Antitumor effects of a dual-specific lentiviral vector carrying the *Escherichia coli* purine nucleoside phosphorylase gene

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Abstract. The *Escherichia coli* purine nucleoside phosphorylase/Fludarabine phosphate (ePNP/Fludara) suicide system has several drawbacks, such as side-effects and the low efficiency of ePNP expression. In this study, we evaluated the antitumor effects of the dual-specific 8HSEs-hTERTp-ePNP/Fludara suicide system under hyperthermia *in vitro* and *in vivo*. Luciferase activities from the 8HSEs-hTERT and CMV promoters were compared using the dual luciferase assay in SW480 (high hTERT expression) and MKN74 cells (hTERT-negative) in the presence and absence of hyperthermia. Then, we investigated the effects of overexpressing the suicide gene ePNP using 8HSEs-hTERT-driven lentiviral vectors with Fludara on *in vitro* cell viability, side-effects, apoptosis, cycle distribution, colony formation and *in vivo* xenograft tumor growth. At 43˚C, luciferase activity from the 8HSEs-hTERT promoter was significantly increased in SW480 cells, but not in MKN74 cells. Importantly, luciferase activities from the 8HSEs-hTERT promoter were much higher than from the CMV promoter in hTERT-expressing SW480 cells under heated conditions. The *in vitro* quantitative analysis showed a 4-fold higher ePNP protein expression from the 8HSEs-hTERT promoter at 43˚C than at 37˚C in SW480 cells and the ePNP mRNA expression in SW480 cells at 43˚C was also higher than at 37˚C. Conversely, ePNP mRNA and protein expression were low, almost absent, in hTERT-negative MKN74 cells with or without hyperthermia. After Fludara addition, cell cytotoxicity assays showed that the significant inhibitory effect of the 8HSEs-hTERTp-ePNP on SW480 cells was dose- and time-dependent with hyperthermia. The 8HSEs-hTERTp-ePNP/Fludara suicide system significantly inhibited SW480 cell viability, colony formation, cell cycle progression and induced apoptosis *in vitro*, but also induced significant bystander effects, especially under the heated conditions. At the protein level, the suicide system significantly promoted Bax, caspase-3 and p53 expression and suppressed Bcl-2 expression. In sections from mouse xenografts, TUNEL assays showed that the suicide system reduced xenograft growth and induced SW480 apoptosis. These results indicated that the combinatorial cancer- and heat-specific promoter system has great potential for improving the efficacy of cancer treatment with hyperthermia. The 8HSEs-hTERTp-ePNP/Fludara system may serve as a powerful strategy for cancer gene therapy combined with hyperthermia.

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Introduction

The *Escherichia coli* purine nucleoside phosphorylase/fludarabine (ePNP/Fludara) suicide system, originally described by Sorscher *et al* (1), has been demonstrated to have powerful killing and bystander effects (2). Differing from human or mammalian PNP, this bacterial PNP enzyme converts the low-toxic prodrug Fludara into a very toxic metabolite, 2-fluoroadenine (F-Ade). F-Ade impairs DNA, RNA and protein synthesis (3), killing both dividing and non-dividing cells. However, several drawbacks to PNP/Fludara suicide system remain to be resolved, including the side-effects and low efficiency of ePNP gene expression.

Hyperthermia, an inducible antitumor treatment, has gained acceptance for cancer therapy in breast and colorectal carcinomas as well as malignant melanomas (4-6). Recent studies have shown that hyperthermia not only sensitizes tumor cells to radiation and chemotherapy, but also activates HSP70 expression in some cells. This makes the combination of hyperthermia and gene therapy possible. Furthermore, hyperthermia can augment the effects of therapeutic genes in a controlled range (7). Hyperthermia-induced HSP70 activation is regulated at the transcriptional level (8) and depends on heat shock elements (HSEs), which are short sequences in the HSP70 promoter that are essential for heat inducibility. The introduction of HSEs into a gene transfer vector makes

it possible to provide special control over exogenous gene expression in a locally heated tumor (8).

The human telomerase reverse transcriptase (hTERT) promoter has been widely used to drive the specific expression of therapeutic genes for cancer treatment. The hTERT promoter is significantly weaker than many commonly used viral promoters, such as the cytomegalovirus (CMV) early promoter and the simian virus 40 (SV40) early promoter (9). This limitation caused us to hypothesize that the combination of heat shock elements (HSEs) with the hTERT promoter in a recombinant lentiviral vector may significantly increase the transcriptional activity of the hTERT promoter, improving the efficiency of ePNP gene expression in a locally heated tumor.

Therefore, we designed and constructed a recombinant lentiviral vector carrying the ePNP gene under the control of the 8HSEs-hTERT promoter, which ensured targeted and powerful gene expression in tumor cells. We expect that administration of this recombinant lentiviral vector together with the prodrug Fludara could provide a new strategy for clinical therapy of solid tumors together with hyperthermia.

Materials and methods

Reagents. Fludarabine phosphate (Fludara) was obtained from Sigma-Aldrich (St. Louis, MO, USA) and dissolved in phosphate-buffered saline (PBS). Rabbit monoclonal antibodies specific for Bax, Bcl-2, caspase-3, p53, Fas, cyclin D1 and β-actin were obtained from Epitomics (Burlingame, CA, USA); rabbit monoclonal antibodies specific for 3FALG were obtained from Sigma-Aldrich. TurboFect™ transfection reagent was acquired from Thermo Fisher Scientific (Waltham, MA, USA), and Dual-Glo Luciferase assay system from Promega (Madison, WI, USA).

Cell culture and in vitro hyperthermia. Human colorectal cancer SW480 and gastric cancer MKN74 cells were obtained from Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). SW480 and MKN74 cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS), 100 IU/ml penicillin, 100 mg/ml streptomycin and 2 mM L-glutamine. Cells were grown at 37˚C in a humidified atmosphere containing 5% CO_2. Cells were seeded into cell culture dishes and incubated at 37˚C for 24 h. Afterwards, cells were transferred to a cell culture incubator that was pre-adjusted to 43˚C for 1 h every 48 h (data not shown) (10). After incubation for the desired time, cells were transferred back to the 37˚C incubator and incubated for several hours to recover from the heat treatment.

Construction of plasmid vectors. Preparation of plasmids was accomplished using the Omega plasmid midi kit (Omega, Norcross, GA, USA). The 295-bp promoter region (11) of human TERT [GenBank accession no. AN097365] was amplified by PCR from human genomic DNA, using the following primer pairs: TERT promoter (forward, 5'-CTAGCTAGC CACAGACGCCCAGGACCGCGCTTC-3'; reverse, 5'-CCC AAGCTTCCACGTGCGCCCACGTGCGCCCAC-3'. The 295-bp fragment was inserted into pGL4.2 (Promega) upstream of the luciferase reporter gene and verified by DNA sequencing to generate pGL4.2-hTERTp. Eight heat shock elements (8HSEs) with optimized AGAACGTTCTAGAAC sequences (10,12) alternately separated by 5 bp were generated by oligonucleotide ligation. The 8HSEs fragments were inserted upstream of the luciferase reporter in pGL4.2-hTERTp to generate pGL4.2-8HSEs-hTERTp.

The 588-bp CMV early promoter was amplified by PCR from pLVX-EGFP-3FLAG (GeneChem, Shanghai, China), using the following primer pairs: forward, 5'-CGCCTCGAG CCGCCTGGCTGACCGCCCA-3'; reverse, 5'-GCCAGATCT GCCATGGTTCGAATTCAAAT-3'. The amplified 588-bp fragment was cloned into pGL-4.2 to generate pGL4.2-CMVp.

Dual luciferase assay. SW480 and MKN74 cells were seeded 5,000 cells per well into each well of a 96-well plate. The next day, cells were transfected with 150 ng luciferase reporter plasmid (pGL4.2-8HSEs-hTERTp or pGL4.2-CMVp) and 30 ng of pGL-4.74 (containing the TK promoter; Promega) as an internal control using the TurboFect™ Transfection reagent (Thermo Fisher Scientific) according to the manufacturer's instructions. After 10-h transfection, the mixture was replaced with fresh medium. After 48 h, cells were subjected to heat treatments. Luciferase assays were performed 6 h later using the Dual-Glo Luciferase assay system (Promega). Briefly, 100 µl of Dual-Glo Luciferase assay reagent was added to each well, followed by the addition of 100 µl Dual-Glo Stop & Glo reagent. *Renilla* luminescence was normalized to the internal control vector pGL-4.74 luminescence. Promoter activities were measured as relative luminescence units (RLU) where the value of the firefly luciferase luminescence of pGL-4.2 was divided by the *Renilla* luciferase pGL-4.74 from the same well.

Establishment of stable lentivirus-transfected cell lines. Two recombinant lentiviruses pLVX-8HSEs-hTERTp-ePNP-3FLAG (8HhP) and a negative control pLVX-Ubi-3FLAG (CON) containing the ubiquitin promoter were purchased from GeneChem. The recombinant lentiviral vector (pLVX-8HSEs-hTERTp-ePNP-3FLAG) including the hTERT promoter modified by the artificial 8HSEs, the ePNP gene and three consecutive FLAG sequences was constructed using conventional recombinant techniques. SW480 and MKN74 cells were transduced with the 8HhP and CON lentiviruses following standard techniques. After 72 h, lentivirus-carrying clones were selected for 15 days in medium containing 1 mg/ml puromycin (Life Technologies, Grand Island, NY, USA).

Quantitative reverse transcription polymerase chain reaction (qRT-PCR). SW480, MKN74 and the stably transfected SW480-8HhP, SW480-CON, MKN74-8HhP and MKN74-CON cells were subjected to an RNA Fast 200 kit (Pioneer Biotech, Xi'an, China) to isolate total RNA according to the manufacturer's instructions. cDNA was synthesized from 1 µg of DNase I-treated total RNAs with PrimeScript™ RT Master Mix (Takara, Dalian, China) according to the manufacturer's instructions. An aliquot (1 µl) of the cDNA was then subjected to qRT-PCR analysis of ePNP mRNA using SYBR® Premix Ex Taq™ II (Takara). The primers were as follows: ePNP (product: 64 bp) forward, 5'-TGGGTCACGGTATGG GTATC-3'; reverse, 5'-CCGAAATCGGTGATCAGTTC-3'. β-actin (product: 151 bp) forward, 5'-CTTAGCACCCCTGGC

CAAG-3'; reverse, 5'-GATGTTCTGGAGAGCCCCG-3'. The ePNP and β-actin qPCR reaction mixture was denatured at 94˚C for 3 min followed by 40 cycles of 94˚C for 30 sec, 57˚C for 30 sec, and 72˚C for 30 sec. All reactions were performed in triplicate and the relative ePNP mRNA level in each cell line was calculated by the $2^{-\Delta\Delta Ct}$ method, where ΔCt = Ct (ePNP) - Ct (β-actin).

Protein extraction and western blots. Cells treated with or without Fludara were lysed on ice with NP-40 buffer containing 40 mM Tris-HCl (pH 6.9), 2 mM ethylenediaminetetraacetic acid (EDTA, pH 8.0), 100 mM sodium fluoride, 150 mM NaCl, 10 mM sodium pyrophosphate, 1% Tergitol type NP-40, 2 mM sodium orthovanadate, 1% Triton X-100, 1.0 mM phenylmethanesulfonyl fluoride (PMSF), and 1X protease inhibitor mini-tablet (Roche, Basel, Switzerland). Lysates were then clarified by centrifugation at 12,000 x g for 10 min at 4˚C. Protein concentration was estimated using the Pierce Protein Estimation system (Thermo Fisher Scientific) according to the manufacturer's protocol. Next, equal amounts (30 μg) of protein were heated at 95˚C for 5 min in 5X Laemmli sample buffer, separated on a 10% SDS-PAGE gel and transferred onto polyvinylidene difluoride (PVDF) membranes by the wet transfer method. Non-specific binding was blocked for 1 h at 37˚C using 10% fat-free milk in TBS containing 0.1% Tween-20. The membranes were blotted with anti-Bax, Bcl-2, caspase-3, p53, Fas, cyclin D1, β-actin or anti-3FALG primary antibodies at a dilution of 1:2,000. After washing three times with TBST, horseradish peroxidase-conjugated secondary antibodies (1:5,000 dilution; Proteintech, Chicago, IL, USA) were added at 27˚C for 1 h. Immunoreactive bands were visualized using SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific) and exposed using the ChemiDoc XRS+ (Bio-Rad, Hercules, CA, USA).

Cell viability assay. Cell viability was detected using the cell counting kit-8 (CCK-8, Dojindo Molecular Technologies, Inc., Kumamoto, Japan). Briefly, 2,000 parental and stably transduced cells were inoculated into 96-well plates in a final volume of 200 μl growth medium. The hyperthermia group was treated at 43˚C after 24 h. After treatment and incubation, each plate was subjected to the CCK-8 assay by adding 10 μl of CCK-8 solution to each well, and the plate was further incubated for 2 h at 37˚C. The absorbance at 450 nm was measured with an EnSpire™ Multilabel Reader 2300 (Perkin-Elmer Inc., Waltham, MA, USA). The relative cellular survival rate was calculated using the following formula: $(A_{sample} - A_{blank})/(A_{control} - A_{blank}) \times 100\%$ (13).

Flow cytometric apoptosis and cell cycle distribution assays. SW480, SW480-8HhP, SW480-CON, MKN74, MKN74-8HhP and MKN74-CON cells were seeded into 6-well plates and the hyperthermia group was treated at 43˚C for 1 h every 48 h. For apoptotic analysis, apoptosis detection kit (Becton-Dickinson, Franklin Lakes, NJ, USA) was used to analyze apoptosis rates according to the manufacturer's instructions. For cell cycle analysis, cells growing in logarithmic phase were fixed with 75% ethanol for 24 h at -20˚C, incubated with RNase A and Triton X-100 at 37˚C for 30 min, and then incubated with propidium iodide at room temperature for 30 min. Cells were examined by flow cytometry, and CellQuest software (Becton-Dickinson) was used to conduct data acquisition and analysis.

Colony formation assay. SW480, SW480-8HhP, SW480-CON, MKN74, MKN74-8HhP and MKN74-CON cells (200 cells/well) were suspended in 2 ml media and seeded into 6-well plates. The hyperthermia group was also treated at 43˚C for 1 h every 48 h, after treatment with or without Fludara, cells were fixed and stained with 1% crystal violet and cell colonies were counted under an inverted microscope. Statistical comparison was conducted using Student's t-test.

In vivo gene expression and analysis of antitumor effects. BALB/c nude mice were used according to the guidelines for administration to lab animals, issued by the Ministry of Science and Technology (Beijing, China). Tumors were established by subcutaneous inoculation with SW480 and MKN74 cells (100 μl containing $1x10^7$ cells). Tumor volume was calculated using the empirical formula V = 0.52 x [(shortest diameter)^2 x (longest diameter)]. When the tumor volume reached 100 mm^3, tumor-bearing mice were randomly divided into six groups of 6 mice each: pLVX-Ubi-3FALG+PBS (SW480-CON+PBS), pLVX-Ubi-3FALG+Fludara+37˚C (SW480-CON+Fludara+37˚C), pLVX-Ubi-3FALG+Fludara +43˚C (SW480-CON+Fludara+43˚C), pLVX-8HSEs-hTERTp-ePNP-3FALG+PBS (SW480-8HhP+PBS), pLVX-8HSEs-hTERTp-ePNP-3FALG+Fludara+37˚C (SW480-8HhP+Fludara+37˚C) and pLVX-8HSEs-hTERTp-ePNP-3FALG+Fludara+43˚C (SW480-8HhP+Fludara+43˚C). The mice in each group were intratumorally administered 100 μl serum-free medium containing $1x10^8$ pfu pLVX-Ubi-3FALG or pLVX-8HSEs-hTERTp-ePNP-3FALG. Three days later, lentivirus administration was repeated. Fludara (10 mg/kg) dissolved in 0.5 ml PBS was injected intraperitoneally three times daily for three consecutive days, beginning 48 h after the administration of recombinant lentivirus. This schedule was counted as a single course and three consecutive courses were administered (14). On the 11th and 13th day post-inoculation, mice were placed in a 43˚C water bath for 1 h. The feet of the mice were tied to four nails on a board to ensure the transplanted tumors were immersed in the water bath (10). Tumor size and growth were monitored and measured using a caliper at regular intervals. Mice were sacrificed two weeks after the second virus injection and xenograft tumors were harvested, measured and photographed. Six mice in each group were sacrificed and the tumor specimens were subjected to histopathological analysis and TUNEL staining.

TUNEL assay. Paraffin-embedded tissue slides were prepared from the xenograft tumors. TUNEL staining was detected by the DeadEnd™ Fluorometric TUNEL system (Promega) according to the manufacturer's instructions. Cells were fixed and permeabilized with PBS containing 4% paraformaldehyde and 0.25% Triton X-100. Fluorescence was measured after incubation with FITC-labeled TUNEL. After TUNEL staining, the specimens were immersed with a DAPI solution (Sigma-Aldrich) to stain nuclei. Fluorescence staining was viewed by laser scanning confocal microscopy (FV300, Olympus, Tokyo, Japan) (15).

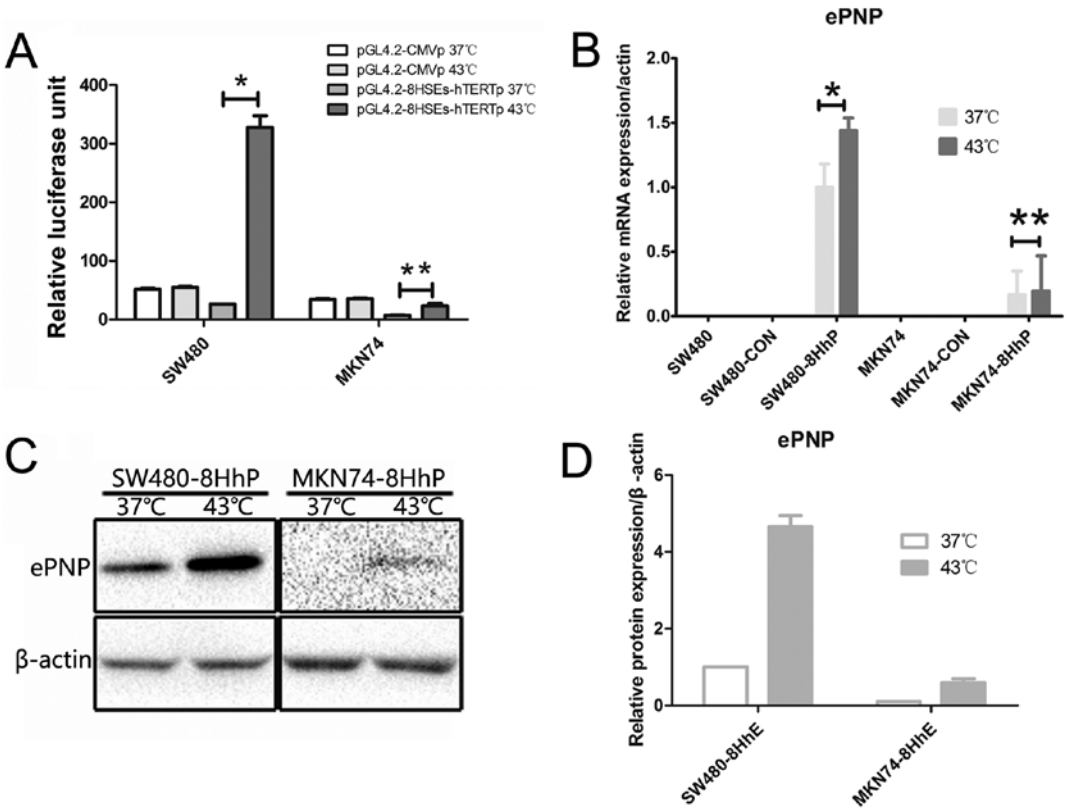

Figure 1. Hyperthermia- and tumor-specificity of the synthetic 8HSEs-hTERT promoter. (A) Luciferase activity from the synthetic 8HSEs-hTERT and CMV promoters at 37˚C or 43˚C in SW480 (hTERT-high expressing) and MKN74 cells (hTERT-negative). *P<0.05, pGL4.2-8HSEs-hTERTp 43˚C vs. pGL4.2-8HSEs-hTERTp 37˚C in SW480 cells; **P>0.05, pGL4.2-8HSEs-hTERTp 43˚C vs. pGL4.2-8HSEs-hTERTp 37˚C in MKN74 cells. (B) Quantitative PCR analysis of ePNP mRNA expression regulated by the synthetic 8HSEs-hTERT promoter at 37˚C or 43˚C in SW480 cells and MKN74 cells. *P<0.05, 37˚C vs. 43˚C in SW480-8HhP cells; **P>0.05, 37˚C vs. 43˚C in MKN74-8HhP cells. (C) Western blot analysis of ePNP expression regulated by the synthetic 8HSEs-hTERT promoter at 37˚C or 43˚C in SW480 cells and MKN74 cells. (D) Integrated optical density was measured to evaluate ePNP expression [from (C)] relative to β-actin.

Statistical analysis. Results are shown as means ± standard error. Differences were evaluated with unpaired two-tailed Student's t-tests with unequal variance for multiple comparisons using the SPSS software version 19.0 (SPSS Inc., Chicago, IL, USA). P<0.05 was considered statistically significant. All experiments were independently repeated at least three times.

Results

Hyperthermia inducibility of the synthetic 8HSEs-hTERT promoter. To determine the hyperthermia inducibility of the synthetic 8HSEs-hTERT promoter after heat treatment, we transfected SW480 and MKN74 with pGL4.2-8HSEs-hTERTp or pGL4.2-CMVp. Transfected cells were incubated at either 43˚C or 37˚C for 1 h and the resultant luciferase activity was measured. Treatment at 43˚C for 1 h significantly increased the luciferase activity of SW480 but not MKN74 cells (P<0.05 and P=0.219, respectively, Fig. 1A). Contrastingly, 43˚C treatment resulted in no apparent differences in pGL4.2-CMVp luciferase activity among these cell lines. Moreover, after the 43˚C treatment, the 8HSEs-hTERT promoter luciferase activity was significantly higher than the CMV promoter. These results demonstrated that hyperthermia could significantly enhance the transcriptional activity of the 8HSEs-hTERT promoter in SW480 cells, which endogenously express high hTERT levels. These data suggested that the 8HSEs-hTERTp promoter might be more efficient and specific than the CMV promoter for tumor targeting with hyperthermia.

Overexpression of ePNP in tumor cells using an 8HSEs-hTERTp-driven expression vector. We tested ePNP expression in hTERT-high expressing SW480 cells and hTERT-negative MKN74 cells (10) using an 8HSEs-hTERTp-driven expression vector at 37˚C or 43˚C. ePNP expression was confirmed by qRT-PCR and western blotting (Fig. 1B and C) in SW480-8HhP and MKN74-8HhP cells. SW480-8HhP cells showed very high levels of ePNP mRNA at 43˚C or 37˚C, but MKN74-8HhP only showed low levels of ePNP mRNA at 43˚C or 37˚C. Similarly, ePNP protein levels were significantly increased in SW480-8HhP cells under heat (Fig. 1D). However, ePNP protein levels were very low at 43˚C in MKN74-8HhP cells, and almost absent at 37˚C (Fig. 1C and D). As a heterologous gene, ePNP protein and mRNA were negative in SW480, MKN74, SW480-CON and MKN74-CON cells at 43˚C or 37˚C. These data indicated that both ePNP mRNA and protein were expressed in hTERT-high expressing SW480-8HhP cells, especially following heat application.

Sensitivity of infected cells to Fludara. To test whether ePNP could sensitize cancer cells to Fludara, we tested the proliferation of the different cell lines using the CCK-8 method to

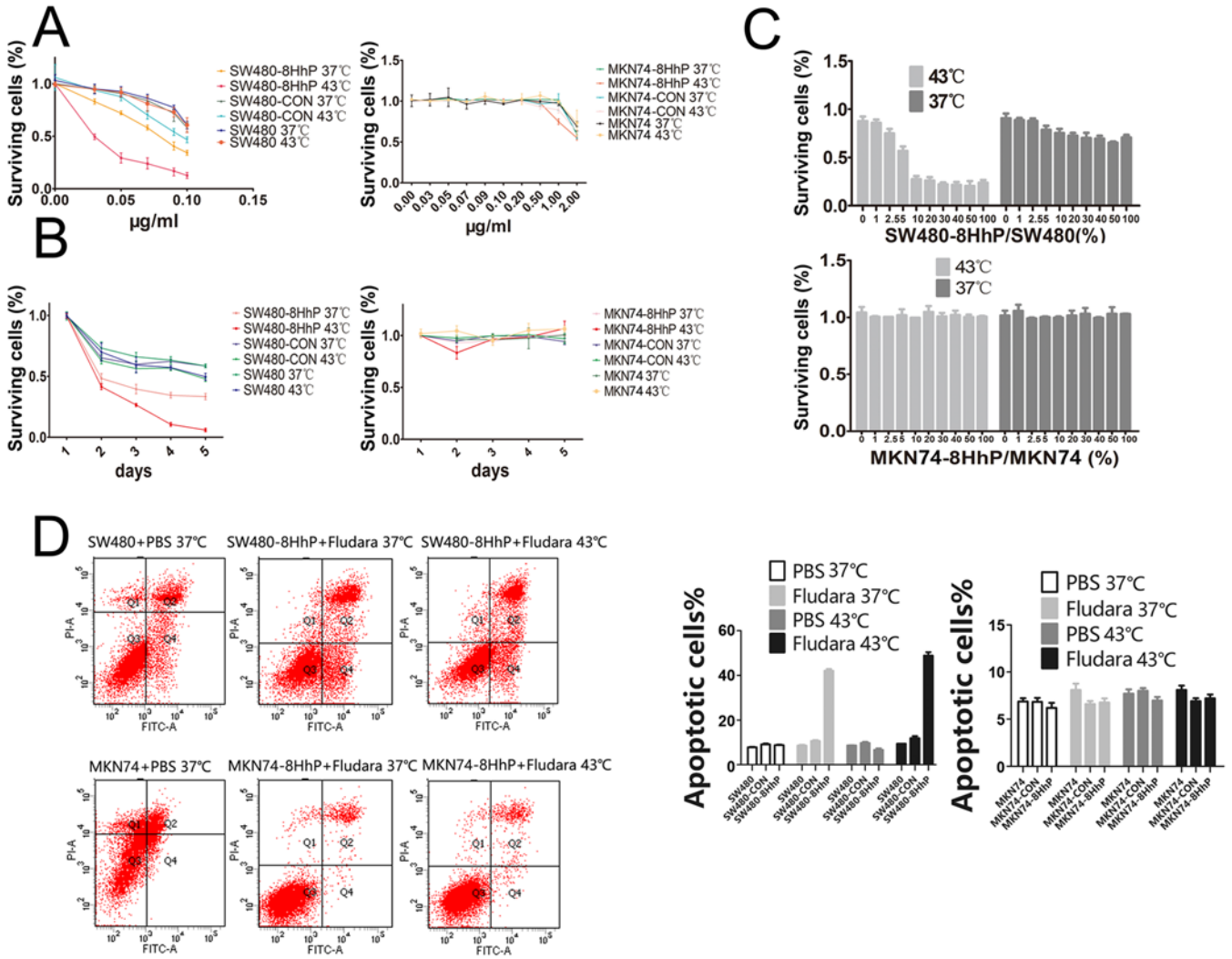

Figure 2. Cytotoxicity of the suicide system ePNP/Fludara regulated by the synthetic 8HSEs-hTERT promoter. (A) Sensitivity of SW480, MKN74 and the corresponding infected cells to Fludara at 37˚C or 43˚C by CCK-8 assay. (B) Growth curves of SW480, MKN74 and the corresponding infected cells to Fludara at 37˚C or 43˚C by CCK-8 assay. (C) Bystander effect of SW480, MKN74 and the corresponding infected cells to Fludara at 37˚C or 43˚C by CCK-8 assay. (D) Apoptosis rates of SW480, MKN74 and the corresponding infected cells to Fludara at 37˚C or 43˚C by flow cytometric analysis. *P<0.05, 37˚C vs. 43˚C in SW480-8HhP+Fludara.

calculate IC_{50} values for each line, which was calculated as the drug concentration that inhibited growth by 50%. When incubated with different concentrations of Fludara (ranging from 0 to 2 μg/ml), parental, CON- and 8HhP-infected cells were resistant at 37˚C. In contrast, SW480-8HhP cells were susceptible to Fludara at 43˚C (IC_{50}=0.02924), lower than SW480-8HhP cells at 37˚C (IC_{50}=0.07618), SW480 and SW480-CON at 37˚C or 43˚C (Fig. 2A). Conversely, hTERT-negative MKN74 cells showed a higher IC_{50} at 37˚C or 43˚C (Fig. 2A). At a concentration of 0.05 μg/ml Fludara, the sensitivity of the infected SW480 cells was time-dependent (Fig. 2B). The data showed that hyperthermia significantly reduced SW480-8HhP cell viability compared with either SW480 or SW480-CON cells, under both normal and heated conditions. However, this result was not replicated in MKN74-8HhP cells (Fig. 2B), because MKN74 cells do not express hTERT, and thus, the lentiviral vector did not induce ePNP expression in these cells (Fig. 2B). These results indicated that ePNP sensitized cancer cells to Fludara only in hTERT-high expressing SW480-8HhP cells, especially under heated conditions.

High level of bystander effect to Fludara. In the absence of ePNP-positive cells, the growth of parental cells was not affected even when the concentration of Fludara was 0.1 μg/ml. At a concentration of 0.05 μg/ml Fludara, a significant bystander effect could be detected, even at a very low proportion (5%, Fig. 2C) of ePNP-positive SW480-8HhP cells at 43˚C. We detected a weaker bystander effect at the same proportion of ePNP-positive SW480-8HhP cells at 37˚C. Furthermore, no obvious bystander effect was detected in MKN74-CON and MKN74-8HhP cells at 43˚C or 37˚C. These results indicated that a significant bystander effect was found in SW480-8HhP cells under heated conditions (Fig. 2C).

Effects of Fludara on SW480 apoptosis. To further elaborate the causes of cell growth inhibition, we detected apoptosis rates by flow cytometry. The results showed that 0.05 μg/ml Fludara treatment increased the rate of spontaneous apoptosis in the infected cells compared with control cells (Fig. 2D) in hTERT-high expressing SW480 cells, but not in MKN74 cells. The greatest increase in the number of apoptotic cells

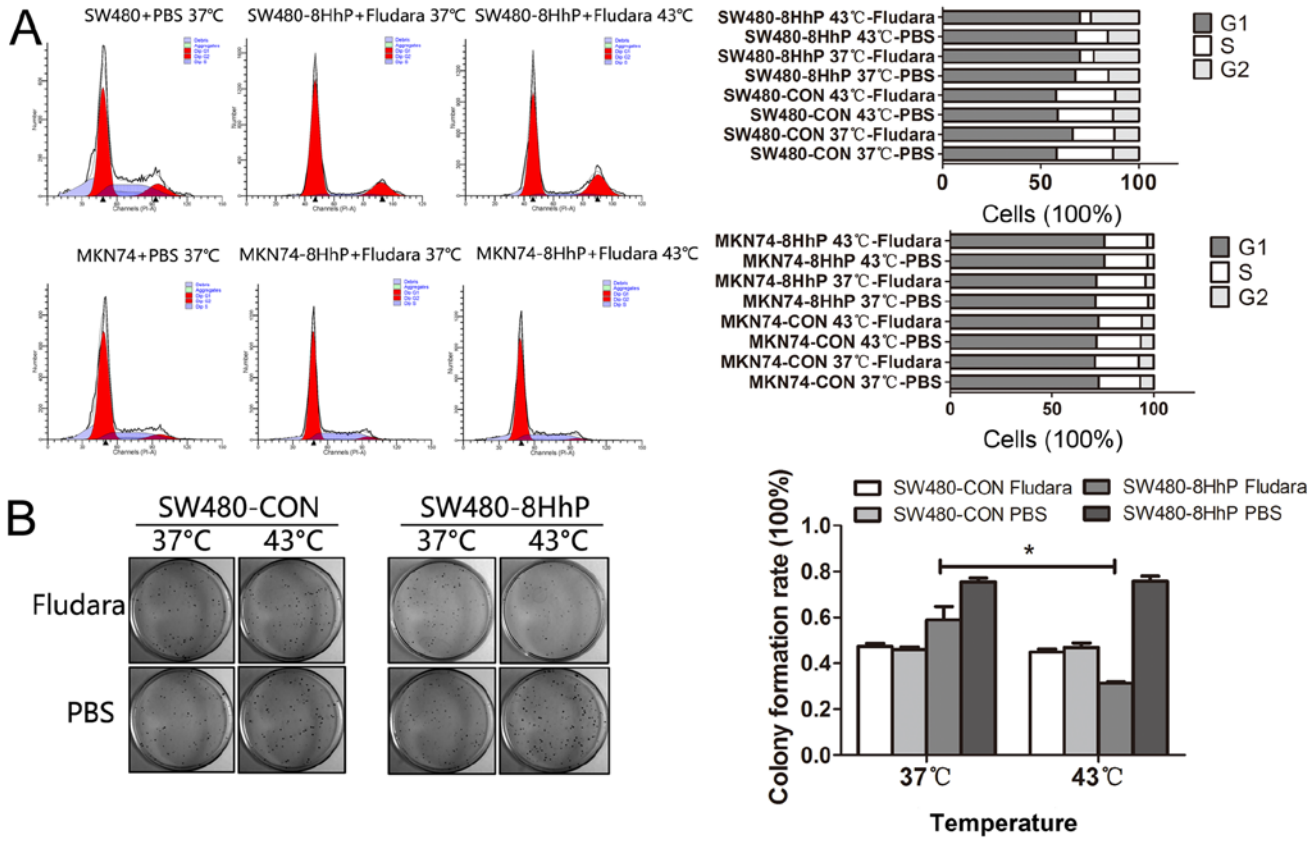

Figure 3. Cytotoxicity of the suicide system ePNP/Fludara regulated by the synthetic 8HSEs-hTERT promoter. (A) Cell cycle distribution of SW480, MKN74 and the corresponding infected cells to Fludara at 37˚C or 43˚C by flow cytometric analysis. (B) Colony formation rates of SW480, MKN74 and the corresponding infected cells to Fludara at 37˚C or 43˚C. *P<0.05, 37˚C vs. 43˚C in SW480-8HhP+Fludara.

was in SW480-8HhP, and the ratio was 48.9% at 43˚C and 42.1% at 37˚C. These results indicated that the suicide system ePNP/Fludara inhibited cell proliferation by causing apoptosis, and the 8HSEs-hTERT promoter could enhance the effect only in hTERT-high expressing cells under heated conditions.

Effects of Fludara on SW480 cell cycle profiles. To explore the mechanism whereby the suicide system ePNP/Fludara inhibited cell proliferation, we studied the effect on the cell cycle. The targeted cells were treated with 0.05 μg/ml Fludara at 43˚C or 37˚C, and cellular DNA content was measured by flow cytometry. The data obtained from these studies demonstrated that ePNP/Fludara induces a G_2 cell cycle arrest only in SW480-8HhP cells (Fig. 3A). At 72 h, flow cytometric analysis of SW480-8HhP cells showed an ~2-fold increase in the percentage of cells in G_2-M phase (22.89%) compared with cells infected with CON vector (12.32%; Fig. 3A) under normal conditions. The addition of hyperthermia produced an additional increase in G_2-M cells to 24.5% in SW480-8HhP cells. Conversely, few differences were observed between MKN74-CON and MKN74-8HhP cells at 43˚C or 37˚C. These results indicated that the suicide system ePNP/Fludara inhibited cell proliferation by causing a G_2 cell cycle arrest, and the 8HSEs-hTERT promoter could enhance the effect only in hTERT-high expressing cells under heated conditions.

Effects of Fludara on SW480 colony formation. We next evaluated the colony formation ability of SW480 cells expressing ePNP. After exposure to 37˚C or 43˚C treatments, SW480 cells were treated with Fludara. The Fludara plus 43˚C treatment significantly reduced SW480-8HhP colony formation (P<0.05, Fig. 3B). However, 43˚C treatment exhibited no effect in all other cells when combined with either PBS or control vector. The 43˚C treatment caused a significant colony formation decrease only in SW480-8HhP cells after exposure to Fludara when compared with PBS, control vector, 37˚C or MKN74 cells (data not shown). These results indicated that the suicide system ePNP/Fludara inhibited colony formation, and the 8HSEs-hTERT promoter could enhance the effect only in hTERT-high expressing cells under heated conditions, demonstrating cancer- and heat-specificity.

Effects of in vitro ePNP/Fludara on Bax, caspase-3, p53 and cyclin D1 expression. To investigate the molecular events associated with ePNP/Fludara-mediated cell cycle arrest and apoptosis, we assessed whether recombinant 8HhP-regulated ePNP/Fludara could affect the expression of Bax, Bcl-2, caspase-3, p53, Fas and cyclin D1 under heated conditions *in vitro*. A significantly variable upregulation of the apoptotic proteins Bax, caspase-3 and p53, and downregulation of the anti-apoptotic protein Bcl-2 were achieved when ePNP/Fludara was included in SW480 cells (Figs. 4 and 5, *P<0.05, P<0.05). Conversely, significant changes in expression by ePNP/Fludara did not occur in the hTERT-negative MKN74 cells. Only Cyclin D1 protein was not detected in SW480, SW480-CON and SW480-8HhP cells. These results indicated that changes

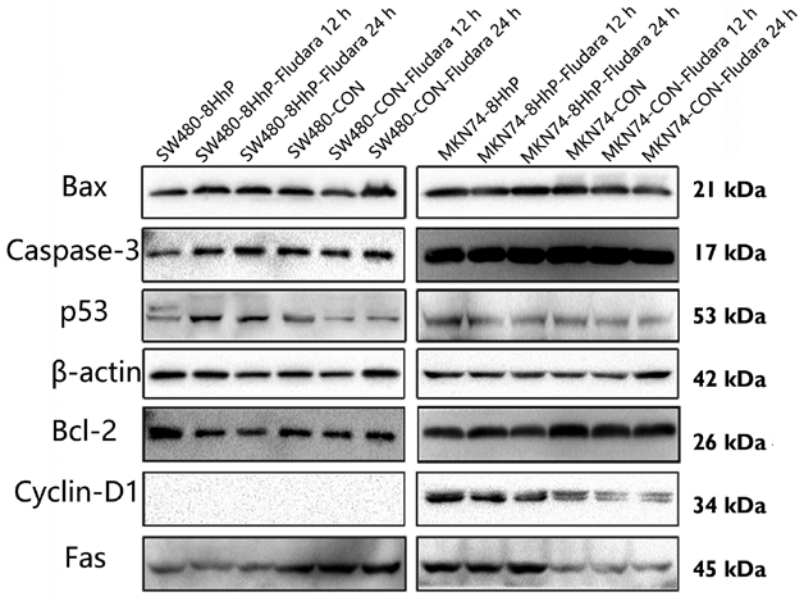

Figure 4. Effects of the suicide system ePNP/Fludara on the regulation of Bax, caspase-3, p53 and cyclin D1 proteins *in vitro*. Western blot analysis of Bax, Bcl-2, caspase-3, p53, Fas and cyclin D1 expression in SW480 and MKN74 cells.

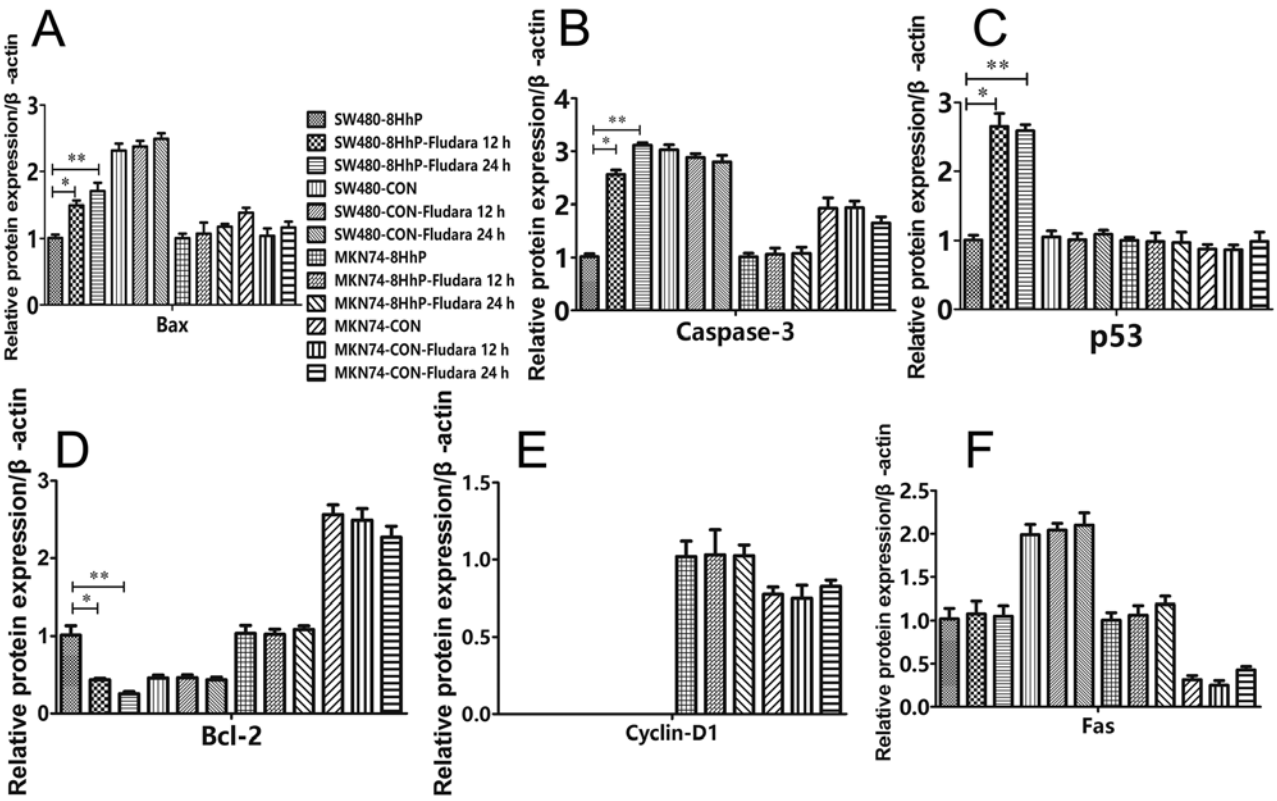

Figure 5. Integrated optical densities were measured to evaluate (A) Bax, (B) caspase-3, (C) p53, (D) Bcl-2, (E) cyclin D1 and (F) Fas proteins expression relative to β-actin. *P<0.05, SW480-8HhP vs. SW480-8HhP+Fludara 12 h; **P<0.05, SW480-8HhP vs. SW480-8HhP+Fludara 24 h.

in the expression of apoptosis-regulating proteins was occurring in the ePNP/Fludara treated cells.

Effects of lentiviral-mediated gene therapy on a mouse xenograft model. SW480 and MKN74 cells were subcutaneously implanted in BALB/c nude mice. Mice were monitored every two days for tumor growth and after 8 days, xenograft tumors formed and grew to 50-100 mm^3. Then, we intratumorally injected lentiviruses ($1x10^8$ pfu) in 100 μl of serum-free medium at three sites per xenograft tumor and repeated the injections on day 4. Two weeks after the second virus injection, mice were sacrificed and xenograft tumors were collected,

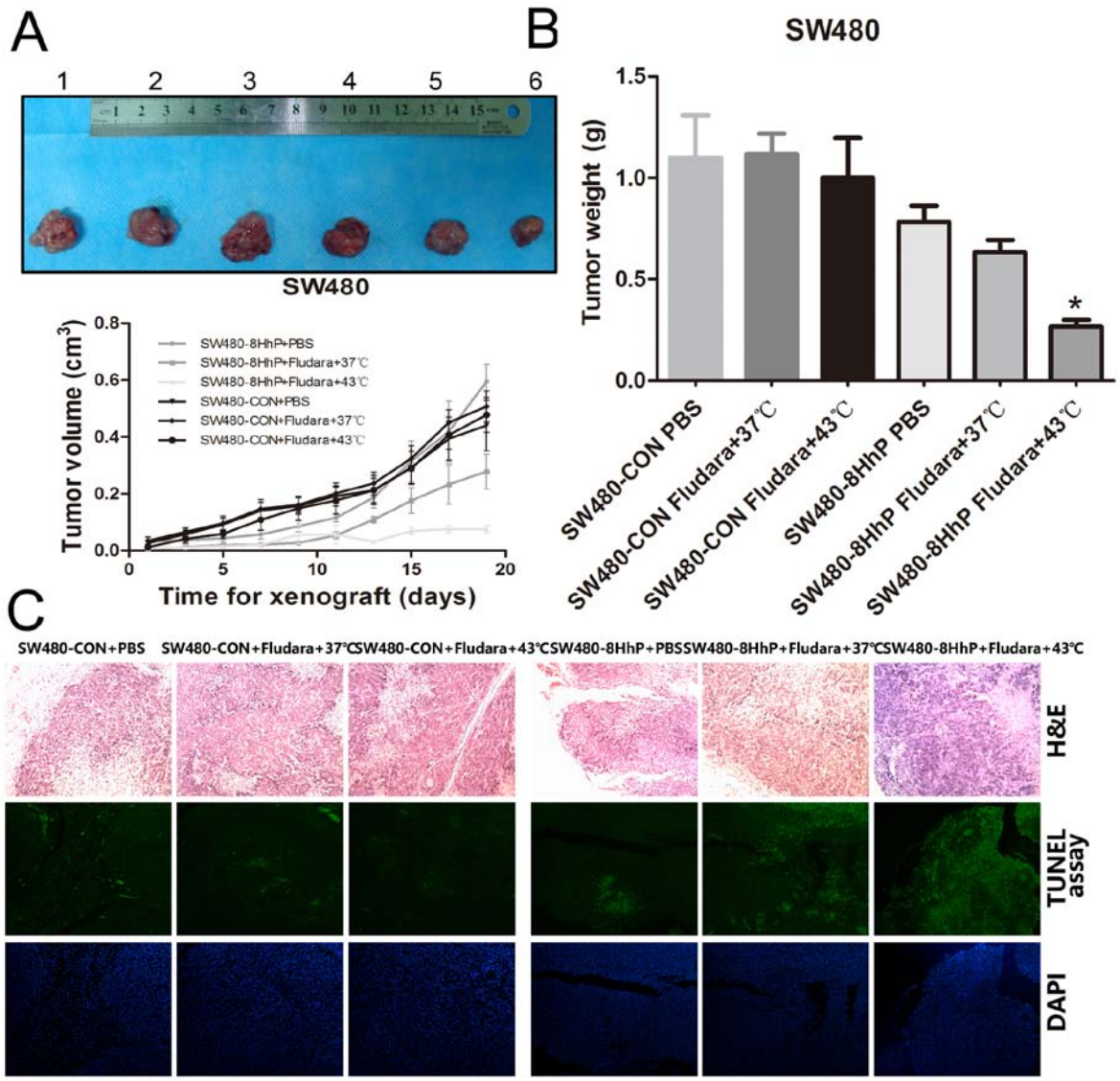

Figure 6. *In vivo* antitumor effects of the 8HhP recombinant lentivirus in SW480 cells at 37˚C or 43˚C. (A) Xenograft growth curves of SW480 cells infected by the 8HhP or CON recombinant lentiviruses and treated with Fludara at 37˚C or 43˚C. (B) Xenograft weights at the end of the experiment. *P<0.05, SW480-8HhP+Fludara+43˚C vs. SW480-8HhP+Fludara+37˚C. (C) TUNEL analysis revealing xenograft apoptosis of SW480 cells infected by the 8HhP or CON recombinant lentiviruses and treated with Fludara at 37˚C or 43˚C.

measured and photographed. Compared with PBS at 37˚C and the control lentivirus groups, the size and weight of the tumors were significantly lower in the SW480-8HhP+Fludara+43˚C group (P<0.05; Fig. 6B). Conversely, this it did not occur in MKN74 cells; the size and weight of tumors showed no significant differences in any of the MKN74 groups (Fig. 7B). TUNEL assay data also showed that the suicide system ePNP/Fludara regulated by the recombinant 8HhP lentivirus induced more apoptosis in SW480 xenograft tumors (Fig. 6C). No obvious differences in apoptosis were found in the MKN74 xenograft tumors (Fig. 7C). These results demonstrated that ePNP/Fludara induced apoptosis and the recombinant lentivirus 8HhP had tumor- and heat-specificity *in vivo*.

Discussion

In this study, we explored rational strategies for combining an ePNP/Fludara system controlled by a novel chimeric promoter with hyperthermia. We cloned the suicide gene ePNP into a recombinant lentiviral vector to express the ePNP gene with high efficiency and enhanced target specificity in tumor cells. When the prodrug Fludara was added, massive cytotoxicity was induced. Both *in vitro* and *in vivo* experiments confirmed the efficiency and specificity of this system.

We found several advantages to the 8HSE-hTERT promoter system for cancer therapy. First, the combination of ePNP/Fludara gene therapy with hyperthermia presents possible therapeutic advantages in the treatment of solid malignancies. Second, combining gene therapy and hyperthermia has the potential to overcome many of the limitations of hyperthermia alone. Third, 8HSEs elements can enhance the activity of hTERT promoter under heated conditions but do not dampen its specificity to cancer cells. HSEs have been demonstrated to be necessary for the heat inducibility of the HSP70B promoter and have been widely used in gene therapy as an inducible tumor-specific promoter (16). The precise mechanism of this

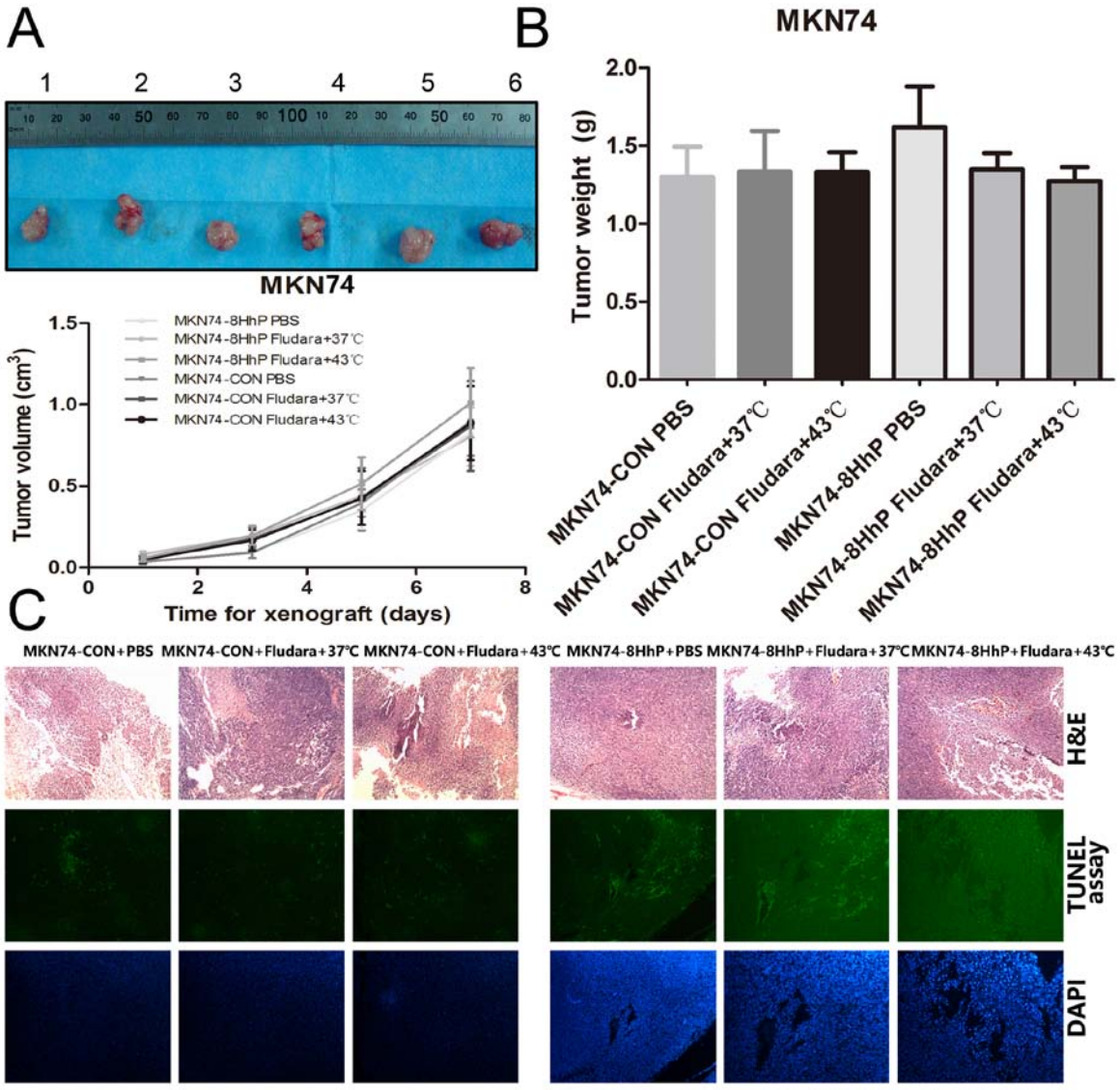

Figure 7. *In vivo* antitumor effects of the 8HhP recombinant lentivirus in MKN74 cells at 37˚C or 43˚C. (A) Xenograft growth curves of MKN74 cells infected by the 8HhP or CON recombinant lentiviruses and treated with Fludara at 37˚C or 43˚C. (B) Xenograft weights at the end of the experiment. (C) TUNEL analysis revealing xenograft apoptosis of MKN74 cells infected by the 8HhP or CON recombinant lentiviruses and treated with Fludara at 37˚C or 43˚C.

response and the transcription factors that interact with HSE elements have yet to be elucidated. However, it is known that complexes of transcription factors and accessory proteins, including heat-shock inducible transcription factor (heat shock factor 1, HSF1), promote gene expression by binding to HSE motifs (17). In this study, we demonstrated that eight consecutive HSEs improve the induction response to hyperthermia, which may be explained simply because eight target sequences provide efficient binding sites for HSF1.

The PNP/Fludara system also has several advantages for treating colorectal cancer. First, PNP converts Fludara into a highly cytotoxic metabolite F-dAe, which is an inhibitor of both ribonucleoside reductase and DNA polymerase-A and effectively kills both dividing and non-dividing cells (18). By contrast, the common suicide system HSV-TK/GCV can kill dividing cells but not senescent or slowly-dividing cells (19). Second, overexpression of ePNP can activate the prodrug Fludara and kill almost entire populations of tumor cells, even when as few as 5% of the cells express the ePNP gene (20). Hong *et al* showed that human glioma tumors in mice were inhibited by adenoviral delivery of ePNP followed by systemic treatment with the clinically approved compound Fludara in different proportions of ePNP expressing cells (21). In this study, we observed activity of the PNP/Fludara system in SW480 cells when only 5% of the cells expressed the ePNP gene. Furthermore, the bystander effect of the PNP/Fludara system was mostly absent in hTERT-negative MKN74 cells. This indicated that the bystander effects exhibited dependence on both the level of prodrug administered and ePNP expression *in vitro* (Fig. 2). Third, fludarabine has been studied extensively, and pharmacokinetics of the agent in animal models are well defined. Conversely, MeP-dR, another prodrug of ePNP, has to be chemically synthesized in laboratories because there is no readily available source. Finally, analysis of the mechanisms of cell death of hepatocellular carcinoma expressing the suicide gene showed that PNP/Fludara induced apoptosis through p53

accumulation in p53-positive cells compared to p53-negative cells (22). Our results indicated that PNP/Fludara-induced apoptosis, might depend not only on p53 accumulation, but also on other factors, such as Bax and caspase-3. The precise apoptotic mechanisms induced by PNP/Fludara remain to be determined. Furthermore, we detected a G_2/M arrest in PNP/Fludara-treated cells, as shown previously, suggesting the PNP/Fludara suicide system might induce irreversible DNA damage (23). Together, this study revealed that the mechanism by which PNP/Fludara inhibits the proliferation of SW480-8HhP cells might be related to inducing apoptosis and G_2/M arrest under heated conditions.

The synergistic effect of ePNP and Fludara, bringing greater efficacy than the two alone, has important clinical significance; in particular, the ability to use low doses of Fludara should translate to reduced side effects and clinical improvements in quality of life. This is particularly relevant for patients with ePNP who had previously been or who were undergoing chemotherapy with Fludara. The reduction in the therapeutic dose of ePNP also decreases the amount of Fludara required for each patient. The data obtained in this study warrant further confirmation *in vivo* and *in vitro*. It has been reported that synergy between ePNP and docetaxel against prostate cancer cells led to a decrease in tumor load, both in the prostate and at distant sites in immunocompetent mice (24). However, interactions between PNP and docetaxel are not yet fully understood (25). A better understanding of these interactions will help design new studies in patients who have experienced pre-existing treatment.

To understand the interaction between PNP and Fludara, molecular studies were performed. Apoptosis has been shown to play a significant role in the cell death triggered by some suicide systems (26). The most effective apoptotic stimulus occurred when PNP interacted with Fludara, which was reflected in the cell killing observed in our studies. Pro- or anti-apoptotic proteins and caspases that regulate apoptosis have been observed in several suicide system treatments (27). Overall, the pro-apoptotic proteins Bax, cleaved caspase-3 or -9 were upregulated the and anti-apoptotic protein, Bcl-2 was downregulated when suicide systems were implemented (28). Strong expression of both caspase-3 and Bcl-2 in responses to the suicide system suggests that pathways involving caspases and the Bcl-2 family may be active in these systems (29). This is the first study to identify changes in apoptosis-related proteins in response to PNP/Fludara in SW480 cells. However, our results do not rule out that PNP/Fludara may be acting through numerous processes ultimately leading to an upregulation of pro-apoptotic or a downregulation of anti-apoptotic proteins (30).

To investigate *in vivo* our *in vitro* observation that the 8HSEs-hTERTp-ePNP/Fludara system was capable of inducing heat- and tumor-specific killing effects, we tested tumor growth following heat treatment in a mouse xenograft model, injected with a heat-inducible lentiviral vector. In addition to tumor-specific killing effects, 8HSEs-hTERTp-ePNP/Fludara system exhibited optimal tumor xenograft growth inhibition under heated conditions. Supporting these observations, TUNEL assays showed that the 8HSEs-hTERTp-ePNP/Fludara system induced obvious apoptosis following hyperthermia in SW480 xenografts. These results suggest that the 8HSEs-hTERTp-ePNP/Fludara system might be a promising method of modulating gene expression during treatment by clinical hyperthermia.

In conclusion, we have demonstrated that the 8HSEs-hTERTp-ePNP/Fludara suicide system efficiently kills hTERT-expressing tumor cells *in vivo* and *in vitro*. Inclusion of 8HSEs-hTERTp in the recombinant lentivirus vector significantly improved the antitumor effects and specificity to heat treatment of the ePNP/Fludara system. Furthermore, we have demonstrated that the 8HSEs-hTERTp-ePNP/Fludara suicide system induced antitumor effects by promoting apoptosis and a G_2 arrest. Our study suggests that by combining hyperthermia with gene therapy, the 8HSEs-hTERTp-ePNP/Fludara system, may serve as a powerful strategy for tumor gene therapy under hyperthermia.

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