visualized using an LSAB kit (DAKO) with 3,3'-diaminobenzidine (DAB) as the chromogen.  $\gamma$ -H2AX signal was detected using Alexa Fluor 594 (Invitrogen) and observed using an Eclipse TE300 fluorescence microscope (Nikon, Tokyo, Japan).

### *Cell cycle analysis by flow cytometry*

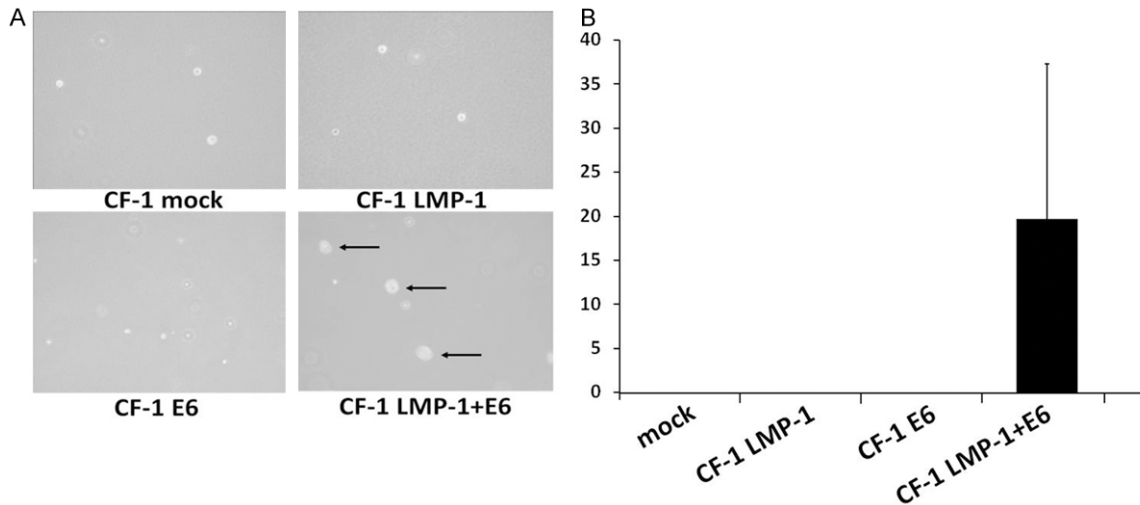
A total of  $10^5$  transfected MEFs were washed with PBS and fixed with ice-cooled 100% ethanol for 2 h, washed again with PBS, and stained with DAPI for 30 min. DNA content of the cells was analyzed using a flow cytometer (MAQS Quant Analyzer; Miltenyi Biotec, Bergisch Gladbach, Germany).

### *Detection of apoptosis*

Apoptotic cells were detected by TdT-mediated dUTP nick end labeling (TUNEL). The cells were plated on chamber slides (Nunc, Roskilde, Denmark) at a concentration of  $10^5$  cells/slide and fixed with 4% PFA for 15 min. After washing with PBS, slides were treated with 0.3% hydro-



## Transformation by LMP-1 and E6 co-expression



**Figure 3.** Anchorage-independent growth of CF-1 LMP-1+E6 cells. (A, B) MEFs expressing LMP-1, E6, LMP-1+E6, or mock were suspended in 0.7% agarose in 6-well plates ( $1 \times 10^4$  cells/well), and colony formation was observed for 4 weeks. Arrows in (A) indicate colony formation. Magnification,  $100 \times$ . The number of colonies was counted 4 weeks after plating. Data in (B) are the mean number of colonies  $\pm$  SDs from three independent experiments.

gen peroxide to block endogenous peroxidase. After washing with PBS, TUNEL staining was performed using the in situ Apoptosis Detection kit (Takara) and visualized using DAB as the chromogen.

### Western blot analysis

Total cell protein extracts were obtained using RIPA lysis buffer supplemented with a cocktail of protease inhibitors (Roche). Protein concentration was measured using the Bradford protein assay (Bio-Rad). Protein samples (25  $\mu$ g) were separated by electrophoresis on 12% sodium dodecyl sulfate-polyacrylamide gels. Separated proteins were transferred to polyvinylidene difluoride membranes (Immobilon-P, Millipore). Protein-bound membranes were blocked in 5% nonfat milk solution (0.1% Tween 20 in PBS) and incubated in one of the following primary antibodies: anti-RB, anti-Chk1, anti-p27, anti-p53 and anti- $\beta$  actin (Cell Signaling Transduction). Antibody binding was detected using enhanced chemiluminescence (Amersham Pharmacia).

### Luciferase assay

Cells were transfected with the reporter plasmids NF- $\kappa$ B-luc and pGL4.74 at 48 h after siRNA transfection. Total amount of transfected DNA and siRNA were equalized by the addition of empty vectors or non-specific siRNAs,

respectively. After 24 h, cell lysates were assayed for firefly luciferase activity using the dual luciferase assay system (Promega). Relative activities were calculated using renilla luciferase activity as an internal control for transfection.

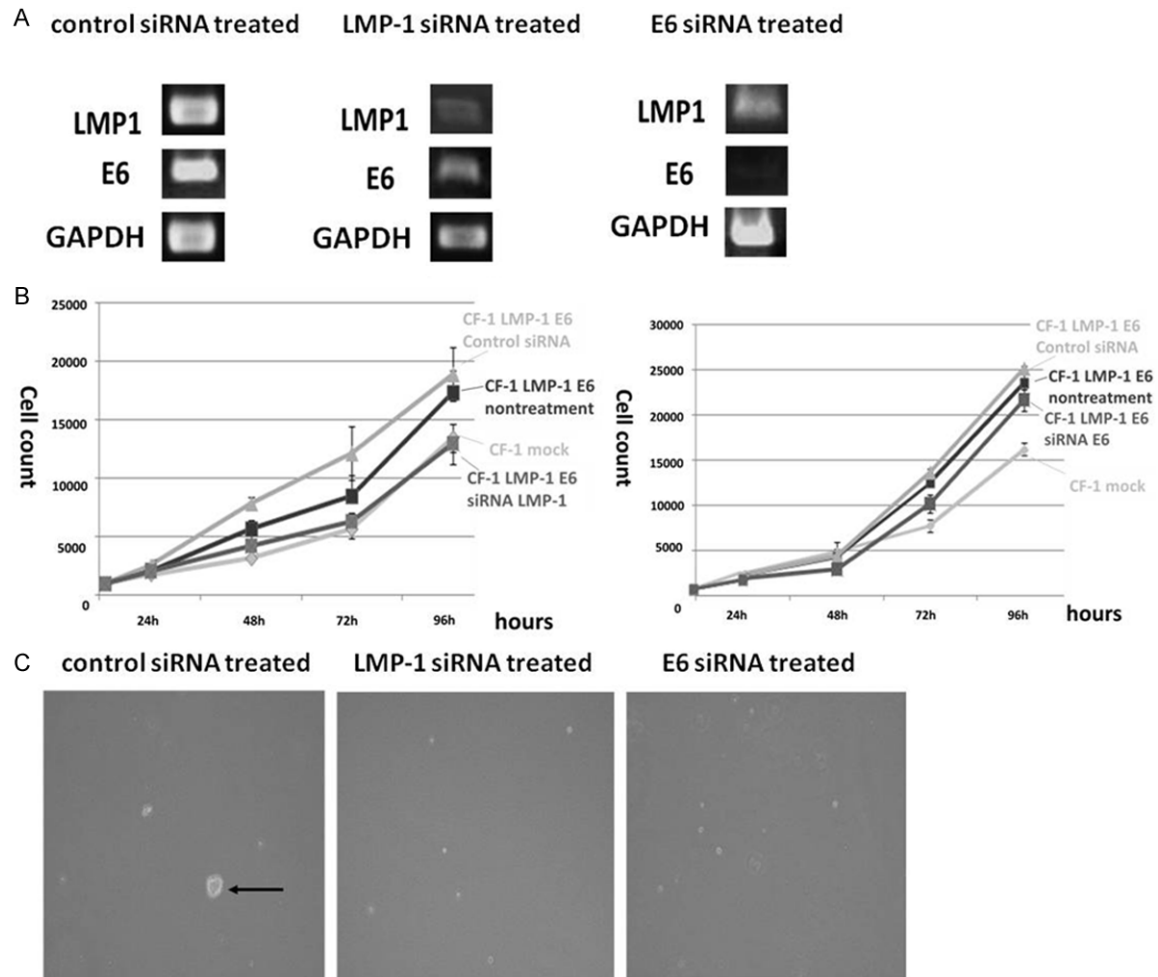
### Soft agar colony formation assay

Base agar (0.5% agar,  $1 \times$  DMEM/F12, and 10% FBS) was added to 6-well plates and allowed to solidify, followed by top agar (0.35% agarose,  $1 \times$  DMEM/F12, and 10% FBS) containing  $1 \times 10^4$  trypsinized cells. After the top agar was solidified, a small amount of  $1 \times$  DMEM/F12 with 10% FBS was added to each well. Cells were incubated at  $37^\circ\text{C}$  in a humidified incubator, and the culture medium was changed every 3 days. Colony formation was assessed observing cells under an inverted microscope for 28 days.

### Animal experiments

Animal experiments were performed in accordance with the guidelines for animal treatment, housing, and euthanasia of the Animal Experiment Committee of the University of the Ryukyus. Four week-old female BALB/cScl-nu/nu nude mice were purchased from Japan SLC (Shizuoka, Japan). At 5-7 weeks of age, mice were injected subcutaneously in the back with  $2 \times 10^6$  retrovirally-infected MEFs. After 12

## Transformation by LMP-1 and E6 co-expression



**Figure 4.** Effects of LMP-1 or E6 gene knockdown on cell proliferation and anchorage-independent growth of CF-1 LMP-1+E6 cells. **A:** CF-1 LMP-1+E6 and CF-1 mock cells were transfected with siRNA for LMP-1, E6, or control siRNAs. The cells were analyzed by RT-PCR for expression of LMP-1, E6 and GAPDH mRNAs. **B:** CCK8 analysis of proliferation of CF-1 LMP-1+E6- and CF-1 mock cells transfected with LMP-1, E6, or control siRNAs. Data are means  $\pm$  SDs from three independent experiments. **C:** Colony formation in soft agar by CF-1 LMP-1+E6 cells transfected with LMP-1, E6, or control siRNAs. Forty-eight hours after siRNA transfection, cells were suspended in 0.7% agarose and observed for 4 weeks for colony formation. The arrow indicates a colony. Magnification, 100  $\times$ .

weeks, the mice were euthanized by intra-abdominal injection of sodium pentobarbital, and tissue samples were collected for molecular and histological analyses.

### Histological examination

Tumors from the nude mice were fixed in 10% phosphate-buffered formalin, embedded in paraffin, and sectioned at a thickness of 4  $\mu$ m. The sections were dewaxed with xylene and rehydrated in a graded ethanol series. Sections were stained with either hematoxylin and eosin, periodic acid Schiff, or Gomori's silver impregnation method.

### Statistical analysis

Data were analyzed by unpaired Student's *t* tests and presented as means  $\pm$  SDs. *P* < 0.05 was considered statistically significant.

### Results

#### LMP-1 and HPV gene co-expression induces cell proliferation

To assess the transformation activity of dual expression of EBV and high-risk HPV genes, EBV LMP-1 and EBNA-1, and HPV-16 E6 were introduced into CF-1 MEFs using retroviruses to generate stable cell lines expressing viral genes

## Transformation by LMP-1 and E6 co-expression

**Table 2.** Tumor formation in nude mice injected with MEFs expressing EBV and/or HPV gene products

	Tumor formation in nude mice
Mock	1/12
CF-1 LMP-1	0/12
CF-1 E6	0/7
CF-1 LMP-1+E6	21/21

singly or in pairs (**Figure 1A**). LMP-1 expressing clone and cell lines co-expressing LMP-1 with E6 had higher rates of cell proliferation than single HPV gene-expressing clones or CF-1 mock cells (**Figure 1B**). In contrast, cell lines expressing EBNA-1, or co-expressing EBNA-1 and E6 had lower proliferation rates than CF-1 mock (data not shown). We next analyzed the cell cycle using the S-phase marker PCNA and DNA content. PCNA immunostaining was increased in LMP-1-expressing clones, particularly CF-1 LMP-1+E6 cells indicating an increase in cells in S-phase (**Figure 1C, 1D**). Cell cycle analysis revealed a higher population of cells in the S/G2/M phase in MEFs expressing LMP-1 and LMP-1+E6 than in MEFs expressing E6 alone or the mock controls (**Figure 1E**). These results suggested that LMP-1 or LMP-1+E6 co-expression induced MEF proliferation, whereas expression of EBNA-1 did not. We speculated that LMP-1 and high-risk HPV E6 may play a critical role in transformation; therefore, we focused more on the clones showing increased cell proliferation rate than the mock in our subsequent analysis.

### *Genotoxic stress in cells co-expressing LMP-1 and E6 did not trigger apoptosis*

In general, transformed cells have a reduced requirement for mitotic growth factors, are resistant to apoptosis, and survive under serum-free conditions, which typically induce apoptosis because DNA damage by intracellular generation of reactive oxygen species (ROS) [20-22]. We examined apoptosis in MEFs expressing EBV and/or HPV genes grown in serum-free medium. Although the serum-free treatment induced several apoptotic CF-1 mock or CF-1 E6 cells, there were only a few apoptotic CF-1 LMP-1 or CF-1 LMP-1+E6 MEFs (**Figure 2A, 2B**).

### *Viral gene expression induces DNA damage but escape from apoptosis*

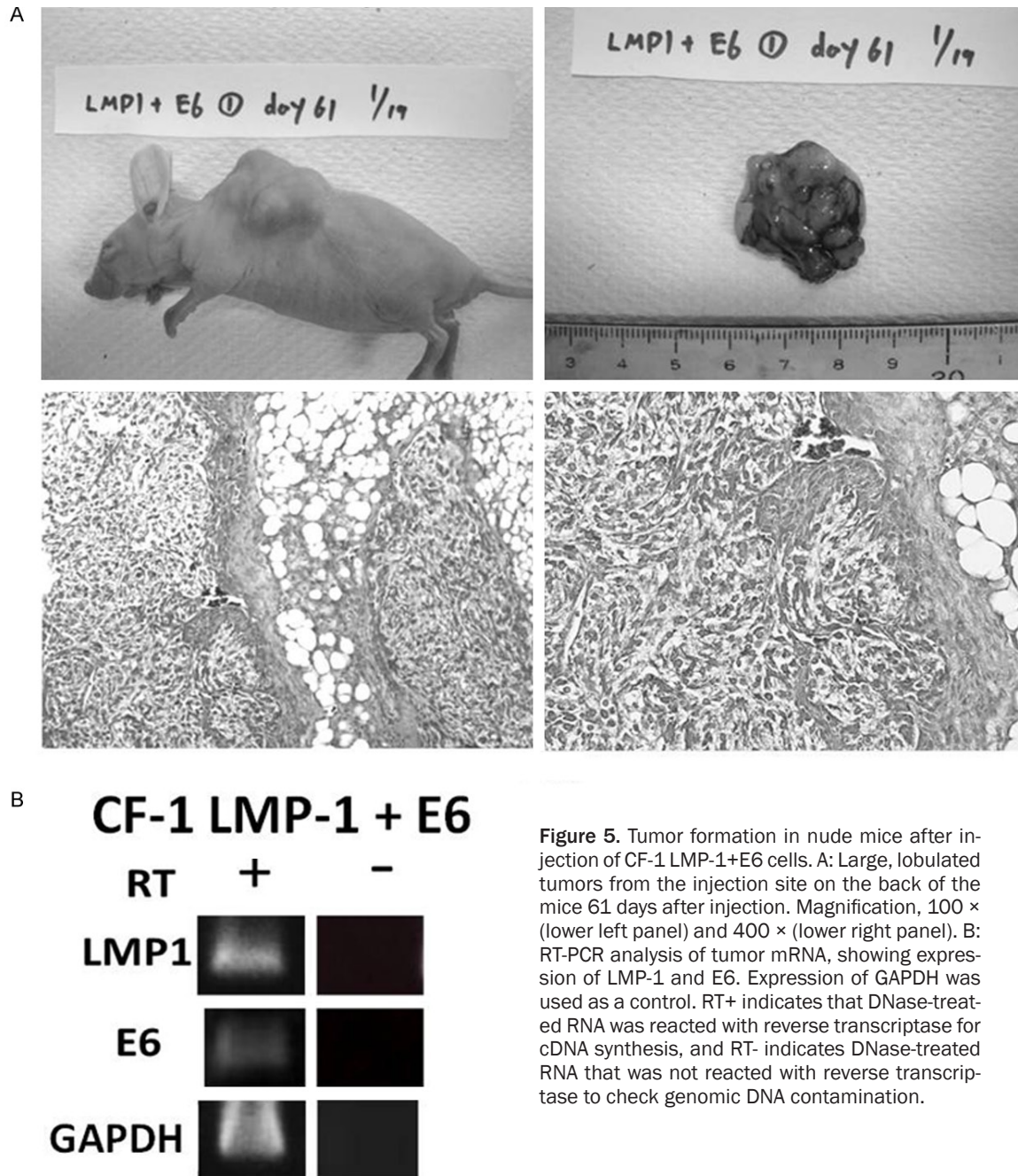
DNA damage is a potent trigger for apoptosis and viral oncoproteins cause DNA damage through several mechanisms: increased replicative stress from high rate of proliferation [23, 24], inappropriate entry into mitosis caused by constitutive entry into S-phase [25], and ROS generation [26]. DNA damage induces DNA damage-associated repair proteins, such as  $\gamma$ H2AX. We used  $\gamma$ H2AX to assess the degree of DNA damage in our EBV and HPV cell lines. The  $\gamma$ H2AX signal was very weak in CF-1 mock, whereas cells expressing LMP-1 alone or in combination with E6 showed significantly greater induction of  $\gamma$ H2AX consistent with increased DNA damage (**Figure 2C, 2D**). Although viral gene expression induces DNA damage and results in cell senescence [25, 26], viral oncoproteins may act in part by suppressing DNA damage response (DDR) [27, 28]. Since cells expressing LMP-1 and co-expressing LMP-1 and E6 were highly proliferative and not apoptotic in spite of the increased load of DNA damage, we postulated that these viral proteins may suppress DDR.

### *Anti-apoptotic effect by dual LMP-1+E6 expression correlates with the suppression of DDR*

Under genotoxic stress, DDR associated proteins are induced or activated by the presence of double-strand breaks in nuclear DNA. Activation of DDR induces p53-dependent cell cycle arrest, apoptosis, or senescence [29, 30]. We assessed the effect of LMP-1 and/or E6 on the levels of DDR proteins. In CF-1 E6 cells, p53 was decreased and RB was markedly increased, whereas the reverse occurred in CF-1 LMP-1 cells (**Figure 2E**). These findings indicate that LMP-1 and E6 have reciprocal effects on p53 and RB. In contrast, LMP-1+E6 co-expression reduced the levels of both p53 and RB indicating synergistic effect of LMP-1 and E6 on DDR. Furthermore, CF-1 LMP-1+E6 had reduced levels of Chk1 and p27 (**Figure 2E**). These results strongly suggest that the DDR machinery was impaired in CF-1 LMP-1+E6 cells. On the basis of our observation that dual LMP-1+E6-expressing MEFs showed increased cell proliferation, resistance to apoptosis and low expression levels of DNA damage checkpoint



## Transformation by LMP-1 and E6 co-expression



**Figure 5.** Tumor formation in nude mice after injection of CF-1 LMP-1+E6 cells. A: Large, lobulated tumors from the injection site on the back of the mice 61 days after injection. Magnification, 100 × (lower left panel) and 400 × (lower right panel). B: RT-PCR analysis of tumor mRNA, showing expression of LMP-1 and E6. Expression of GAPDH was used as a control. RT+ indicates that DNase-treated RNA was reacted with reverse transcriptase for cDNA synthesis, and RT- indicates DNase-treated RNA that was not reacted with reverse transcriptase to check genomic DNA contamination.

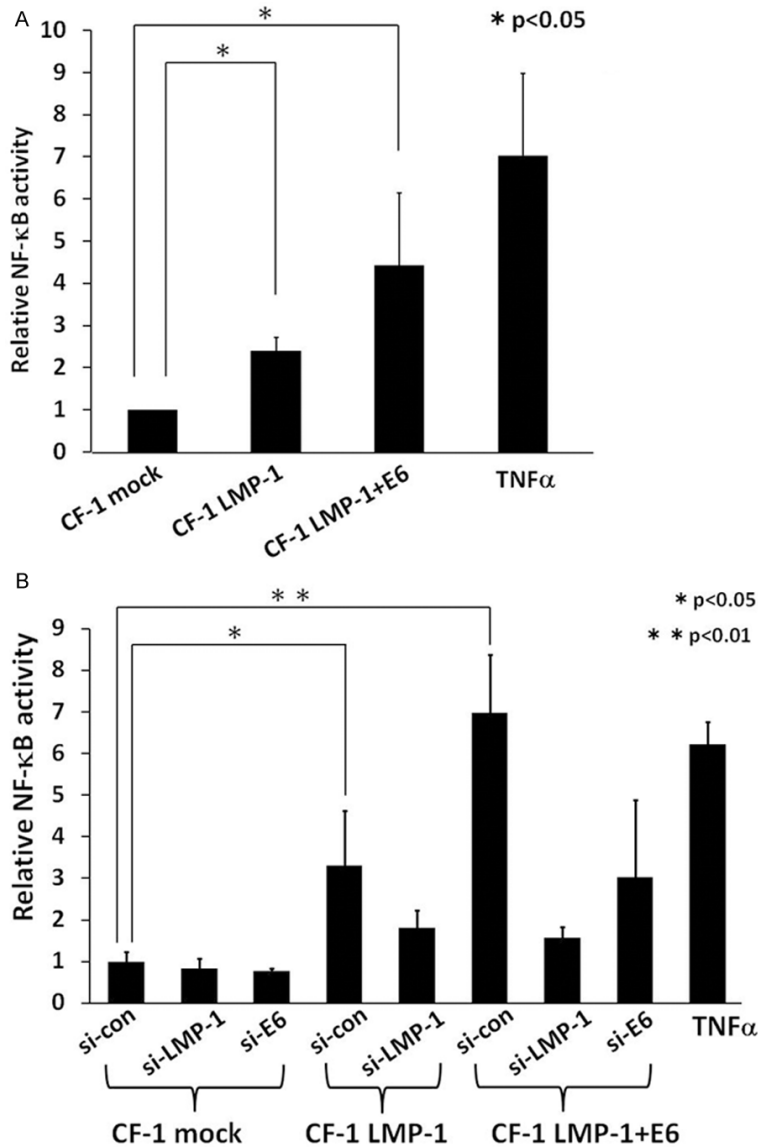
associated proteins, we hypothesized that dual LMP-1+E6 expression in MEFs may induce transformation.

### *LMP-1 and E6 co-expression induces anchorage-independent growth*

*In vitro*, malignant cells have characteristic phenotypes, such as unregulated cell proliferation, colony formation because of anchorage-independent growth, ability to invade or infiltrate, and higher telomerase activity [31, 32]. We analyzed the effects of LMP-1 and E6 on

anchorage-independent growth using soft agar colony formation assay. Cells expressing either LMP-1 or E6 or mock vector did not form colonies during the 4- week observation period (**Figure 3A, 3B**). In contrast, CF-1 LMP-1+E6 cells formed a mean of 20 colonies per  $10^4$  plated cells (**Figure 3B**), indicating that approximately 0.2% of these cells had the ability to grow in an anchorage-independent manner. E6 was much more effective than E7 in this regard because CF-1 LMP-1+E7 cells formed very few colonies (data not shown).

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**Figure 6.** NF-κB activity in MEFs expressing LMP-1, and LMP-1+E6, and its suppression by knockdown of LMP-1 or E6. A: NF-κB activity relative to mock-transduced MEFs determined by luciferase assays in MEFs expressing EBV and/or HPV genes. CF-1 TNFα are MEFs treated with TNFα as a positive control. Data are means ± SDs from three independent experiments. \*P < 0.05. B: Luciferase assays of NF-κB activities in CF-1 LMP-1, CF-1 LMP-1+E6 and CF-1 mock cells transfected with control or LMP-1- and E6-specific siRNAs. CF-1 TNFα represents MEFs treated with TNFα as a positive control. Data are means ± SDs from three independent experiments. \*P < 0.05. \*\*P < 0.01.

### *LMP-1 or E6 depletion inhibits increased proliferation and anchorage-independent growth*

To assess the requirement for LMP-1 and E6 co-expression on cell proliferation and anchorage-independent growth, we used siRNAs to deplete either LMP-1 or E6 in CF-1 LMP-1+E6

cells. The specificities of the siRNAs for LMP-1 and E6 are demonstrated in **Figure 4A**. Knockdown of LMP-1 in CF-1 LMP-1 (data not shown) or CF-1 LMP-1+E6 cells reduced the cell proliferation rate to the level of CF-1 mock cells, whereas knockdown of E6 had no effect (**Figure 4B**). These findings indicate that the increase in cell proliferation was mainly because of LMP-1 with only minimal contribution from E6. We also examined the effect of LMP-1 or E6 knockdown on the anchorage-independent growth of CF-1 LMP-1+E6 cells. Knockdown of either LMP-1 or E6 resulted in the loss of colony formation in soft agar indicating that both genes were required for anchorage-independent growth and suggesting that co-operation between LMP-1 and E6 was involved in some aspects of cell transformation.

### *LMP-1 and E6 co-expression induces tumor formation in nude mice*

The effects of LMP-1 and E6 on tumorigenicity *in vivo* were assessed by injection of  $5 \times 10^6$  cells of each cell line into 5- to 7-week-old nude mice. After 30 weeks, no tumor was detected in mice injected with either CF-1 LMP-1 or CF-1 E6 cells and a single tumor was found in 12 mice injected with the CF-1 mock cells (**Table 2**). In contrast, all mice injected with CF-1 LMP-1+E6 cells developed tumors at the injection site

within only 2 weeks (**Table 2** and **Figure 5A**). The tumors were 10-20 mm in diameter, showed irregular shapes, and were ulcerated with a hemorrhagic and necrotic center. The borders between the tumors and adjacent normal tissues were macroscopically and microscopically inconspicuous (**Figure 5A**) suggest-

ing that the tumors were infiltrating the subcutaneous tissue. Histologically, the tumor cells were polygonal with enlarged nuclei and were arranged irregularly in the myxomatous stroma with a sarcomatous appearance (**Figure 5A**). Tumor cell at the periphery of the lesions were highly proliferative, but the lack of expression of the epithelial cell markers-EMA and AE1/AE3, indicated that there was no epithelial differentiation (data not shown). RT-PCR analysis of tumor mRNA identified LMP-1 and E6 expression several weeks after injection, indicating that their expression remained stable (**Figure 5B**). In contrast, there was no evidence of any EBV or HPV gene expression in the single tumor of CF-1 mock-injected mouse (data not shown) indicating that this tumor was most likely from the spontaneous transformation of MEF [33, 34].

### *NF-κB signaling is associated with cell transformation by LMP-1 and E6 co-expression*

LMP-1 induces resistance to apoptosis and immortalization in human B lymphocytes through activation of NF-κB signaling [35]. Therefore, we examined NF-κB signaling in our CF-1 cell line. NF-κB activity in CF-1 LMP-1+E6 cells was approximately 2-fold higher than that in CF-1 LMP-1 cells and almost 5-fold higher in CF-1 mock cells (**Figure 6A**). siRNA knockdown of LMP-1 in CF-1 LMP-1+E6 cells reduced NF-κB activity to near the level observed in CF-1 mock cells, whereas E6 knockdown had less of an effect (**Figure 6B**). It is consistent with an association between NF-κB activity in CF-1 LMP-1+E6 cells and some transformation properties, such as increased cell proliferation, cell survival under genotoxic stress and anchorage-independent growth.

### **Discussion**

Our results demonstrate synergy between LMP-1 and HPV-16 E6 in the malignant transformation of primary mouse fibroblasts. LMP-1 and E6 co-expression was essential for transformation of primary MEF cells because CF-1 LMP-1+E6 showed both the *in vitro* properties of transformation as well as tumor formation in nude mice, which is ultimate proof for transformation. On the contrary, single viral gene expressing clones were not able to cause tumorigenesis in nude mice.

Although many aspects of viral oncogenesis have been clarified using immortalized rodent cell lines, such as Rat-1 and NIH3T3, we chose to use primary MEFs for these experiments because tumor formation in primary cells is closer to the clinical model than the experimental model of transformation using immortalized cells. One study reported that LMP-1 and CDK4 expression in MEFs increases proliferation, anchorage-independent growth, telomerase activation, and immortalization; however, these cells did not form tumors in nude mice, indicating that co-expression of LMP-1 and CDK4 was not sufficient for transformation [36]. In our experiments, CF-1 LMP-1+E6 cells showed not only *in vitro* properties of transformation and tumor formation in nude mice, but also DDR suppression and NF-κB activation. Knockdown of either LMP-1 or E6 using siRNA reduced proliferation, anchorage-independent growth (**Figure 4B** and **4C**), and NF-κB activity (**Figure 6B**). These results demonstrate that synergism between E6 and LMP-1 in the transformation of primary MEF is associated with NF-κB activation.

LMP-1 activates the NF-κB pathway by binding with TRAFs and TRAF6 [37], and also activates the MAPK pathway through JNK and p38 [38]. In our study, serum-free cultures of CF-1 LMP-1 and CF-1 LMP-1+E6 cells were resistant to apoptosis. Because NF-κB signaling increases proliferation, suppresses apoptosis [39], and regulates ROS synthesis [40], it suggests that LMP-1 may transform MEFs through NF-κB signaling. However, LMP-1 expression alone, which activates NF-κB signaling, did not result in colony formation in soft agar or tumor formation in nude mice, whereas co-expression of LMP-1 with E6 led to anchorage-independent growth and tumor formation in nude mice. Furthermore, the NF-κB activity of CF-1 LMP-1 and E6 with siRNA treatment of LMP-1 or E6 was still higher than that of CF-1 mock (**Figure 6B**) suggesting that transformation is not correlated with the increased NF-κB activity. Thus, although LMP-1 expression is associated with increased cell proliferation and survival through the NF-κB pathway, NF-κB activation in this context is not sufficient to transform primary MEFs.

Single E6 expression was not able to transform MEFs and CF-1 E6 cells became senescent after 40 passages (data not shown). These

## Transformation by LMP-1 and E6 co-expression

results suggest that suppression of p53 alone was not sufficient to transform E6-expressing MEFs, which entered replicative senescence even though p53 was downregulated. Because RB protein was upregulated in CF-1 E6 cells (**Figure 2E**), it may compensate for the reduction in p53 and regulate cell cycle progression and apoptosis under genotoxic stress. Kanda et al reported that HPV-16 E7±E6 immortalized primary cells in culture, although these cells did not form tumors in nude mice [9]. Together with our results, this suggests that E6 or E7 was not sufficient to cause transformation in primary MEF.

In our study, a distinct difference was found in the induction of DDR components against viral gene expression between transformable clones and incapable clones. All the viral genes expressed in this study induced DNA damage, but only LMP-1 and E6 co-expression suppressed DDR and induced transformation. DNA damage is a common effect of viral gene expression and replicative stress, which activates the DDR [23, 24] and perturbs cell cycle control [25] or ROS generation [26]. Our data suggest that DNA damage was induced in CF-1 LMP-1 and CF-1 LMP-1+E6 cells showing hyperproliferation by replicative stress, whereas CF-1 E6 cells showing decreased amount of p53 caused DNA damage by disruption of cell cycle control. Despite DNA damage, viral oncoproteins may inactivate the DDR by direct suppression of DDR checkpoint kinases [24, 41] or suppression of downstream signaling components such as p53 [27, 42]. Expression of HPV oncoproteins in undifferentiated keratinocytes increases activation of some DDR components such as ATM, Chk1 and Chk2 [43]; however, LMP-1 expression inhibits ATM signaling through transcriptional down-regulation [44]. In our model, E6 suppresses p53 and increases Chk1, whereas LMP-1 decreases the level of Chk1 possibly through the suppression of ATM expression. The decrease in the levels of DDR components, such as p53, RB, Chk1, and p27 in CF-1 LMP-1+E6 cells indicates a synergistic effect of LMP-1 and E6. DDR inactivation by viral oncoproteins is linked to malignant transformation through increased somatic mutation and aneuploidy [44, 45]. Analysis of human precancerous and cancerous lesions shows that oncogene activation induces both DNA damage and DDR in precancerous lesions,

whereas DDR is suppressed in cancerous lesions despite the presence of DNA damage [46, 47]. These studies demonstrated that a functional DDR is an important defense against tumorigenesis [46, 47]. Our data suggest that the synergistic transforming ability of LMP-1 and E6 was related to profound suppression of the DDR.

LMP-1 and E6 co-expression transformed MEFs more effectively than CF-1 LMP-1+E7. It has been suggested that the p53 pathway has a predominant role in regulating the senescence of murine cells under genotoxic stress [48, 49], whereas the murine RB pathway contributes to cellular senescence to a lesser extent [50]. In our model, the difference of tumorigenicity in nude mice between CF-1 LMP-1+E6 and CF-1 LMP-1+E7 may be explained as p53 degradation by E6 may induce more severe effect to cell cycle control or induction of DDR than RB degradation by E7.

In conclusion, our results indicate that, in addition to NF- $\kappa$ B signaling activation by LMP-1 expression in primary MEFs, inhibition of a tumor suppressor gene, such as p53 or RB, is required for transformation and the transformation is associated with suppression of DDR.

To our knowledge, this is the first use of a simple system to show the synergistic transforming activity of HPV-16 E6 and EBV LMP-1. Regarding the geographic differences in the prevalence of EBV, LMP-1 may be an oncogenic factor in areas where oral cancer is highly associated with EBV infection [15, 18, 19]. However, in addition to LMP-1 expression, our study suggests that an additional oncogenic factor, such as HPV-16 E6 or EBNA-1, may be strongly associated with oncogenic transformation in EBV and HPV co-infected cells. We have demonstrated this synergistic mechanisms using co-expression of HPV and EBV genes in primary MEFs, but the precise molecular mechanisms remain to be clarified.

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### Disclosure of conflict of interest

None.

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