The Epstein–Barr virus nuclear antigen-1 may act as a transforming suppressor of the *HER2/neu* oncogene

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Received 5 September 2002; accepted 16 October 2002

First published online 7 November 2002

Edited by Julio Celis

Abstract **It is known that the *HER2/neu* proto-oncogene is associated with a wide variety of human cancers and considered to be an attractive target for developing anti-cancer agents. We report here for the first time that the Epstein–Barr virus nuclear antigen-1 (EBNA1) suppresses the *HER2/neu* oncogene expression at the transcriptional level. Recombinant clones of EBNA1 were subcloned and stably transfected into *HER2/neu*-overexpressing human ovarian cancer SKOV3.ip1 cells. These EBNA1-containing clones down-regulated the endogenous production of p185$^{HER2/neu}$. In addition, the EBNA1-expressing stable transfectants showed reduced growth rate, low soft agarose colony-forming ability and tumorigenic potential as compared with the parental line. These data suggest that EBNA1 may act as a transforming suppressor of the *HER2/neu* oncogene.**

Key words: HER2/neu; EBNA1; Tumorigenicity; Ovarian cancer

1. Introduction

The loss of control in the genetic regulation of the *HER2/neu* (also known as c-*er*bB-2) proto-oncogene is associated with many types of human cancers. The progress of malignancy and metastasis due to *HER2/neu* overexpression has been demonstrated both in vitro and in vivo. The malignant phenotypes of *HER2/neu*-overexpressing cancer cells can be repressed by targeting *HER2/neu*, which suggests that *HER2/neu* itself is a good target for developing cancer gene therapy (reviewed in [1,2]). Therefore, down-regulation of *HER2/neu* oncogene expression may be an effective approach to the treatment of the *HER2/neu*-overexpressing human cancers [3,4].

Many *HER2/neu* oncogene suppressors have been reported, including p53, Rb, SV40 large T antigen, E1A, c-Myc, c-Myb, c-Cbl, and PEA3 gene product [5–13]. During research on the transcriptional suppression of the *neu* promoter by the N-terminal domain of the SV40 large T antigen [8], we discovered that the EBNA1-containing plasmid vector pCEP4 alone was able to significantly repress the *neu* promoter activity in a transient transfection assay. In this paper, we further characterized this newly discovered *HER2/neu* oncogene suppressor, that is, the Epstein–Barr virus nuclear antigen-1 (EBNA1). EBNA1, the major nuclear antigen of the oncogenic human herpes virus, Epstein–Barr virus (EBV) [14], is a multifunctional phosphoprotein of 641 amino acid residues [15]. It is the only viral protein consistently expressed in all EBV-infected cells and is necessary for replication and the maintenance of the EBV extrachromosomal episomes of the latent form of EBV [16,17]. These functions result from the specific binding of EBNA1 to DNA at the *ori*P site of the EBV genome [17,18]. EBNA1 plays an important role in the deregulation of cellular proliferation [19,20]. In the present paper, to further elucidate the mechanism for suppression of *HER2/neu*-mediated transformation by EBNA1 and explore its clinical applications in human cancer gene therapy, we established a construct with the EBNA1 coding region that can effectively repress the *HER2/neu* gene function in vitro and in vivo. These results suggest that EBNA1 gene therapy aimed at down-regulation of *HER2/neu* may provide a new useful approach in the treatment of *HER2/neu*-overexpressing ovarian cancer.

2. Materials and methods

2.1. Plasmids and bacterial strains

The pNeu*Eco*RVCAT plasmid, in which a DNA segment containing the *neu* promoter was fused with the CAT reporter [21], was used in all the CAT assays. pCMVβ, a plasmid containing the bacterial β-galactosidase gene under the control of the cytomegalovirus (CMV) long terminal repeat, was used to monitor transfection efficiency. Plasmid pcDNA3 (Invitrogen) was an expression vector containing the CMV promoter and the neomycin-selection marker. pCEP4 was a plasmid which contained the EBNA1 gene and an ampicillin-resistance gene marker. pRSVCAT was a plasmid which contained the Rous sarcoma virus (RSV) promoter-driven CAT reporter and an ampicillin-resistance gene marker. All plasmids were amplified in *Escherichia coli* DH5α and purified using a Wizard Maxipreps Kit (Promega, Madison, WI, USA).

2.2. Construction of plasmids containing wild-type and mutant EBNA1 genes

A frame-shift mutant of EBNA1 was generated by cutting the pCEP4 plasmid DNA sequence with *Avr*II. This was followed by a T4 DNA polymerase filling-in reaction and blunt-end ligation (inserting four bases at the cutting site) to form pCEP4-*Avr*II. The mutant pcDNA3-EBNA1-*Avr*II was constructed in the same way. The 2.2-kb wild-type EBNA1 gene was cut out of the plasmid pCEP4 with *Stu*I and *Msc*I and subcloned into the *Eco*RV site of the plasmid vector pcDNA3 to form pcDNA3-EBNA1. All recombinant clones were

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0014-5793/02/$22.00
PII: S0014-5793(02)03658-X

verified by sequence analysis using a Sequenase V2.0 Kit (USB-Amersham).

2.3. Cell lines, culture and transfection

The SKOV3.ip1 human ovarian carcinoma cell line was established from ascites that developed in a *nu/nu* mouse injected i.p. with SKOV3 cells. NIH3T3 and SKOV3.ip1 cells were grown in a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM)/F12 medium supplemented with 10% fetal bovine serum (FBS, Gibco-BRL, Grand Island, NY, USA). The cells were incubated at 37°C in a humidified, 5% CO_2 atmosphere. NIH3T3 cells were used as the recipient cells for all DNA constructs in all transient transfection experiments. The calcium phosphate precipitation method was used for transient transfection [22]. Briefly, 5×10^5 cells were plated in a 100-mm-diameter tissue culture dish overnight; 10 μg of recombinant EBNA1 or its mutant, 10 μg of pNeu*Eco*RVCAT and 2 μg of pCMVβ were co-precipitated and added to the cells. After 20 min at room temperature, the cells were incubated in 3% CO_2 for 12–16 h at 37°C. The cells then were washed twice with PBS and fresh medium was added. This was followed by incubation in 5% CO_2 at 37°C for 36–48 h.

2.4. CAT assay and β-galactosidase assay

Wild-type and mutant EBNA1-transfected NIH3T3 cells were harvested with a cell scraper after transfection. They were washed with PBS followed by resuspension in 100 μl of 0.25 M Tris (pH 8). Four cycles of freeze–thaw–vortex alternating between liquid nitrogen and 37°C were used to lyse the cells. Cell debris was spun down in a microfuge, and 100 μl of cell extract was collected; 10–20 μl of the extract was tested for β-galactosidase activity [23] to monitor transfection efficiency. Cell extracts were added to a reaction buffer containing 650 μl of 0.1 M phosphate buffer (pH 7.3), 50 μl of 30 mM $MgCl_2$, and 50 μl of 3.34 M β-mercaptoethanol. Then, 750 μl of ONPG (0.13 g/100 ml phosphate buffer) was added, and the reaction mixture was incubated at 37°C until a yellow color was observed. The reaction was stopped with 500 μl of 1 M Na_2CO_3, and the optical density at a wavelength of 410 nm was measured. Normalized quantities of cell extracts were then used for the CAT assay [24]. The cell extract was heated to 60°C for 10 min and then added to the CAT reaction mixture [25]. Reaction time was adjusted according to the transfection efficiency of each experiment, but was not more than 4 h. The reaction mixture was extracted with ethyl acetate, dried in a Speed-Vac, redissolved in ethyl acetate, and separated on a silica gel thin-layer chromatography (TLC) plate (Whatman). The TLC plate was then exposed to X-ray film. The spots corresponding to the positions of the [^{14}C]chloramphenicol and the acetylated products were examined by phospho-imager.

2.5. Stable transfection of EBNA1 into SKOV3.ip1 cells

A liposome system was used for delivering the pcDNA3-EBNA1 construct for stable transfection [26]. The stable transfectants were derived from the parental SKOV3.ip1 cells. The EBNA1-expressing plasmid pcDNA3-EBNA1 was transfected into the *HER2/neu*-overexpressing recipient ovarian cancer line SKOV3.ip1 to generate the EBNA1-expressing stable transfectants, which were named SKOV3.ip1-EBNA1 (ip1-EBNA1-W1 to -W30 and ip1-EBNA1-WL1 to -WL30). The control lines were generated by transfecting pcDNA3

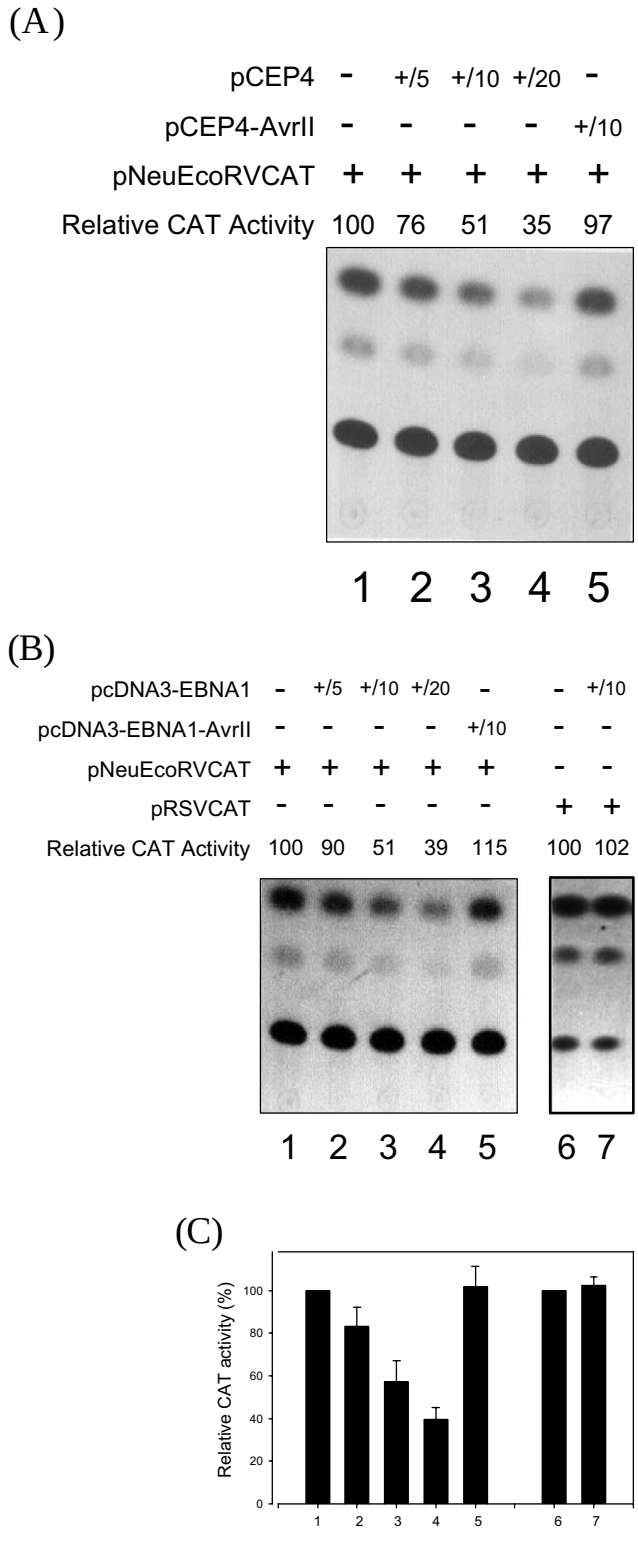

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Fig. 1. Transcriptional and dose-dependent repression of *neu* promoter by EBNA1. A: Dose-dependent repression of *HER2/neu* promoter by pCEP4. A constant amount (10 μg) of pNeu*Eco*RVCAT construct was co-transfected into NIH3T3 cells with either 5 μg (lane 2), 10 μg (lane 3), 20 μg (lane 4) of pCEP4 DNA or 10 μg of the mutant pCEP4-*Avr*II (lane 5) test construct. The relative CAT activity without pCEP4 (lane 1) is defined as 100%. B: Transcriptional and dose-dependent repression of *HER2/neu* promoter by pcDNA3-EBNA1. A constant amount (10 μg) of pNeu*Eco*RVCAT construct was co-transfected into NIH3T3 cells with increasing amounts of pcDNA3-EBNA1, which contains the full-length cDNA of EBNA1. The relative CAT activities with 5 (lane 2), 10 (lane 3) and 20 μg (lane 4) of the test constructs and the control EBNA1 frame-shift mutant construct (lane 5) were 90%, 51%, 39% and 115%, respectively. The relative CAT activity without EBNA1 is defined as 100% (lane 1). For lanes 6 and 7, NIH3T3 cells were transfected with 10 μg of the pRSVCAT construct, which contains the CAT reporter driven by RSV promoter sequences. In lane 6, the CAT activity of the control transfection with the pRSVCAT plasmid is defined as 100%. Lane 7 shows the CAT activity detected after co-transfection with 10 μg of pcDNA3-EBNA1 (102%). C: A bar plot showing the relative CAT activities of panel B. The error bars indicate the standard deviation of the relative CAT activities, which were the means of three independent transfections for which the standard deviation was less than 15%. The total amount of transfected DNA was kept constant by adding the appropriate amount of carrier DNA.

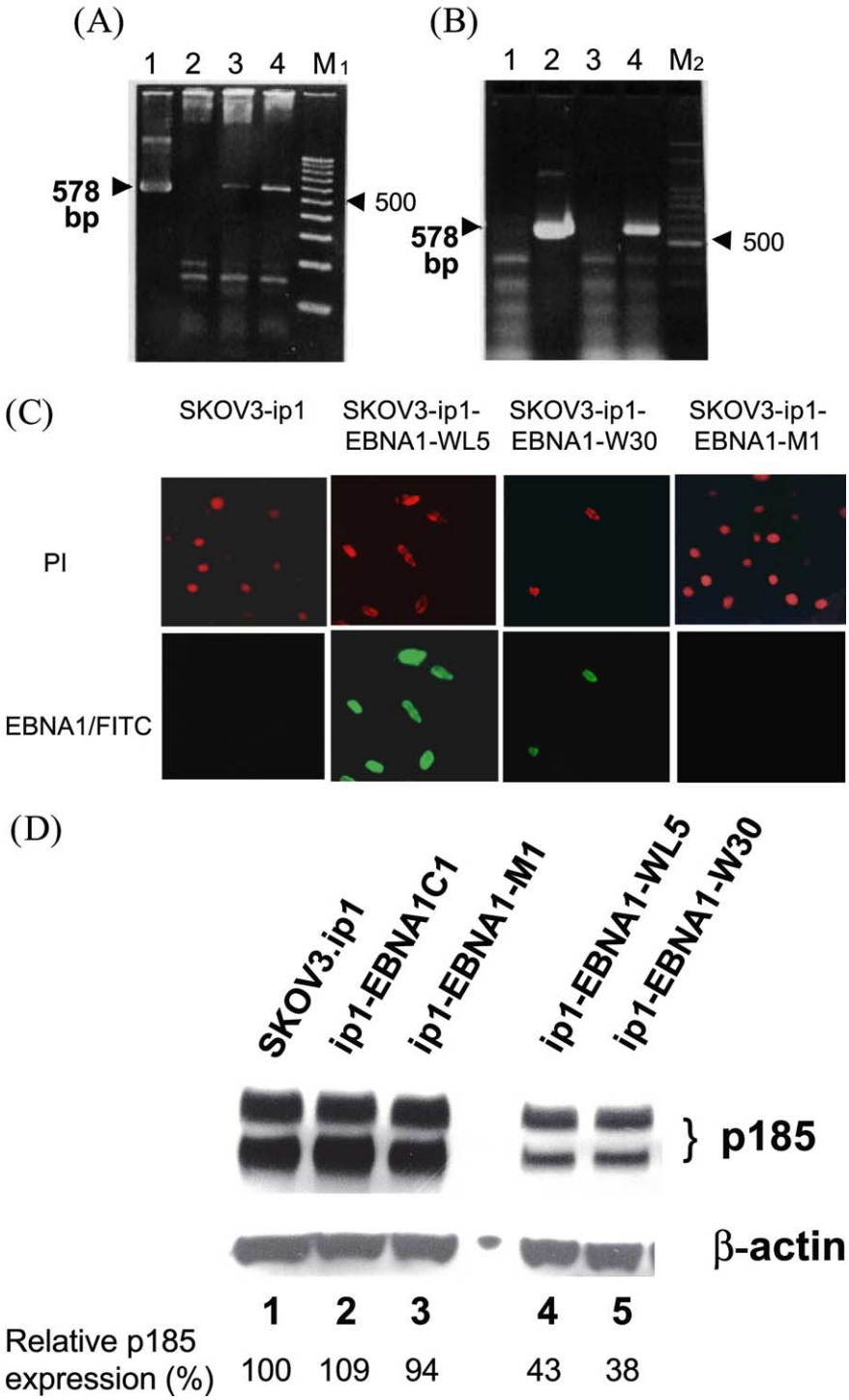

Fig. 2. Stable integration and expression of EBNA1 in SKOV3.ip1 cells resulting in decreased levels of the p185 encoded by *HER2/neu*. A: Genomic PCR analysis of DNAs from ip1 and two stable transfectants, ip1-EBNA1-M1 and ip1-EBNA1-W30. The primers used in this experiment were EBNA1-454S and EBNA1-CAS as described in Section 2. Lane 1, pcDNA3-EBNA1 plasmid as the positive control; 2, parental SKOV3.ip1 as negative control; 3, stable transfectant ip1-M1; 4, ip1-W30. An arrowhead indicates the expected PCR product (578 bp). 100-bp ladder DNA markers (lane M_1) are shown to the right. B: RT-PCR analysis of parental and EBNA1-transfected SKOV3.ip1 cells. Lane 1, parental SKOV3.ip1 cells; 2, pcDNA3-EBNA1 plasmid as the positive control; 3, ip1-EBNA1-M1 cells; 4, ip1-EBNA1-W30 cells. The primers used in this experiment were EBNA1-454S and EBNA1-CAS. An arrowhead indicates the expected RT-PCR product (578 bp). M_2, 100-bp ladder DNA marker. C: Detection of EBNA1 expression in EBNA1-transfected ip1 lines using an indirect immunofluorescence analysis technique. The PI staining in the upper row shows the location of the cell nucleus. The lower row shows that EBNA1 is expressed in the nuclei of the EBNA1-transfected lines, but not in the nuclei of the parental ip1 and mutant ip1-EBNA1-M1 lines. Original magnification was 200×. D: Western blot analysis of *HER2/neu*-encoded p185 protein of parental and pcDNA3-EBNA1-transfected SKOV3.ip1 cells. Lane 1, parental SKOV3.ip1 cells; 2, vector-only control ip1-EBNA1-C1 cell line; 3, the EBNA1 frame-shift line; 4,5, ip1-EBNA1-WL5 and ip1-EBNA1-W30, expressing the full-length EBNA1 stable lines. The *HER2/neu*-encoded p185 protein is shown on the right, and β-actin was used as the protein loading control.

vector only (ip1-EBNA1-C1) and pcDNA3-EBNA1-*Avr*II mutant (ip1-EBNA1-M1 to -M4) into ip1 cells, respectively. The G418-resistant clones were selected in medium containing 800 μg of G418 per ml for 4–6 weeks and expanded to cell lines.

2.6. Genomic polymerase chain reaction (PCR) and reverse-transcribed (RT)-PCR analysis

For genomic PCR analysis, 2 μg of genomic DNA from each of the respective cell lines was used. For RT-PCR analysis, the RNeasy™ Kit (Qiagen) and Superscript™ Kit (Gibco-BRL) were used to extract the total RNA and to do the reverse transcription. The primers used in these genomic PCR and RT-PCR experiments were EBNA1-454S (sense: 5′-GGATCCATGGGTGATGGAGGCAGG-3′) and EBNA1-CAS (antisense: 5′-AAGATATCACTCCTGCCCTTC-3′).

2.7. Indirect immunofluorescence analysis

Due to the low level of expression of EBNA1, an indirect immunofluorescence analysis technique was used to detect the EBNA1 protein. Briefly, cells were cultured on poly-L-lysine-coated cover-slips overnight and then fixed with 3% paraformaldehyde in PBS for 15 min at room temperature. After thorough washing with phosphate buffered saline (PBS), cells were incubated with 1% Triton X-100 at room temperature for 15 min in PBS. Then, the cells were incubated with mouse anti-EBNA1 mAb (1:10 v/v in PBS; Chemicon, MAB8173) at 37°C for 1 h. The cells were then washed again in PBS, and incubated with affinity-purified goat anti-mouse IgG (1:1000 v/v in PBS; Calbiochem, Germany) at 37°C for 45 min. Then, the cells were incubated in the dark with anti-goat fluorescein isothiocyanate (FITC)-conjugated antibody (1:200 v/v, Santa Cruz Biotechnology) at 37°C for 45 min. Unbound antibodies were removed by washing with PBS, after which the cells were further incubated with propidium iodide (10 μM) and RNase A (50 μg/ml) at 37°C for 45 min. Both types of cells were visualized and photographed using a standard fluorescence microscope (Axiophot, Zeiss, Germany).

2.8. Western blot analysis and growth rate analysis by MTT assay

To observe the change of the $p185^{HER2/neu}$ protein expression level in parental and different EBNA1 stable lines, Western blot analysis was performed as described in [4]. In addition, the growth rates of parental, wild-type and mutant EBNA1 stable lines were analyzed by MTT assay as described in [4].

2.9. Anchorage independence assay

A soft agarose growth assay was carried out as follows: Briefly, the wild-type and mutant EBNA1 lines and their parental ip1 cells (1×10^3 cells/well) were plated in 24-well plates in culture medium containing 0.35% agarose (Gibco-BRL, Gaithersburg, MD, USA) overlying a 0.7% agarose layer. The cells were then incubated at 37°C for 3–4 weeks, after which the plates were stained with *p*-iodonitrotetrazolium violet (1 mg/ml) overnight at 37°C. Colonies greater than 100 μm in diameter were counted for each dish. Each soft agarose assay was performed in triplicate.

2.10. Animals and tumorigenicity assay

4–6-week old female pathogen-free Balb/cAnNCrj-*nu/nu* athymic nude mice, obtained originally from Charles River Laboratory (Yokohama, Kanagawa, Japan), were bred in the Animal Center of the National Defense Medical Center, Taipei, Taiwan, ROC. The care and use of the animals were in accordance with institutional guidelines. For the tumorigenicity assay, the wild-type and mutant EBNA1 transfectants and the control ip1 cells in log-phase growth were trypsinized, washed twice with PBS and centrifuged at $1500\times g$. The viable cells were counted, and 1.2×10^6 viable cells in 0.3 ml of serum-free medium were injected s.c. into the right flanks of female homozygous *nu/nu* mice under aseptic conditions. Tumor volumes were estimated as the product of three-dimensional caliper measurements (longest surface length and width; tumor thickness). The growth of tumors was monitored once a week.

3. Results

3.1. Transcriptional and dose-dependent repression of neu promoter by EBNA1

Repression of the *neu* promoter by EBNA1 was dose-dependent (Fig. 1A, lanes 2–4). However, the pCEP4-*Avr*II, a frame-shift mutant of the EBNA1 gene in pCEP4, had no effect on CAT activity (Fig. 1A, lane 5). It appears that the pCEP4 plasmid, which contains the EBNA1 gene, has an efficient repression effect on *neu* gene expression (Fig. 1A). Therefore, the pCEP4 plasmid was used for further analysis. To further study the interactions of EBNA1 gene products with the *neu* promoter, we subcloned the EBNA1 from pCEP4 plasmid into the expressing plasmid vector pcDNA3 resulting in the pcDNA3-EBNA1 recombinant construct. A frame-shift mutant of pcDNA3-EBNA1, named pcDNA3-EBNA1-*Avr*II, was also constructed and used as a negative control. Increasing amounts of pcDNA3-EBNA1 were co-transfected with pNeu*Eco*RVCAT (Fig. 1B,C, lanes 2–4). Inhibition of the gene expression directed by the *neu* promoter

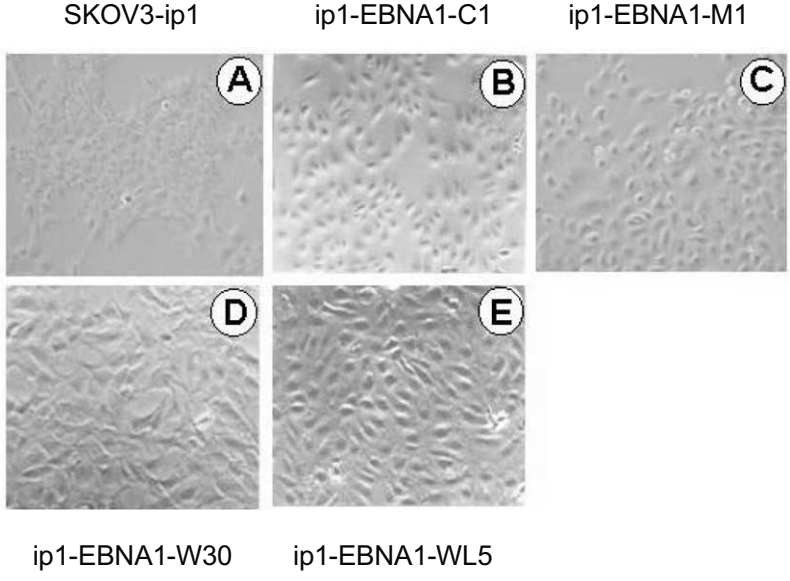

Fig. 3. Morphology change of SKOV3.ip1 cells after being stably transfected with EBNA1. A: SKOV3.ip1 cells. B: ip1-EBNA1-C1 cells. C: ip1-EBNA1-M1 cells. D: ip1-EBNA1-W30. E: ip1-EBNA1-WL5 cells. Panels A–E, 200× magnification.

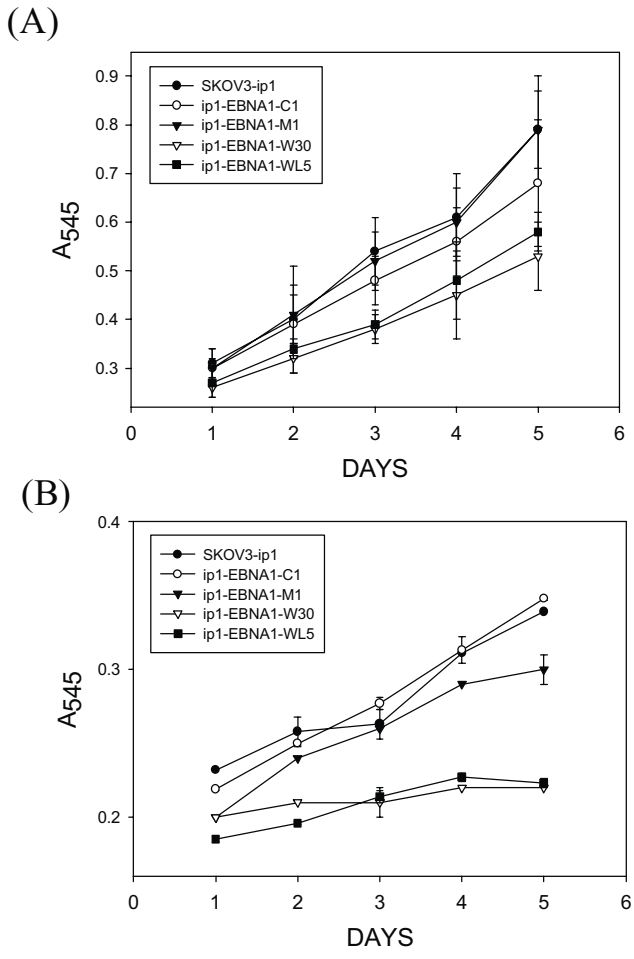

Fig. 4. Growth rate analysis of parental and EBNA1 stably transfected SKOV3.ip1 cells. Results of the MTT metabolic assay after plating 1×10^4 cells into microtiter plates with (A) 10% FBS/DMEM, (B) 0.1% FBS/DMEM. Triplicate experiments were run for each cell line.

was dependent on pcDNA3-EBNA1 concentration, with around 50% repression observed at a pcDNA3-EBNA1:pNeu*Eco*RVCAT ratio of 1:1 (Fig. 1B,C, lanes 1–4). No inhibition was observed in the presence of the pcDNA3-EBNA1-*Avr*II mutant (Fig. 1B,C, lane 5). The repression effect of pcDNA3-EBNA1 is specifically on the *neu* promoter and there is no effect on other promoters such as the RSV promoter (compare lanes 3, 6 and 7 in Fig. 1B,C).

3.2. Inhibited expression of HER2/neu-encoded p185 in EBNA1-expressing ovarian carcinoma transfectants

To see the in vivo effect of EBNA1 on *HER2/neu* gene expression, in this study we used the EBNA1-expressing stable transfectants ip1-EBNA1-WL5 and ip1-EBNA1-W30, and the vector-only (ip1-EBNA1-C1) and EBNA1 frame-shift control lines (ip1-EBNA1-M1 and -M4). To ensure that the exogenous EBNA1 gene had integrated into the genome of the transfectants, genomic PCR analysis was performed. Fig. 2A shows the results of genomic PCR analysis for the parental ip1 cell line and two representative stable transfectants, ip1-EBNA1-W30 and ip1-EBNA1-M1. As expected, the parental ip1 cells did not contain any EBNA1 sequence, whereas the two EBNA1 transfectants had acquired the transfected EBNA1 DNA. The size of the major band (578 bp) was consistent with the length of the designed sequence of the EBNA1 clone (Fig. 2A). Moreover, using RT-PCR EBNA1 mRNA was detected in the ip1-EBNA1-W30 stable lines but not in the parental ip1 cells or the two control lines ip1-EBNA1-C1 and ip1-EBNA1-M1 (Fig. 2B). PI staining and immunofluorescence further showed that the expression of EBNA1 protein was co-localized with the nucleus in the ip1-EBNA1-W30 and -WL5 lines, but not in the parental ip1 and mutant ip1-EBNA1-M1 lines (Fig. 2C), indicating that EBNA1 protein was indeed expressed in the nucleus. To examine whether expression of EBNA1 in ip1-EBNA1 stable transfectants can inhibit *HER2/neu* expression, immunoblot analysis for the *HER2/neu*-encoded p185 protein was performed. The p185 proteins were dramatically reduced in both the EBNA1 transfectants (Fig. 2D, lanes 4 and 5), but not in the vector control or the mutant (Fig. 2D, lanes 2 and 3).

3.3. EBNA1 expression in EBNA1 transfectants can suppress the transformed phenotypes induced by HER2/neu

To determine the effect of EBNA1 expression on the transforming ability of *HER2/neu*-transformed SKOV3.ip1 cells, the two EBNA1 transfectants ip1-EBNA1-W30 and -WL5, the parental ip1 cells, and the ip1-EBNA1-C1 and -M1 control lines were assayed for some of the known transforming parameters. The biological effects of EBNA1 expression on *HER2/neu*-transformed cells were first examined in culture. The highly transformed morphology of parental ip1 cells and the two control lines, ip1-EBNA1-C1 and -M1, was essentially unchanged. These cells were rounded, piled up and did not exhibit contact inhibition (Fig. 3A–C). However, the highly transformed morphology of the ip1 cells was markedly altered by EBNA1 transfection (Fig. 3D,E). The EBNA1-transfectants, ip1-EBNA1-W30 and -WL5, exhibit a non-transformed, flattened morphology and a contact-inhibited growth pattern. These results indicate that EBNA1 gene products can specifically reverse the transformed morphology of *HER2/neu*-transformed ip1 cells.

3.4. In vitro suppression of SKOV3.ip1 cell transformation by EBNA1 expression

The EBNA1-expressing SKOV3.ip1 lines were used to examine the effect of EBNA1 expression on the *HER2/neu* overexpressing ovarian cancer cells in vitro, by assaying growth properties and colony formation in soft agarose. The growth curves of the EBNA1-expressing ip1-EBNA1-W30 and -WL5 lines and the three control lines indicated that EBNA1 expression markedly reduced the growth rate of these ovarian cancer cells in 0.1% FBS media, but slightly in 10% FBS media (Fig. 4A,B). To test the influence of the EBNA1 proteins on anchorage-independent growth, which is a good indicator of transforming ability, all the control lines and the EBNA1 stable lines were assayed for their ability to grow in soft agarose. Fig. 5 shows that the *HER2/neu*-overexpressing ip1 cells and the ip1-EBNA1-C1 and -M1 lines exhibited high efficiency in forming soft agarose colonies, whereas the colony-forming efficiencies of the ip1-EBNA1-W30 and -WL5 lines were strikingly reduced. These data suggest that EBNA1 proteins can suppress the transforming effect of the *HER2/neu* overexpression in ovarian cancer cells and inhibit cell growth and anchorage-independent growth.

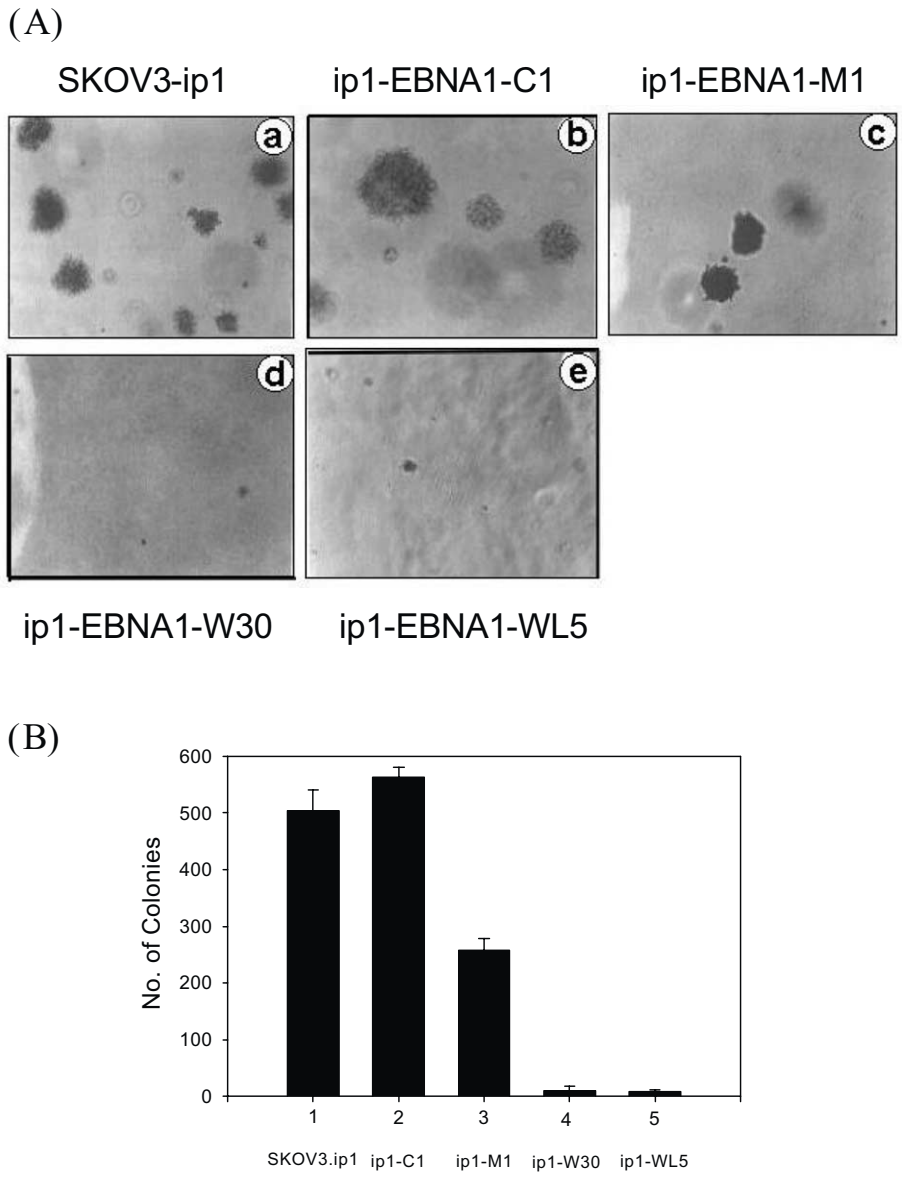

Fig. 5. Soft-agarose colony formation assay of parental and EBNA1 stably transfected SKOV3.ip1 cells. Parental and EBNA1 stably transfected ip1 cells (1×10^3 cells/well) were plated in 24-well plates. A: a, parental ip1 cells; b, ip1-EBNA1-C1 cells; c, ip1-EBNA1-M1 cells; d, ip1-EBNA1-W30 cells; e, ip1-EBNA1-WL5 cells. B: Colonies greater than 100 μm in diameter were counted for each dish. Triplicate experiments were run for each cell line. Error bars indicate the standard deviation of number of colonies/well.

3.5. *EBNA1 as a tumor suppressor gene for HER2/neu-overexpressing human ovarian carcinoma SKOV3.ip1 cells*

A critical test for EBNA1-mediated transformation suppression in ovarian cancer cells is the ability of EBNA1 to suppress tumor formation in vivo. Therefore, a tumorigenicity assay was performed by s.c. injecting *nu/nu* mice with 1.2×10^6 cells from the parental ip1 line and from either the two ip1-EBNA1 stable lines or the two control lines, ip1-EBNA1-C1 and ip1-EBNA1-M1 (Fig. 6). Injections of the parental SKOV3.ip1 and the control ip1-EBNA1-C1 cells produced tumors earlier than the ip1-EBNA1-transfectant lines and the tumor burdens were very large at 4656 ± 2061 mm^3 and 4861 ± 1392 mm^3, respectively, by 70 days post-injection. Injections of the ip1-EBNA1-M1 cells produced tumors a little later than the parental ip1 and ip1-EBNA1-C1 injections, but 70 days after injection the tumor burdens were still large at 3372 ± 1065 mm^3. By contrast, the tumor-suppressing function of EBNA1 was dramatic. Injections of the ip1-EBNA1-W30 and -WL5 cells did not produce tumors even at 70 days post-injection, thus clearly demonstrating that EBNA1 can suppress the tumorigenic potential of the ovarian carcinoma SKOV3.ip1 cells.

4. Discussion

Overexpression of the *HER2/neu* gene is correlated with enhanced tumorigenicity, enhanced metastatic potential, and poor patient prognosis in many types of cancer [27,28]. In this report, we have demonstrated that the EBNA1 gene product can repress *HER2/neu* gene expression at the transcriptional level by targeting the *HER2/neu* gene promoter (Fig. 1). Therefore, it is likely that the reduced $p185^{HER2/neu}$ expression

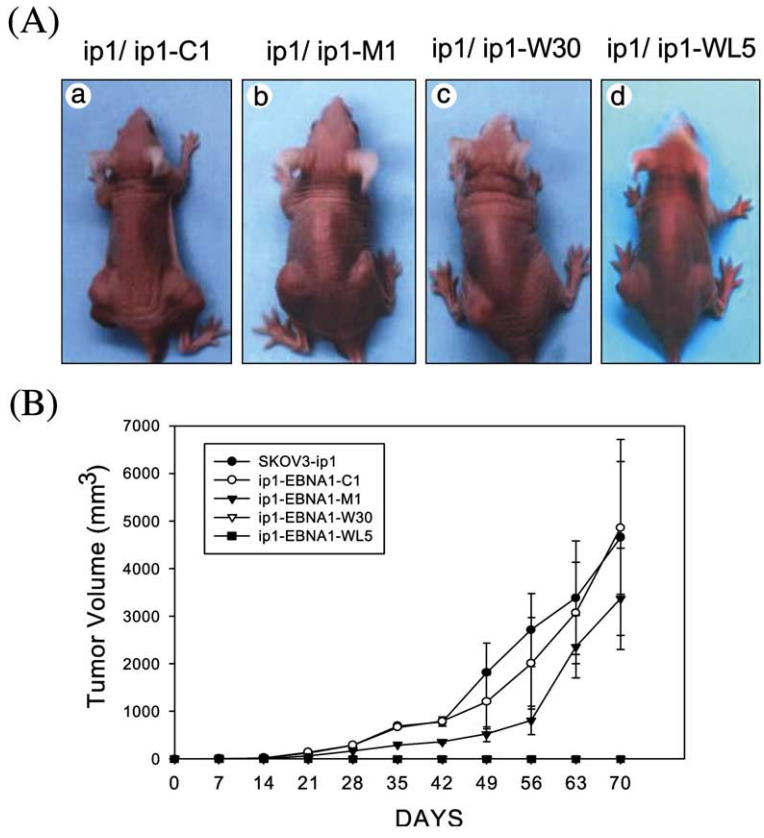

Fig. 6. In vivo tumorigenicity of parental and stable lines. Viable cells (1.2×10^6 cells) were inoculated subcutaneously (s.c.) into nude mice. A: For each group, the parental ip1 cells were injected s.c. into the left flank and the stable lines were injected s.c. into the right flank. (a, ip1-EBNA1-C1; b, ip1-EBNA1-M1; c, ip1-EBNA1-W30; d, ip1-EBNA1-WL5). B: Tumor volumes were estimated as the product of three-dimensional caliper measurements. Data shown here are the average of three individual nude mice. The vertical bars indicate the standard deviation.

in the ip1-EBNA1-expressing cell lines (Fig. 2) is due to transcriptional repression of the overexpressed *HER2/neu* gene. We have also shown that the EBNA1 can act as a transformation suppressor in *HER2/neu*-overexpressing human cancer cells in vitro (Figs. 3–5), and reduce tumorigenicity in vivo (Fig. 6). These results indicate that the EBNA1 gene can function as a tumor suppressor gene for *HER2/neu*-overexpressing human cancer cells.

It has been reported previously that two viral oncoproteins, adenovirus-5 *E1a* gene product (Ad-5 E1A) and SV40 large T antigen (SV40LT), can repress the *HER2/neu* oncogene expression [7–9,29]. Here we report a third viral oncoprotein, EBNA1, that can also act as a transforming suppressor of the *HER2/neu* oncogene. It has been shown that the repression of the *HER2/neu* gene by Ad-5 E1A and SV40LT is mediated by interacting with the transcriptional co-activator p300 [30,31]. However, the mechanism of the repression of the *HER2/neu* gene by EBNA1 remains to be investigated. At present, EBNA1 is known to be involved in a variety of cellular functions, including DNA-binding [32], RNA-binding [19], nuclear localization [33], and transcriptional activation [34]. Since EBNA1 is structurally and functionally different from Ad-5 E1A and SV40LT, then EBNA1 must presumably repress *HER2/neu* oncogene expression via a distinct pathway. We speculate that the repression of *HER2/neu* by EBNA1 may be mediated by an indirect protein–protein interaction mechanism because no EBNA1-specific binding sequences are found on the *HER2/neu* promoter element. Evolutionarily, it will be of interest to know why certain viral oncoproteins, such as Ad-5 E1A, SV40LT and EBNA1, have a suppressing effect on oncogenic transformation.

Acknowledgements: The authors wish to thank Dr. Su-Chien Chang (Department of Biochemistry, National Defense Medical Center, Taipei, Taiwan, ROC) for reading the manuscript and Dr. Mien-Chie Hung (M.D. Anderson Cancer Center, Houston, TX, USA) for kindly providing human ovarian cancer SKOV3.ip1 cells and plasmid pNeu*Eco*RVCAT. This work was supported by the National Science Council (NSC89-2318-B-016-001-M51 and NSC89-2320-B-016-103) and the National Defense Ministry (DOD-89-38 and DOD-90-64), ROC, granted to M.C.K.

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