

High-level transgene expression mediated by the *piggyBac* transposon enhances transgenic therapeutic effects in cervical cancer xenografts

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Abstract. The *piggyBac* (PB) transposon is a recently identified, active and flexible transgene vector, combining the advantages of non-viral gene delivery with genomic integration and persistent transgene expression. In this study, we utilized the PB transposon to carry the herpes simplex thymidine kinase (HSV-tk) and red fluorescent protein (mRFP1) reporter genes into the HeLa cervical cancer cell line or tumor xenografts of cervical cancer. Our data showed that HSV-tk and mRFP1 were expressed in HeLa cells and tumor xenografts three weeks after intratumoral injection. The mRNA and protein levels of HSV-tk and mRFP1 were increased by using the PB transposon vector. Our system also demonstrated that sensitivity of transfected HeLa cells to the pro-drug ganciclovir (GCV) was enhanced *in vitro* and *in vivo*. Furthermore, our data indicated that the enhanced transgenic therapeutic effect was strongly associated with high-level transgene expression mediated by the PB transposon. Our results suggest that applying the PB transposon

in HSV-tk gene delivery and GCV treatment is a promising gene therapy strategy in the treatment of cervical cancer.

Introduction

According to the Global Cancer Facts and Figures published by the American Cancer Society in 2007, 1 in 8 deaths worldwide is due to cancer, accounting for 7.9 million deaths in 2007; furthermore, an estimated 12 million people received cancer diagnoses (1). Uterine cervical cancer is the second most common cancer occurring in females worldwide and is the leading cause of death among women with cancer in developing countries. Despite the existence of effective screening methods, an estimated 555,094 new cases of cervical cancer occurred worldwide in 2007, with 309,808 people dying due to this disease (1).

The conventional therapies for cervical cancer, such as surgery, chemotherapy and radiotherapy, help to reduce the mortality of patients with early-stage tumors. However, the above mentioned methods have been proven to induce some clinical complications. According to the International Federation of Gynecology and Obstetrics (FIGO) the recurrence rate of cervical cancer is 10-20% at stages Ib-IIa and 50-70% in locally advanced cases (FIGO stages IIb-IVa) (2). Radiotherapy is effective for some recurrent cervical cancer patients, but radiation-induced pelvic insufficiency fracture is a frequent complication (3). The role of chemotherapy in patients with recurrent or metastatic tumors is merely palliative (4), as it causes strong side effects such as vomiting, diarrhea and bone marrow suppression. Despite these innovations, one third of patients with invasive cervical cancer have died from recurrent or metastatic tumors. Currently, we lack effective measures to treat uterine cervical cancer; therefore, there is an urgent need to develop new therapeutic strategies (5).

The field of gene therapy promises a number of innovative treatments that are likely to become important in preventing deaths from cancer. Recently, several strategies

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Abbreviations: AAVs, adeno-associated viruses; ECL, enhanced chemiluminescence; FIGO, International Federation of Gynecology and Obstetrics; GCV, ganciclovir; GVHD, graft-versus-host disease; HSV-tk, herpes simplex thymidine kinase; mRFP1, red fluorescent protein; PB, *piggyBac*; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PET, positron emission tomography; SB, Sleeping Beauty

Key words: *piggyBac* transposon, herpes simplex virus, thymidine kinase, non-viral vector, gene delivery, cervical cancer

have been investigated, such as oncogene inactivation, tumor suppressor gene replacement, inhibition of angiogenesis, drug sensitization (suicide genes), antisense strategies, and genetic immunotherapy (cytokines, co-stimulatory molecules, polynucleotide vaccination) (6). However, all *in vitro* and animal studies to date have not resulted in any clinical benefits. Many of the clinical trials have been limited by inefficient gene transduction and low tumoricidal effects (7). The poor transgene efficiency of vectors may be the main reason for this, therefore, the efficiency and safety of transgene vectors needs to be improved.

For long-term therapeutic benefit, vectors that can stably integrate into the genome, and thus provide long-term expression of transgene constructs in cells, are desired (8). The integrating vectors for gene delivery include viral or non-viral vectors. So far, integrating viral vector systems include retroviruses, adeno-associated viruses (AAVs) and lentiviruses. However, those viral vectors have several disadvantages: i) there is a risk of generating active viral particles through *in vivo* recombination; ii) the antigenicity of coat proteins can induce immune reactions to viral vectors that can inhibit gene transfer efficacy (9) and trigger unwanted host inflammatory and immune responses (10); iii) there are strict limits on therapeutic load size as AAVs can only accommodate DNA inserts of up to 4.7 Kb (11); and iv) there is a requirement for cells to be actively dividing for successful retroviral infection and gene integration.

Integrating non-viral vector systems, which are represented by transposons, possess the capacity to stably integrate into the genome, and thus provide long-term expression of transgene constructs in cells (8). Recently, transposons such as *Sleeping Beauty* (SB) (12), *Tol2* (13), *Frog Prince* (14) and *piggyBac* (PB) (15-18) have been widely used to deliver therapeutic genes in mammalian cells. Among them, the PB transposon is the most effective for stable gene integration in mammalian systems. Wu and co-workers also demonstrated that there was no inhibition of overproduction courtesy of the PB transposon, which is advantageous in preclinical development of transposon-based gene therapy (19). Ding and co-workers designed a binary co-transfection assay system consisting of both a donor plasmid containing the transposon, and a helper plasmid containing the transposase (15). However, the potential to improve the clinical outcome of this system still needs to be investigated in a variety of cancer models.

In this study we utilized a PB transposon system to carry a 'suicide' gene, the herpes simplex virus thymidine kinase (HSV-tk) gene, into the HeLa cervical cancer cell line or tumor xenografts of cervical cancer *in vivo*. We also estimated the expression levels of the HSV-tk gene in different clones after cell sorting and a single clone was selected.

Materials and methods

Plasmid construction. To generate the recombinant plasmid PB [Act-RFP, HSV-tk] DS, which we designated pPB/TK, we used the polymerase chain reaction (PCR) to amplify HSV-tk cDNA from pORF-HSVtk (Invivogen, San Diego, CA, USA) with the forward primer, 5'-ATT GGA TCC GCT CCG GTG CCC GTC AGT GG-3' and the reverse primer

5'-CTA ACT AGT TCA GTT AGC CTC CCC CAT CTC C-3'. The resulting PCR fragment was cloned into a pMD18-T vector (Takara Bio Inc., Dalian, China) and subsequently subcloned into the *Bam*HI-*Spe*I sites of the PB[Act-RFP]DS (pPB) vector (15), containing the mRFP1 expression cassette (kindly provided by Xiaohui Wu, Fudan University, Shanghai, China). Correct construction and orientation of fragments was identified by PCR and restriction enzyme analysis. The primers used to identify HSV-tk were: 5'-CCT GTG GTG CCT CCT GAA CT-3' (forward primer); and 5'-GTT GCT ATG GCC GCG AGA AC-3' (reverse primer). A correct clone yielded a 421 bp product via PCR. In order to detect mRFP1 by PCR, the forward primer used was 5'-GGA CGG CGA GTT CAT CTA CA-3', and the reverse primer 5'-TTG ACC TCG GCG TCG TAG TG-3' with a correct clone yielding a 191 bp product.

Complex formation and administration. *In vitro* (jetPEI™) and *in vivo* transfection (*in vivo*-jetPEI) reagents were purchased from Polyplus Transfection (Illkirch, France). The transfection was performed following standard protocols with slight modifications. For the *in vitro* experiments, jetPEI/DNA complexes were prepared at a ratio of 5:1 (PEI nitrogen:DNA phosphate; N/P ratio). Briefly, 1.5 µg pPB/TK and 1.5 µg pPBbase (15) was added to 100 µl of 150 mM NaCl; and add 6 µl of 7.5 mM jetPEI was added to 94 µl 150 mM NaCl. The two solutions were mixed then incubated for 15 min at room temperature before transfection. In the *in vivo* experiments, *in vivo*-jetPEI/DNA complexes were prepared by adding 3.2 µl of 150 mM *in vivo*-jetPEI derivative in a 5% glucose solution and vortex mixed with 10 µg pPB/TK and 10 µg pPBbase in a final volume of 100 µl in a 5% glucose solution. The glucosylated *in vivo*-jetPEI/DNA mixture (N/P ratio = 8) was incubated for 15 min at room temperature, then intratumorally injected into xenografted mice.

Transposition assays. The human cervical carcinoma cell line HeLa, was obtained from the Shanghai Institute of Cell Biology of Chinese Academy of Sciences (Shanghai, China). Cells were cultured in DMEM medium supplemented with 10% (v/v) fetal bovine sera (FBS), 100 U/ml penicillin and 100 mg/ml streptomycin (all from Gibco-BRL, Grand Island, New York, NY, USA). Cells were maintained at 37°C in a humidified incubator containing 5% CO₂. One day prior to transfection, 2x10⁵ cells were seeded into individual wells of 6-well plates. The transfection was performed when cells were 60% confluent. The 200-µl jetPEI/DNA mixture was added to cells in the medium drop-wise with gentle swirling. Following overnight incubation, the medium was replaced with DMEM containing serum and antibiotics. Cells were subcultured every three days for three months.

Tumor model. Six-week old female BALB/c nude mice were purchased from the Animal Center of the Chinese Academy of Science (Shanghai, China) and maintained in specific pathogen-free conditions. To generate cervical cancer tumor xenografts, HeLa cells (5x10⁶ cells in 0.1 ml of medium per mouse) were inoculated subcutaneously into the right flanks of mice. Animals were inspected and weighed every three



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 imal study in this research was performed with approval of Animal Ethics Committee of Obstetrics and Gynecology Hospital, Fudan University in accordance with The Guide for the Ethical Treatment of Laboratory Animals from the Ministry of Science and Technology of the People's Republic of China (Publication No. 2006-398).

Western blot analysis. The level of thymidine kinase (TK) and red fluorescent protein (mRFP1) was evaluated by Western blot analysis. Cell lysates were prepared from untransfected HeLa cells or cells transfected with pPB/TK, pPB or pORF-HSVtk. At 24 h post-transfection, cells were harvested and resuspended in phosphate-buffered saline (PBS). Total protein was loaded per lane on a 5-10% SDS-PAGE gel (Invitrogen) and transferred to a nitrocellulose membrane (Bio-Rad). The membrane was blocked with PBS containing 0.05% Tween-20 and 5% non-fat dry milk overnight at 4°C. After two washes in PBS with 0.05% Tween-20, anti-HSV1-TK goat polyclonal antibody (1:200; Santa Cruz Biotechnology, Inc., CA, USA) and anti-mRFP1 rabbit polyclonal antibody (1:1000; MBL International Corp., USA) was added for 1.5 h at room temperature. After a further three washes, a secondary antibody was added for 1.5 h at room temperature. Using an enhanced chemiluminescence (ECL) kit (Amersham Life Science, Little Chalfont, UK), the membrane was developed on Kodak film in a dark room. The film was then scanned for quantitation of the TK signal with β -actin used as an internal control.

Flow cytometric analysis. One day after transfection, 5×10^4 HeLa cells from each untransfected or transfected culture was harvested by trypsinization and washed with PBS. Flow cytometric analysis was performed on a FACSCalibur, with data analyzed using CellQuest software (Becton-Dickinson, USA) and presented as the percentage of mRFP1-positive cells.

Cytotoxicity assays in vitro. A cytotoxicity assay was performed on untransfected HeLa cells and those transfected with pPB/TK, pPB and pORF-HSVtk. Cells were seeded onto 96-well plates at a density of 1×10^4 cells/well. To certain wells 0.1, 1, 10 or 100 μ g/ml of GCV (Roche, USA) was added. Five days later, the number of the surviving cells was analyzed by the MTT method using a Cell Titer 96 Aqueous Non-Radioactive Cell Proliferation Assay (Promega, USA). All experiments were carried out in triplicate.

Animal studies. Following the two-week injection of mice with HeLa cells, they were intratumorally injected with 10 μ g pPB/TK and 10 μ g PBase (PB/TK group) to observe the long-term expression of the transgene. The group inoculated with 10 μ g pORF-HSVtk (TK group) was used as controls. Mice were sacrificed 2, 7, 14 and 21 days post-injection and the subcutaneous tumor removed in order to detect HSV-tk gene expression by Western blot analysis and mRFP1 gene expression by flow cytometric analysis, as described previously.

To test the *in vivo* sensitivity of the pPB/TK transfected cells to GCV, 20 mice were assigned to four groups, so that the mean tumor volume in each group was comparable. The

mice were intratumorally injected with 10 μ g pPB/TK and 10 μ g PBase (PB/TK group), 10 μ g pPB and 10 μ g PBase (PB group), 10 μ g pORF-HSVtk alone (TK group) in 5% dextrose, or 5% dextrose alone (control group) two weeks after tumor inoculation. Every mouse was intra-tumorally injected with 100 μ l *in vivo*-jetPEI/DNA complexes once a day on days 1, 4 and 7. Twenty-four h after the first injection, 25 mg/kg GCV was intraperitoneally injected twice daily for seven days. Tumor size was measured twice a week, and tumor volume was estimated using the formula: $V = \text{length (mm)} \times \text{width}^2 (\text{mm}^2)/2$. Three weeks after the last injection, mice were euthanized and necropsies performed. The tumors were weighed to determine the tumor inhibitory rate, calculated using the following formula: Inhibitory rate (%) = $[(\text{tumor weight of control group} - \text{tumor weight of experimental group}) / \text{tumor weight of control group}] \times 100$.

Whole-body imaging. After transfection of pPB/TK and intraperitoneal injection of GCV, anesthetized mice were imaged with a NightOWL LB981 *in vivo* optical imaging system (Berthold Technology Co., Germany). For mRFP1 detection, an anesthetized mouse was placed in a darkened instrument chamber and images obtained using an exposure time of 1 sec. For visualization of mRFP1, an excitation filter of 530/20 nm, and an emission filter of 600/20 nm was used. Gray-scale and pseudocolor images were merged using the WinLight32 software. Mice in the control group were also examined.

Apoptosis and cell cycle analysis. To analyze the effect of the HSV-tk/GCV system on cervical cancer cells, flow cytometric analysis of DNA content was performed to assess the cell cycle phase. Fresh tumor tissues from each group were obtained and made into homogeneous single-cell suspensions and stained for DNA content using propidium iodide. The computer program Multicycle from Phoenix FlowSystem (USA) was used to generate histograms so as to determine the cell cycle phase distribution. Histopathological analysis was also performed on heart, lung, liver, spleen, kidney and tumor sections. The tissues were fixed in formalin and embedded in paraffin and then stained with hematoxylin and eosin (H&E).

Cell sorting and cell cloning. One day following transfection, $\sim 1 \times 10^7$ pPB/TK transfected cells were harvested by trypsinization and sorted with a FACSCalibur using a 620-nm filter. The mRFP1 positive cells were collected and cloned by limiting dilution in round-bottomed 96-well plates. The mRFP1-positive single clones were selected by fluorescent microscopy. Different single clones were collected to detect HSV-tk gene expression by quantitative RT-PCR and Western blot analysis, and then cytotoxic effects of the HSV-tk/GCV system were assayed. The Western blot analysis and cytotoxicity assays were performed as described above.

Quantitative RT-PCR. Total RNA was extracted from different single HeLa clones using TRIzol reagent following the manufacturer's protocol. After heating at 65°C for 5 min to denature RNA and to inactivate RNases, 2 μ g total RNA was subjected to reverse transcription for cDNA synthesis

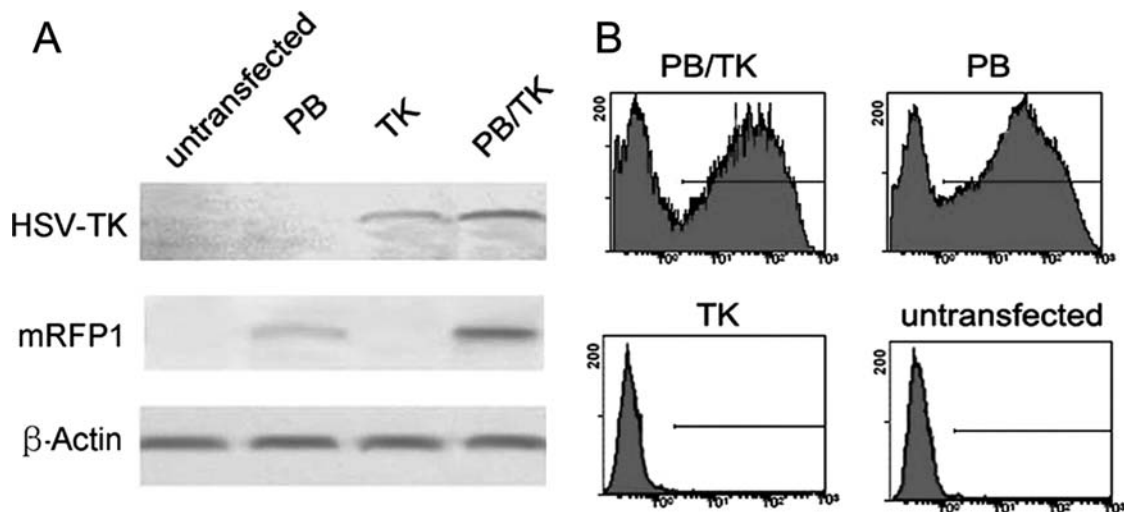


Figure 1. The PB transposon improves the expression of the HSV-tk gene *in vitro*. (A) Expression of the thymidine kinase (TK) protein Western blot analysis of lysate extracted from HeLa cells after transfection. Staining with anti-TK antibody yields a strong signal from pPB/TK-transfected cells, with a very faint band visible in pORF-HSVtk-transfected cells. pPB-transfected cells and HeLa cells alone show no signal for TK. Staining with mRFP1 antibody yields a signal in pPB/TK-transfected cells but not for pORF-HSVtk-transfected cells and untransfected cells. β -actin expression was similar in all four cell lines. (B) Transfection efficiency of the PB transposon in HeLa cells. HeLa cells were transfected with pPB/TK and pPBBase, or pPB and pPBBase, or pORF-HSVtk alone. The percentage of mRFP1-positive cells was measured by FACS 24 h after transfection.

using the SuperScriptTM III First-Strand Synthesis System for RT-PCR kit (Invitrogen, USA). Then 2 μ l cDNA was used as the template for PCR amplification. The primer sequences for amplifying HSV-tk were: 5'-CCT GTG GTG CCT CCT GAA CT-3' (forward primer); and 5'-GTT GCT ATG GCC GCG AGA AC-3' (reverse primer). The relative mRNA level in each sample was calculated after normalizing the values to the level of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene. The primers used for detection of GAPDH were: 5'-ACC ACA GTC CAT GCC ATC AC-3' (forward primer); and 5'-TCC ACC ACC CTG TTG CTG TA-3' (reverse primer). A total of 40 amplification cycles were employed. Quantitative values were obtained from the cycle threshold (Ct) number that indicates exponential amplification of the PCR product (Applied Biosystems 7500 Real-Time PCR System; Applied Biosystems, USA). Using the $2^{-\Delta\Delta CT}$ method (20,21), the data are presented as the fold-change in gene expression relative to pORF-HSVtk-transfected cells.

Inverse PCR and sequencing. PB transposon insertion sites in transformed cells were identified using an inverse PCR technique (22). Genomic DNA (1 μ g) from two transformed single clones was isolated using a Genomic DNA purification kit (Promega, Madison, WI, USA) and then digested with *Hae*III. The *Hae*III-digested, self-ligated fragments were used as the template for the inverse PCR. The primers used to bind the flanking sequence of the right side of the PB transposon were: 5'-CCT CGA TAT ACA GAC CGA TAA AAC ACA TGC-3' (RF1); and 5'-AGT CAG TCA GAA ACA ACT TTG GCA CAT ATC-3' (RR1). The thermal cycling conditions used for the PCR involved denaturation at 93°C for 30 sec, annealing at 57°C for 30 sec, and extension at 65°C for 3 min over 39 cycles. The PCR products were separated by electrophoresis on a 2% (w/v) agarose gel.

Specific bands were excised, and the DNA purified using the QIAquick Gel Extraction Kit (Qiagen, USA). These fragments were cloned into the pMD18-T vector (Takara Bio Inc., China) and sequenced using the dideoxy nucleotide chain termination method with M13 forward and reverse primers on a Beckman CEQ 8000 sequencer. Sequencing results were analyzed using NCBI BLAST searches (www.ncbi.nlm.nih.gov) and the Ensembl human genome database (www.ensembl.org). To detect additional sequence preferences of PB insertion events, five base pairs upstream and downstream of the TTAA target site were analyzed for PB insertions in HeLa cells.

Statistical analyses. Results are expressed as means \pm SD. Statistical comparison of two means were done using the Student's unpaired t-test. A $p < 0.05$ was considered statistically significant.

Results

The PB transposon improves the expression of the HSV-tk gene *in vitro*. We first generated the recombinant plasmid pPB/TK, which contains a PB transposon, the HSV-tk gene and an mRFP1 expression cassette. The pPB/TK and pPBBase plasmids were co-transfected into HeLa cells using the jetPEI transfection reagent to examine the effects of the PB transposon on transgene expression. The same amount of cDNA for pPB and pPBBase, or pORF-HSVtk alone was transfected individually as controls. The untransfected group treated with PBS was also included as a control. The protein levels of TK and mRFP1 were determined by Western blot analysis. A much stronger band corresponding to TK is observed in cell extracts from pPB/TK-transfected cells compared to a very faint band in pORF-HSVtk-transfected cells (Fig. 1A). This was an indication that the PB transposon enhanced HSV-tk

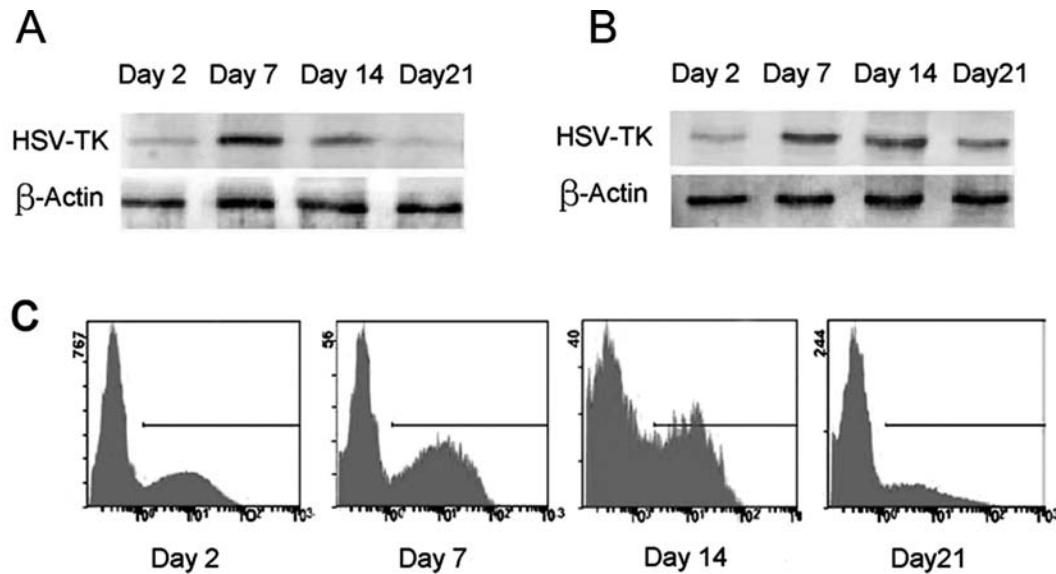


Figure 2. PB transposon improves the expression of the HSV-tk gene *in vivo*. (A and B) Western blot analysis of TK transient expression in subcutaneous tumors on days 2, 7, 14 and 21 after transfection. (A) The expression of TK in the TK group was observed on day 2, peaking on day 7 and diminishing gradually until day 14. (B) The expression of TK in the PB/Tk group was observed on day 2, peaking on day 7, and lasting until day 21. (C) Flow cytometry analysis of mRFP1 positive cells in the PB/Tk group accounted for $37.7 \pm 4.89\%$ of the whole cell population on day 2, up to $53.08 \pm 5.04\%$ on day 7, decreasing to $41.47 \pm 5.56\%$ on day 14 and $26.21 \pm 6.66\%$ on day 21.

expression. The expression of mRFP1 was observed in both pPB/Tk and pPB-transfected cells (Fig. 1A). The flow cytometry results showed that the percentage of mRFP1-positive cells in pPB/Tk and pPB-transfected cells were $67.27 \pm 7.62\%$ and $76.32 \pm 11.15\%$, respectively, whereas pORF-HSVtk-transfected cells and untransfected cells exhibited no measurable mRFP1 expression (Fig. 1B). These results confirmed that the PB transposon could efficiently increase HSV-tk expression in HeLa cells. Additionally, red fluorescence in pPB/Tk- and pPB-transfected cells could not be observed using a fluorescence microscope three months after transfection (data not shown).

The PB transposon improve the expression of the HSV-tk gene in vivo. The transient expression of the HSV-tk gene on days 2, 7, 14 and 21 were analyzed by Western blot analysis, with the results showing that HSV-tk expression in the TK group was observed on day 2, peaking on day 7 and gradually diminishing up until day 14 (Fig. 2A). The HSV-tk gene expression in the PB/Tk group was observed to last longer, up to 21 days (Fig. 2B). These results were confirmed by flow cytometry analysis (Fig. 2C). The mRFP1 positive cells in the PB/Tk group accounted for $37.7 \pm 4.89\%$ of the total cell population on day 2, $53.08 \pm 5.04\%$ on day 7, then decreased to $41.47 \pm 5.56\%$ on day 14 and $26.21 \pm 6.66\%$ on day 21. Additionally, the Student Newman-Keuls method revealed significant differences between PB/Tk groups with day 7 greater than day 2, and day 14 greater than day 21. The proportion of mRFP1 positive cells in the PB/Tk group on day 7 was greater than on days 2 and 14 ($p < 0.05$), and significantly different from those present on day 21 ($p < 0.01$).

The PB transposon enhances the therapeutic effect of HSV-tk/GCV. To test the therapeutic effect of the HSV-tk/GCV

system on transfected HeLa cells, a cytotoxicity assay was performed. The HeLa cells were transfected with pPB/Tk and pPBbase, pPB and pPBbase, or pORF-HSVtk alone, and seeded onto 96-well plates 24 h later at a density of 1×10^4 cells/well. The tested concentrations of GCV were 0.1, 1, 10 and 100 $\mu\text{g/ml}$, with cytotoxic effect measured by the MTT method five days later. As shown in Fig. 3A, the pPB/Tk-transfected HeLa cells were more sensitive to GCV treatment, with an IC_{50} value of 0.32 $\mu\text{g/ml}$, compared with 24.89 $\mu\text{g/ml}$ on pORF-HSVtk-transfected HeLa cells ($P < 0.01$). At 1 $\mu\text{g/ml}$ GCV, $\sim 54.78\%$ of cells were dead in the pPB/Tk-transfected group compared with an approximate 35.46% rate of death in pORF-HSVtk-transfected cells. The non-transfected HeLa cell and pPB-transfected cells were very resistant to GCV treatment with only 3.46% and 11.38% dead cells apparent, respectively, at a GCV dosage up to 10 $\mu\text{g/ml}$ ($P > 0.05$).

To further ascertain whether PB transposon-mediated gene transfer supports more stable HSV-tk gene expression *in vivo* and thus enhanced sensitivity to GCV treatment, the mice with subcutaneous tumors were assigned to four groups: PB/Tk, PB, TK, and control. Every mouse was intratumorally injected with *in vivo*-jetPEI/DNA complexes, followed by GCV administration. Fig. 3B shows that the tumor volume in the PB/Tk group was significantly reduced, while a slight inhibition in tumor growth was observed in the TK group. The weights of tumors were determined to assess the tumor weight inhibitory rate. Three weeks after the last injection, the average tumor weight was 0.53 ± 0.21 g in the PB/Tk group, 1.23 ± 0.35 g in the TK group, 2.13 ± 0.68 g in the PB group and 2.42 ± 0.57 g in the control group. The inhibitory rate of tumors was 77.15% in the PB/Tk group, much higher than that of mice transfected with pORF-HSVtk (34.52%). The difference between the PB/Tk and control

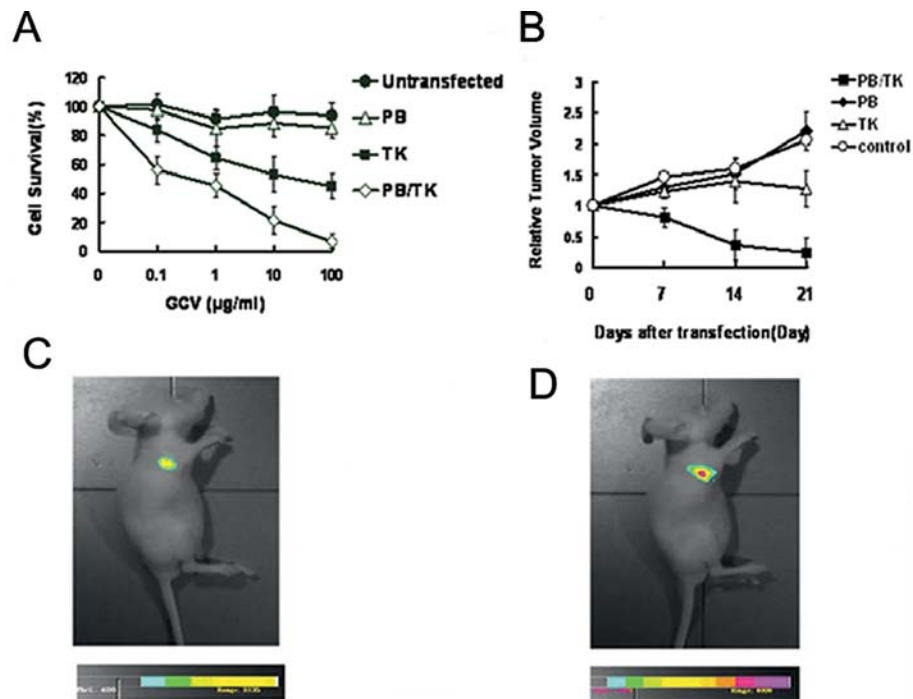


Figure 3. PB transposon enhances the therapeutic effects of HSV-tk/GCV. (A) Cytotoxic effect of GCV on different transfected HeLa cells pPB/TK-transfected cells were seeded onto 96-well plates (1×10^4 cells/well), and subsequently exposed to different concentrations of GCV (0, 0.1, 1, 10 and 100 $\mu\text{g/ml}$) for five days. Cell survival was determined using the MTT assay. Error bars represent the standard deviation of triplicate data. This experiment was repeated twice with similar results. (B) Tumor growth curve. The day after transfection with pPB/TK, pPB, pORF-HSVtk using *in vivo*-jetPEI/DNA, GCV was intraperitoneally injected twice daily for seven days. Tumor volumes were monitored over time. Mice were sacrificed three weeks after transfection. The tumor volume in the PB/TK group was significantly reduced, whilst a slight inhibition in tumor growth was observed in the TK group. Errors bar represent the standard error of the mean. (C and D) Imaging of mice three weeks after *in vivo* transfection. Representative whole-body images of mice taken three weeks after *in vivo* transfection. There is a significant decrease in the expression of mRFP1 in the PB/TK group (C) compared to the PB group (D).

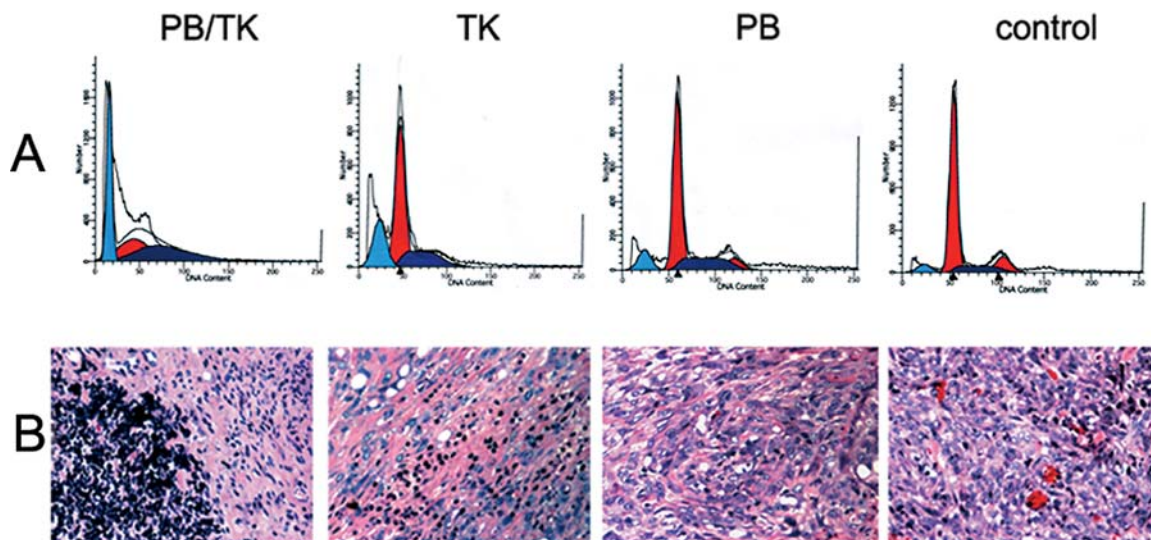


Figure 4. HSV-tk/GCV induced tumor cell apoptosis and cell cycle phase distribution changes *in vivo*. (A) Flow cytometric analysis. Fresh tumor tissues in the PB/TK (panel 1), TK (panel 2), PB (panel 3) and control groups (panel 4) were obtained and made up as a homogeneous single-cell suspension and analyzed for quantitation of DNA fragmentation by flow cytometry incorporating propidium iodide. (B) H&E staining of tumor tissue samples removed from different subgroups the PB/TK group (panel 1) exhibited extensive necrosis and lymphocyte aggregation. The TK group (panel 2) showed minimal necrosis but moderate lymphocyte aggregation around the tumor cells. The PB (panel 3) and control groups (panel 4) exhibited enlarged hyperchromatic and pleomorphic nuclei with irregular nuclear membrane and nucleoli. Magnification is $\times 100$ for all micrographs.

groups had a P-value < 0.01 . Therefore, the growth of tumors was significantly inhibited in mice transfected with pPB/TK,

and the therapeutic effect of the HSV-tk gene was enhanced by the PB transposon delivery system. In all experiments, no



	Apoptosis (%)	Cell cycle phase distribution		
		G0-G1	S	G2-M
PB/TK	41.32±7.68	40.74±8.12	51.84±6.73	7.54±2.13
TK	21.24±7.11	55.54±12.17	34.63±9.53	9.72±3.15
PB	10.65±5.30	69.83±9.02	24.85±6.02	5.95±7.81
Control	8.1±4.38	72.47±8.23	22.43±9.22	5.39±1.53

Fresh tumor tissues from the PB/TK, TK, PB, and control groups were obtained and reconstituted as single-cell suspensions, then analyzed for quantitation of DNA fragmentation by flow cytometry using propidium iodide-stained nuclei.

obvious toxicity was observed in major organs as determined by histopathological analysis (data not shown). The results demonstrated that non-viral gene transfer using a PB transposon resulted in potent inhibition of tumor growth.

In addition, we also monitored transgene expression *in vivo* by assessing mRFP1 reporter gene expression in live mice. After transfection of pPB/TK and intraperitoneal injection of GCV, mice were visualized with an *in vivo* optical imaging system. The expression of mRFP1 was detected in tumor cells, with a significant decrease of mRFP1 expression in the PB/TK treatment group (Fig. 3C) compared to the PB group (Fig. 3D).

HSV-tk/GCV induced tumor cell apoptosis and cell cycle phase distribution changes *in vivo*. To observe the induction of tumor cell apoptosis and cell cycle phase distribution changes induced by the HSV-tk/GCV system *in vivo*, tumor tissues were analyzed for quantitation of DNA fragmentation by flow cytometry (Fig. 4A). The rate of apoptosis for tumor cells in the PB/TK group was 41.32±7.68%, significantly higher than those in the TK (21.24±7.11%) and PB groups (10.65±5.3%; $P<0.05$). The results also showed that the percentage of S-phase tumor cells in the PB/TK group was significantly higher than in the TK, PB and control groups ($P<0.05$; Table I).

We also analyzed sections from those tumor tissues to determine anti-tumor effects of the HSV-tk/GCV system *in vivo*. Paraffin-embedded tissues were sectioned at 4 μ m and stained with H&E for histopathology analysis. These sections exhibited extensive necrosis and lymphocytes were aggregated in the tumor tissue of the PB/TK group (Fig. 4B), whereas only minimal necrosis and moderate lymphocyte aggregation was identified in sections from the TK group (Fig. 4B). Necrosis was absent in tumor samples from both the PB and control groups (Fig. 4B).

High levels of transgene expression enhance the therapeutic effects of the HSV-tk gene. Our results demonstrate that transferring the HSV-tk gene with a PB transposon resulted in inhibition of tumor growth *in vivo*. To confirm whether the enhanced killing effects of GCV on tumor tissues following intratumoral injection of the pPB/TK was due to high levels of HSV-tk expression, we performed a single clone assay. Briefly, one day after transfection, pPB/TK-transfected cells

were sorted by a FACSCalibur and mRFP1 positive cells were collected. The range of mRFP1 positive cells was 59.58-74.82% of the total population. The sorted cells were cloned by limiting dilution, and mRFP1 positive single clones were selected by fluorescent microscopy. Forty-nine different clones were obtained and two of these clones (PB/TK-S16 and PB/TK-S43) were used in further experiments. The total RNA and protein was extracted and measured by quantitative PCR and Western blot analysis. To estimate the fold-change of the HSV-tk mRNA levels among the different clones, quantitative PCR analysis was conducted on RNA extracts from the two clones, with GAPDH was used as a reference gene. The levels of HSV-tk mRNA in the two clones were 1.22-fold (PB/TK-S16) and 182.74-fold (PB/TK-S43) higher than pORF-HSVtk-transfected cells (Fig. 5A). The data indicated that the enhanced transgene RNA signal in different clones may be caused by the production of the PB transposon. Western blot analyses were also performed on total cellular protein to determine relative TK protein levels. Protein extracts were made from clone PB/TK-S16, PB/TK-S43 and cells transiently transfected with pORF-HSVtk. A much stronger band for HSV-tk was observed in clone PB/TK-S43 compared to PB/TK-S16, with the relative protein levels 10.58-fold and 2.37-fold greater, than that of pORF-HSVtk-transfected cells (Fig. 5B). These results confirmed that the PB transposon could efficiently increase HSV-tk expression in HeLa cells.

To determine if the enhanced cytotoxic effect was due to high levels of HSV-tk expression, we performed cytotoxicity assays on clones PB/TK-S16, PB/TK-S43, the pORF-HSVtk-transfected cells and untransfected HeLa cells following GCV treatment. Our data showed that all clones were killed by GCV in a dose-dependent manner with different sensitivities, while HeLa cells were resistant to GCV treatment and remained viable. As shown in Fig. 5C, PB/TK-S43 cells were most sensitive to GCV treatment with an IC_{50} value of 0.36 μ g/ml, compared with PB/TK-S16 and pORF-HSVtk-transfected cells at 8.58 and 9.58 μ g/ml, respectively ($P<0.01$). At a GCV concentration of 10 μ g/ml, about 71.57% of PB/TK-S43 cells were dead compared to 58.65% dead for clone PB/TK-S16, and 55.27% dead in pORF-HSVtk-transfected cells. The parental HeLa cell line was especially resistant to GCV treatment with only 2.36% dead cells when GCV was used at 10 μ g/ml (Fig. 5C).

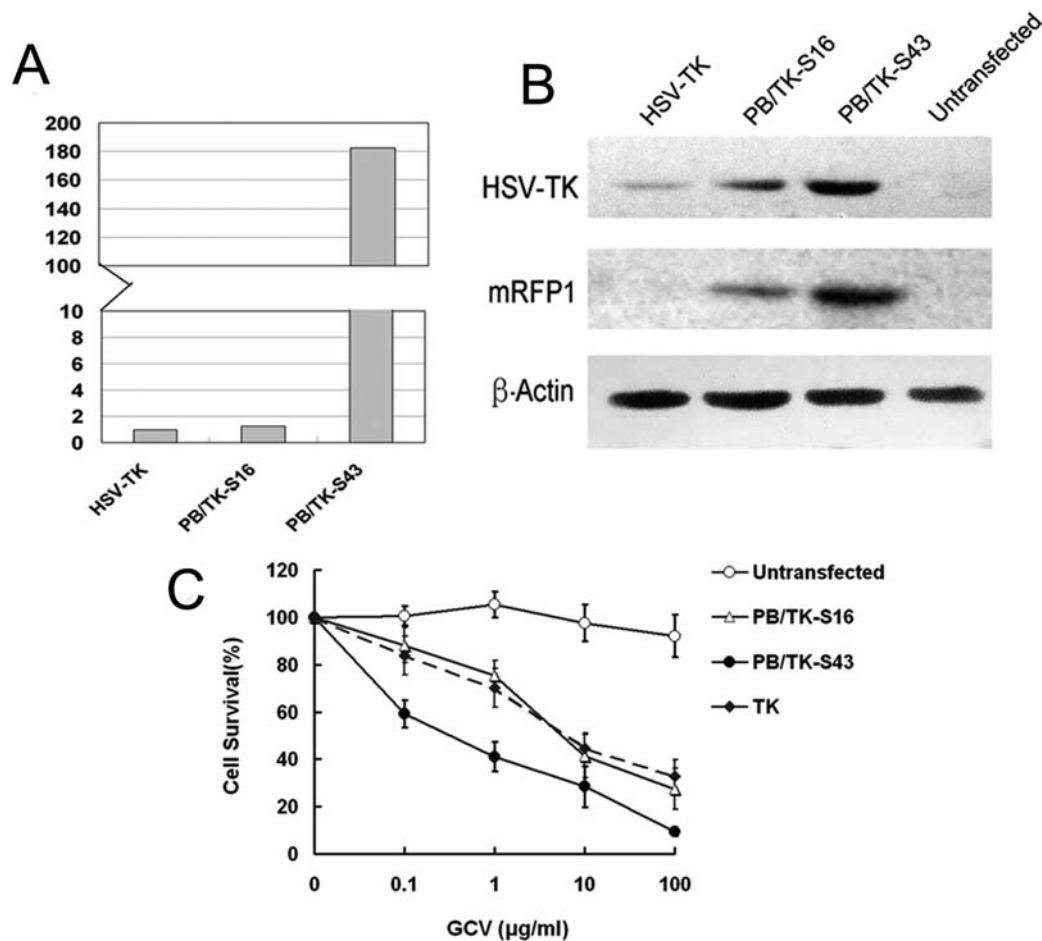


Figure 5. High levels of transgene expression enhanced the therapeutic effects of the HSV-tk gene. (A) Expression of HSV-tk gene at the RNA level. The RNA level of HSV-tk expression was 1.22-fold (PB/TK-S16) to 182.74-fold (PB/TK-S43) greater than pORF-HSVtk-transfected cells. The GAPDH gene was used as a reference. (B) Expression of HSV-tk at the protein level. Western blottings of protein extracts from the clones PB/TK-S16, PB/TK-S43 and pORF-HSVtk-transfected cells demonstrated a much stronger band for the presence of TK in cell extracts from PB/TK-S43 compared to a very faint band in the PB/TK-S16 extracts. Untransfected cells had no detectable signal. The expression β -actin was similar in all cell lines. (C) Enhanced lethality in HeLa cells following transfer of the HSV-tk gene with PB vectors. The *in vitro* sensitivity of these different clones to GCV was evaluated by the MTT method. Cells were seeded into 96-well plates (1×10^4 cells/well), and subsequently exposed to different concentrations of GCV (0, 0.1, 1, 10 and 100 μ g/ml) for five days. The number of remaining viable cells was determined using an MTT assay. Error bars represent the standard deviation of triplicate data. This experiment was repeated twice with similar results.

Table II. PB transposon integration sites in the genome of transfected cells.

Locus no.	Gene sequence adjacent to PBR	Chromosome	GENE/ensemble ID	Insertion locus
A12	TTAAGCTCGAGC	Repeats		
F07	TTAATACCTTTG	6	NT_025741	Intron
F12	TTAAGCAGGAGG	1	NT_167214.1	Intron
F09	TTAAGTAAAGCC	Repeats		
F11	TTAACAGCCAGG	1	NT_004610.19	Intron
G12	TTAACTTCACTA	Repeats		

The sites of integration in the transfected cells were identified by inverse PCR analyses. The six identified insertions confirmed a consensus TTAA-site-specific integration site in the PB transposon, and these insertions were dispersed on different chromosomes.

Confirmation of PB integration in the genome of transfected cells. The sites of integration in the transfected cells were identified by inverse PCR analyses performed using inverted oligonucleotide primers within the right arm of the PB



n. We isolated and identified six different insertion sites, two transfected single clones. Sequence analysis of these six insertions confirmed consensus TTAA site-specific integration of the PB transposon, and those insertions were dispersed on different chromosomes (Table II). These multi-copy insertions appear to have occurred in different locations in the human genome, consistent with the transpositional mechanism of transgene integration.

Discussion

We have used the PB transposon system to carry a 'suicide' gene (HSV-tk) into cervical cancer cells *in vitro* and cervical cancer tumor xenografts *in vivo*. Our results demonstrated high-level HSV-tk gene expression mediated by the PB transposon increased the sensitivity of cervical cancer cells to the pro-drug GCV. The HSV-tk gene was used in our study since the transgene itself does not lead to any effects in transfected human cells but produces a cytotoxic nucleotide analog, which kills target cells after administration of GCV (23). The combination of the HSV-tk suicide gene and GCV is an effective measure in killing targeted cells and has been applied in several clinical areas, such as suicide gene therapy for malignant tumors (24), graft-versus-host disease (GVHD) (25), and PET reporter probes (26). However, its efficacy is not satisfactory, and the main reasons are: i) the transfection efficiency of the HSV-tk gene is low as it has a low expression level *in vivo* (27); and ii) due to the poor efficiency of gene transfer and expression *in vivo*, the clinical benefit of this system is limited, which in turn will require high doses of GCV in treatment. There are concerns that high doses of GCV might induce leukopenia, thrombocytopenia, renal failure and other adverse side effects (28). The above-mentioned disadvantages have resulted in poor clinical application and greatly limited its usage.

To improve the therapeutic potential of the HSV-tk/GCV system, substantial research has been carried out to improve the transfection efficiency and enhance the expression of HSV-tk *in vivo* as well as to increase the level and duration of expression of the gene. Many novel transfection reagents are readily available, but transfection efficiency has not been improved. The transient expression of normal plasmids does not meet clinical requirements which require a long-term, low dose therapy. Thus, recent research has been focused on developing vectors which are highly efficient and able to be integrated into the genome. The viral vector is one type of integrated expression vector encompassing retroviral, adenovirus related vectors and lentiviral vectors. All viral vectors have undergone rapid development, but their poor safety and strong immunogenicity have limited their clinical application.

In this study, an integrating non-viral vector system, the PB transposon, was tested to deliver the HSV-tk gene into HeLa cervical cancer cells and tumor xenografts of cervical cancer. One of the main advantages of the PB transposon as a transgene vector is its large genetic load capacity. Ding and coworkers showed that this PB transposon can accommodate foreign genes up to 14 kb in length, which is larger than what retroviral and AAV vectors, and the SB transposon vectors can hold (15). Some studies have shown that PB is the most

effective transposon, as it has a target integrating ability, enabling it to enter host cells and express in a stable manner with reduced side-effects due to low-immunogenicity and reconstruction *in vivo* (19). We utilized the PB transposon to carry the HSV-tk gene and mRFP1 reporter gene, and then delivered them into HeLa cells and tumor xenografts of cervical cancer. We observed mRFP1 expression in HeLa cells three months after transfection, and mRFP1 could be detected in pPB/TK-transfected tumor cryosections three weeks after *in vivo* transfection. Our results also showed that pPB/TK-transfected cells were more sensitive to GCV treatment compared to pORF-HSVtk-transfected cells ($P<0.01$). The inhibitory rate of tumors in the PB/TK group was much higher than the control group ($P<0.01$) with no obvious toxicity observed in major organs by histopathological analysis. In addition, it was also demonstrated that the PB transposon causes the HSV-tk gene to integrate into the genome at multiple sites, thus TK is present within cells for longer and more stably expressed, and enhanced the target cells susceptibility to GCV. Our data suggests that the PB transposon can be used as a safe and efficient vehicle for stable HSV-tk gene transfer and long-term expression.

We transferred pPB/TK into tumors with the aim of destroying the tumor by administering GCV. Compared with pORF-HSVtk, pPB/TK had a stronger lethal effect with a lower dose of GCV. We detected TK expression in tumor sites at different stages with TK expression in the PB/TK group lasting more than 21 days. In the group transfected with pORF-HSVtk, TK expression peaked after seven days then gradually decreased. As a result, the PB/TK group was more susceptible to GCV and exhibited a better lethal effect. Although the transfection efficiency was about 50%, the tumor sizes in the PB/TK group reduced substantially with GCV administration and some even disappeared. Our results revealed that there was a bystander effect *in vivo* using the HSV-tk/GCV system and the stable expression of TK can enhance this effect to some extent. The molecular mechanisms are not clear, and require further study.

Much evidence has indicated that the HSV-tk gene, when transferred into tumor cells converts the non-toxic pro-drug GCV into its cytotoxic metabolite, which results in the target cell undergoing apoptosis (29,30). Our study showed the apoptosis rate of cells in tumor tissues in the PB/TK group was higher than that in the TK group. One interesting observation in this study is that there were many more tumor cells in the S-phase of the cell cycle in the PB/TK group than the control groups. We also observed similar results in a previous study involving ovarian cancer (31). This suggested that the HSV-tk/GCV system combined with S-phase targeted chemotherapy may enhance suicide gene therapy in cancer.

We also selected mRFP1-positive single clones by cell sorting and limiting dilution culture. Our results showed that every single clone exhibited different fluorescence expression intensity, and different transgene expression was confirmed by quantitative PCR and Western blot analysis. This result is similar to those of Huang and coworkers (32), who used the SB transposon carrying a DsRed reporter gene in human T cells and observed the long-term expression of a transgene. They determined copy numbers of the DsRed gene per-cell

in different transfected clones by Southern blot analysis and found all the clones had 4-19 copies, suggesting that a high level of transgene expression mediated by SB transposons may be due to high copy numbers of the integrated transgene. Furthermore, we compared the GCV-sensitivity among different expressing clones and pORF-HSVtk-transfected cells. The data indicated that the IC₅₀ of pORF-HSVtk-transfected cells was 26.61-fold higher than in the clone PB/TK-S43.

In conclusion, our results demonstrate that non-viral gene transfer using the PB transposon has the potential of persistent transgene expression, and the enhanced therapeutic effect of the HSV-tk/GCV system was probably due to high-level transgene expression mediated by the PB transposon. Because the transposon of this system was inserted into the genome, which can cause other genes to be inactivated in the genome, we must consider the clinical risk of this system. In our study, we delivered pPB/TK by intratumoral injection. The tumor cells (including the normal cells that surround it) integrated with PB/TK may not survive after GCV administration. Thus, there is a low risk of HSV-tk gene tumorigenesis mediated by the PB transposon *in vivo* by local injection. Additionally, the design of a novel transposon system targeted to specific cancer tissues or genes would be a significant advance in transgenesis (33).

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References

- Garcia M JA, Ward EM, Center MM, Hao Y, Siegel RL and Thun MJ: Global Cancer Facts and Figures, http://www.cancer.org/downloads/STT/Global_Cancer_Facts_and_Figures_2007_rev.pdf 2007.
- Benedet JL, Odicino F, Maisonneuve P, Beller U, Creasman WT, Heintz AP, Ngan HY and Pecorelli S: Carcinoma of the cervix uteri. *Int J Gynaecol Obstet* 83 (Suppl. 1): 41-78, 2003.
- Kwon JW, Huh SJ, Yoon YC, Choi SH, Jung JY, Oh D and Choe BK: Pelvic bone complications after radiation therapy of uterine cervical cancer: evaluation with MRI. *AJR Am J Roentgenol* 191: 987-994, 2008.
- Pectasides D, Kamposioras K, Papaxoinis G and Pectasides E: Chemotherapy for recurrent cervical cancer. *Cancer Treat Rev* 34: 603-613, 2008.
- Tao X, Hu W, Ramirez PT and Kavanagh JJ: Chemotherapy for recurrent and metastatic cervical cancer. *Gynecol Oncol* 110: S67-S71, 2008.
- Hughes RM: Strategies for cancer gene therapy. *J Surg Oncol* 85: 28-35, 2004.
- Kanerva A, Lavilla-Alonso S, Raki M, Kangasniemi L, Bauerschmitz GJ, Takayama K, Ristimäki A, Desmond RA and Hemminki A: Systemic therapy for cervical cancer with potentially regulatable oncolytic adenoviruses. *PLoS ONE* 3: e2917, 2008.
- Voigt K, Izsvak Z and Ivics Z: Targeted gene insertion for molecular medicine. *J Mol Med* 86: 1205-1219, 2008.
- Yang Y, Li Q, Ertl HC and Wilson JM: Cellular and humoral immune responses to viral antigens create barriers to lung-directed gene therapy with recombinant adenoviruses. *J Virol* 69: 2004-2015, 1995.
- Baum C and Fehse B: Mutagenesis by retroviral transgene insertion: risk assessment and potential alternatives. *Curr Opin Mol Ther* 5: 458-462, 2003.
- Smith AE: Viral vectors in gene therapy. *Annu Rev Microbiol* 49: 807-838, 1995.
- Ivics Z, Katzer A, Stuwe EE, Fiedler D, Knespel S and Izsvak Z: Targeted Sleeping Beauty transposition in human cells. *Mol Ther* 15: 1137-1144, 2007.
- Kawakami K: Tol2: a versatile gene transfer vector in vertebrates. *Genome Biol* 8 (Suppl. 1): S7, 2007.
- Miskey C, Izsvak Z, Plasterk RH and Ivics Z: The Frog Prince: a reconstructed transposon from *Rana pipiens* with high transpositional activity in vertebrate cells. *Nucleic Acids Res* 31: 6873-6881, 2003.
- Ding S, Wu X, Li G, Han M, Zhuang Y and Xu T: Efficient transposition of the piggyBac (PB) transposon in mammalian cells and mice. *Cell* 122: 473-483, 2005.
- Feschotte C: The piggyBac transposon holds promise for human gene therapy. *Proc Natl Acad Sci USA* 103: 14981-14982, 2006.
- Morales ME, Mann VH, Kines KJ, Gobert GN, Fraser MJ Jr, Kalinna BH, Correnti JM, Pearce EJ and Brindley PJ: piggyBac transposon mediated transgenesis of the human blood fluke, *Schistosoma mansoni*. *FASEB J* 21: 3479-3489, 2007.
- Wilson MH, Coates CJ and George AL: PiggyBac transposon-mediated gene transfer in human cells. *Mol Ther* 15: 139-145, 2007.
- Wu SC, Meir YJ, Coates CJ, Handler AM, Pelczar P, Moisyadi S and Kaminski JM: piggyBac is a flexible and highly active transposon as compared to sleeping beauty, Tol2, and Mos1 in mammalian cells. *Proc Natl Acad Sci USA* 103: 15008-15013, 2006.
- Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C (T)) method. *Methods* 25: 402-408, 2001.
- Schmittgen TD and Livak KJ: Analyzing real-time PCR data by the comparative C(T) method. *Nat Protoc* 3: 1101-1108, 2008.
- Earp DJ, Lowe B and Baker B: Amplification of genomic sequences flanking transposable elements in host and heterologous plants: a tool for transposon tagging and genome characterization. *Nucleic Acids Res* 18: 3271-3279, 1990.
- Fillat C, Carrio M, Cascante A and Sangro B: Suicide gene therapy mediated by the Herpes Simplex virus thymidine kinase gene/Ganciclovir system: fifteen years of application. *Curr Gene Ther* 3: 13-26, 2003.
- Sterman DH, Recio A, Vachani A, Sun J, Cheung L, DeLong P, Amin KM, Litzky LA, Wilson JM, Kaiser LR and Albelda SM: Long-term follow-up of patients with malignant pleural mesothelioma receiving high-dose adenovirus herpes simplex thymidine kinase/ganciclovir suicide gene therapy. *Clin Cancer Res* 11: 7444-7453, 2005.
- Ciceri F, Bonini C, Gallo-Stampino C and Bordignon C: Modulation of GvHD by suicide-gene transduced donor T lymphocytes: clinical applications in mismatched transplantation. *Cytotherapy* 7: 144-149, 2005.
- Ponomarev V, Doubrovina M, Serganova I, Vider J, Shavrin A, Beresten T, Ivanova A, Ageyeva L, Tourkova V, Balatoni J, Bornmann W, Blasberg R and Gelovani Tjvujav J: A novel triple-modality reporter gene for whole-body fluorescent, bioluminescent, and nuclear non-invasive imaging. *Eur J Nucl Med Mol Imaging* 31: 740-751, 2004.
- Rainov NG: A phase III clinical evaluation of herpes simplex virus type 1 thymidine kinase and ganciclovir gene therapy as an adjuvant to surgical resection and radiation in adults with previously untreated glioblastoma multiforme. *Hum Gene Ther* 11: 2389-2401, 2000.



SPANDIDOS n DJ, Wirin D, Shaked A and Busuttil RW: Randomised comparison of ganciclovir and high-dose acyclovir for long-term cytomegalovirus prophylaxis in liver-transplant recipients. *Lancet* 346: 69-74, 1995.

29. Beltinger C, Fulda S, Kammertoens T, Meyer E, Uckert W and Debatin KM: Herpes simplex virus thymidine kinase/ganciclovir-induced apoptosis involves ligand-independent death receptor aggregation and activation of caspases. *Proc Natl Acad Sci USA* 96: 8699-8704, 1999.
30. Beltinger C, Fulda S, Kammertoens T, Uckert W and Debatin KM: Mitochondrial amplification of death signals determines thymidine kinase/ganciclovir-triggered activation of apoptosis. *Cancer Res* 60: 3212-3217, 2000.
31. Jiang W, Xu CJ, Shao ZM, Zhou WJ, Ye B, Tian PK, Zhu JD and Gu JR: Enhanced efficiency and specificity of ovarian cancer gene therapy in rats with a novel non-viral gene delivery system (GE7) via intraovarian artery perfusion approach. *Cancer Gene Ther* 12: 810-817, 2005.
32. Huang X, Wilber AC, Bao L, Tuong D, Tolar J, Orchard PJ, Levine BL, June CH, McIvor RS, Blazar BR and Zhou X: Stable gene transfer and expression in human primary T cells by the Sleeping Beauty transposon system. *Blood* 107: 483-491, 2006.
33. Song JS, Kim CW and Ochoa ER: Sleeping Beauty-mediated suicide gene therapy of hepatocellular carcinoma. *Biosci Biotechnol Biochem* 73: 165-168, 2009.