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Plasmid-based E6-specific siRNA and co-expression of wild-type p53 suppresses the growth of cervical cancer in vitro and in vivo

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Abstract

The E6 protein of the oncogenic HPV-16 functions by interfering with the normal cell cycle control mechanisms, particularly those controlled by p53. In this study, we developed a dual expression plasmid that coexpressed-E6-specific siRNA and wild type p53, and to evaluate its effects on cervical cancer growth. We found that simultaneous expression of pSi-E6-P53 caused a robust suppression of tumor growth when compared to the controls either E6-specific siRNA or p53 alone. In conclusion, our findings demonstrate that a combined strategy of co-expressed E6-specific siRNA and p53 synergistically and more effectively suppressed cervical tumor growth when compared with single treatment.

Keywords

Cervical cancer; E6; p53; Gene therapy; Attenuated *Salmonella typhimurium*

1. Introduction

Cervical cancer is the second most common cancer affecting women worldwide and is the leading cause of cancer mortality in women in developing countries. There are 450,000 new cases and approximately 233,000 deaths per year caused by cervical cancer [1,2]. Infection with high-risk human papillomavirus (HPV), such as types 16, 18, 31 or 33, plays a central role in the development of 99.5% of cervical cancers [3].

The oncogenic function of HPV has been primarily attributed to the expression of early in the viral life cycle of two genes, E6 and E7, the two major HPV oncoproteins. Extensive experimental evidence indicates that the E6 and E7 oncogenes play major roles in the development and maintenance of malignancy in cervical cancer. The E6 viral oncoprotein binds to the host cell's wild-type p53 (wt-p53), while E7 binds to the retinoblastoma (RB) family of tumor suppressor proteins and disrupts the RB/E2F complex, thereby driving cell division [4,5].

Therefore, the E6 and E7 oncogenes represent ideal targets for gene therapy of cervical cancer. RNAi is potentially applicable for the targeting of genes involved in any number of human diseases [6]. In current studies, several therapeutic strategies, including the application of shRNAs have been used specifically to block the translation of several genes. It was reported that shRNAs targeting HPV18 E6 exerted a negative growth effect on HPV18-positive HeLa and SW756 cervical cancer cells [7]. Further, experimental results from HPV16 E6/E7 silencing via siRNA have demonstrated that E6/E7 silencing is associated with both increased promoter DNA methylation and histone methylation [8]. Thus, highly specific and potent HPV E6/E7-siRNAs successfully suppress tumor growth and induce apoptosis in HPV-positive cervical cancer cells [9].

p53 plays a fundamental role in cellular networks associated with genotoxic and cytotoxic stresses, which potentially affect genomic integrity and lead to abnormal cell division. Normally, p53 is tightly regulated by mdm2 and also by ubiquitin-mediated degradation [10]. Activation of p53 can induce downstream target genes involved in cell cycle arrest and apoptosis. The cytotoxic effect of p53 is mediated by transcriptional activation of the cyclin-dependent kinase (cdk) inhibitor, p21cip1/waf1 [11], whereas apoptotic effects are mediated in part by activation of a pro-apoptotic gene product, Bax [12]. The restoration of normal p53 function in cancer cells represents a viable option that might sensitize cells to cancer therapies or induce apoptosis directly [13]. Consequently, the possibility of reactivating the p53 pathway has been extensively studied in several cancers [14].

If E6 expression is abolished, p53 function will be restored along with its target effectors involved in cell cycle arrest and apoptosis [15,16]. This characteristic makes cervical cancer unique among other cancers in which the predominant mechanism for inactivation of p53 is p53-gene mutation. Theoretically, inhibiting E6-P53 binding or clearing E6 in tumor cells would render these cells more susceptible to cell cycle arrest or apoptosis by different stress-based treatments. In certain other cancers, however, active p53 may either enhance or inhibit the response to therapeutic agents [17,18].

Salmonella enterica serovar Typhimurium (*Salmonella Typhimurium*) specifically survives in macrophages that also are involved in targeting tumor tissues. The phoP/phoQ two-component regulatory system [19,20] is a well-characterized transcriptional region required for *Salmonella* pathogenesis. This regulatory system controls the expression of more than 40 different genes that are required for virulence in mice by promoting resistance to innate immune defenses (e.g., resistance to defensins and low pH), inducing specious phagosome formation, contributing to survival in macrophages, and playing a role in nutrient scavenging and lipid modification. The attenuated *Salmonella* strain phoP/phoQ mutant strain has an excellent safety profile [21,22] and a propensity to target tumors [23]. In this study, we have investigated if an attenuated *S. Typhimurium* strain can be used as a vector for delivering pSh-E6-P53 to suppress the growth of a pre-established cervical tumor in vivo.

2. Materials and methods

2.1. Plasmid construction

The pSilencer 1.0-U6 plasmid (Ambion, Austin, TX, USA) was used for DNA vector-based siRNA synthesis. Three siRNA target sequences were selected from different portions of HPV16 E6 and E7 (GenBank Accession No. NC-001526). The synthesized siRNA targeting E6 and E7 were shRNA-E6A (109–127):siRNA-E6B (288–306) and siRNA-E7 (101–119). The eukaryotic expression plasmid pcDNA3.1 was used to construct: the pcDNA3.1-siRNA-E6A (plasmid pSi-E6) encoding siRNA specific to HPV16 E6; the pSi-Scramble vector (containing Scrambled siRNA sequence) which served as a negative control; pcDNA3.1-P53 containing the wild type p53 (wt p53) coding region (i.e. plasmid pP53) [24,25]; and the co-expression plasmid pcDNA3.1-Si-E6-P53 [or plasmid (pSi-E6-P53)] expressing both siRNA-HPV16 E6 and p53 tumor suppressor protein (Fig. 1A).

2.2. Cell culture and transfection

The human cervical cancer cell line, SiHa, was obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China), and grown in Iscove's modified Dulbecco's medium (Hyclone, Logan, UT, USA) with 10% (v/v) fetal bovine serum (Hyclone, Logan, UT). This line contains an integrated human papillomavirus type 16 genome and was transfected with various plasmids using the Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) for an additional 48–72 h before analysis of mRNA and protein levels, cell apoptosis, and cell proliferation.

2.3. Semi-quantitative reverse transcription-PCR (RT-PCR)

The mRNA expression levels of E6, P53 and related genes were examined using semi-quantitative RT-PCR. Cells transfected with pSi-E6, pP53, or pSi-E6-P53 plasmids were collected after 48 h. and total RNA was extracted using the Trizol Reagent (Invitrogen, Carlsbad, CA, USA). Reverse transcription was performed with 5 µg of total RNA purified after DNase I treatment using a commercially available RT-PCR kit (Promega, Madison, WI, USA) according to the manufacturer's instructions.

P53 sense:5'-CCTCCTCAGCATCTTATCCG-3'/antisense:5'-CACAAACACGCACCATA-AGA-3'/E6sense:5'-CAGAGCTGCAAACAACTATACA-3'/antisense:5'-CACCGACCCCTTA-TATTATG-3'

2.4. Western blot analysis

Antibodies against E6 were obtained from Abcam (Cambridge, MA, USA). Anti-bodies against Bcl-2, Bax, cleaved-caspase3, cleaved-caspase9, Caspase8, and Cyto-c were obtained from Cell Signaling Technology (Beverly, MA, USA). Antibodies against P53, P21, and VEGF were obtained from Santa Cruz Biotech, Inc. (Santa Cruz, CA, USA). For western blot analyses, cells were harvested at 48 h after transfection and lysed with lysis buffer (TaKaRa, Tokyo, Japan). After centrifugation at 15,000g for 30 min, the supernatants were analyzed for protein content using Bradford reagent (Bio-Rad, Hercules, CA, USA). After boiling at 100 °C for 10 min, a total of 40 mg of protein mixed with SDS-PAGE buffer were loaded onto 12% SDS-PAGE gel for electrophoresis. The proteins were separated and then transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). The membranes were blocked in 5% nonfat milk for 1 h at 37 °C and were incubated with the primary antibodies diluted 1:1000 with an antibody diluting buffer at 4 °C over-night. After washing thoroughly with TBST buffer three times, the membranes were incubated with HRP-labeled anti-rabbit IgG secondary antibody at 1:2000 dilution (Abcam, Cambridge, MA, USA) for 2 h at 37 °C. The membranes were subsequently

washed thoroughly with TBST buffer. The antibody-bound bands were visualized using ECL reagents (Amersham Pharmacia Biotech, Amersham, UK) to detect protein. The absorbances of the positive bands in the analysis were measured by densitometry using a GIS Analysis System (Tannon, Shanghai, China).

2.5. Flow cytometry

Three days after transfection with the indicated plasmids, SiHa cells (1×10^6) were collected and were resuspended in 100 μ l phosphate-buffered saline (PBS). Then, 5 μ l propidium iodide (PI) (Beckman Coulter, Fullerton, CA, USA) was added and incubated for 30 min at room temperature, in the dark. The cells were assessed via flow cytometry (FCM) to measure the apoptosis rate using an Epics-XL-MCL flow cytometer (Beckman Coulter). H&E and immunohistochemical staining plus CCK-8 and TUNEL assays were carried out as described previously [25].

2.6. CCK-8 assay

Cell proliferation after treatment with the plasmids of interest was measured by CCK-8 assay. SiHa cells were seeded at a density of 4.5×10^5 cells/100 μ l/well in 96-well plates and allowed to attach overnight. Cells were grown under these conditions for 24, 48, and 72 h at 37 °C in a humidified atmosphere in a 5% CO₂ incubator. After the designated time, CCK-8 was added to each well containing 100 μ l of culture medium and the oligopeptide mixture, and the wells were further incubated for 4–5 h at 37 °C. The quantity of formazan dye was measured using 450 nm illumination and a microplate reader (Bio-Rad). All experiments were performed in triplicate.

The percentage of inhibition of cell proliferation was calculated as follows:

$$\% \text{inhibition} = [(\text{OD}_{\text{control}} - \text{OD}_{\text{treated}}) / (\text{OD}_{\text{control}} - \text{OD}_{\text{blank}}) \times 100\%]$$

2.7. Bacterial strains and animals

The attenuated *S. Typhimurium* phoP/phoQ null strain LH430 was generously provided by Hohmann [26]. Each of the plasmids was electroporated into Salmonella. Forty-two female BALB/c nude mice (6 weeks old), weighed 18–20 g, were purchased from the Beijing Institute for Experimental Animals (Beijing, China). All animals were maintained in the Experimental Animal Center of the School of Basic Medical Sciences, Jilin University (Changchun, China). All animal experiments were conducted in accordance with the principles and procedures approved by the University Animal Experimentation Committee.

2.8. Tumor xenografts

Female BALB/c nude mice were randomly split into six groups ($n = 5$) that were injected with 2×10^6 SiHa cells. After 12 days, the mice were injected intravenously with one of the following: (a) Mock (PBS buffer alone), (b) PQ (attenuated *S. Typhimurium* phoP/phoQ), (c) pSi-Scramble (attenuated *S. Typhimurium* phoP/phoQ carrying pSi-Scramble), (d) pSi-E6 (attenuated *S. Typhimurium* phoP/phoQ carrying pSi-E6), (e) pP53 (attenuated *S. Typhimurium* phoP/phoQ carrying pcDNA3.1-P53), or (f) pSi-E6-P53 (attenuated *S. Typhimurium* phoP/phoQ carrying pcDNA3.1-Sh-E6-P53). Mice were killed on day 36 and the tumor blocks were evaluated by RT-PCR, western blot assay, immunohistochemical analyses and TUNEL assay.

2.9. Statistical analyses

Data are expressed as the Mean $\pm$ standard deviation (SD). Statistical comparisons of in vitro and in vivo data were performed using analysis of variance (ANOVA) to determine statistical significance ($P < 0.05$ was deemed to be statistically significant).

3. Results

3.1. Construction of treatment plasmids and the effect on mRNA and protein expression of E6 and wtP53

We have constructed three plasmids (pSi-E6, pP53 and pSi-E6-P53) that are capable of expressing an shRNA that targets the E6 oncogene of HPV type 16, tumor suppressor p53 either alone or in combination (Fig. 1A). Transfection of the pSi-E6-P53 plasmid simultaneously silences E6 mRNA and expresses p53 (Fig. 1B and C). As expected, the E6 mRNA and protein expression decreased significantly in the SiHa cells transfected with either pSi-E6 or pSi-E6-P53. p53 mRNA and protein expression was increased in pP53 and pSi-E6-P53 transfected cells compared to those that received Mock or pSi-Scramble via transfection (see semi-quantitative RT-PCR in Fig. 1B and C and western blot data in Fig. 1D and E).

3.2. Effects of pSi-E6-P53 on cell proliferation and apoptosis and related mechanisms in SiHa cells

3.2.1. Effects on apoptosis—The phenomenon of apoptosis was analyzed by light microscopy, Rhodamine B, TUNEL and flow cytometry (Fig. 2A). Using light microscopy, apoptotic cells were observed surrounding the necrotic area. We observed that Mock and pSi-Scramble groups exhibited normally adherent, polygonal cells with clear nucleoli and fewer cytoplasmic granules. Cell morphology changed significantly, especially in the pSi-E6-P53 group. After 48 h, these cells lost their adherent properties. The cell became round, with reduced cell volume, and exhibited increased cytoplasmic granules, rough irregular edges, crescent chromatin, and nuclear fragmentation.

Cell proliferation in the pSi-E6, pP53, and pSh-E6-P53 groups was significantly diminished compared to the Mock and pSi-Scramble groups, as determined by Rhodamine B staining. Among the SiHa cell groups treated with pSi-E6, pP53, or pSi-E6-P53, the lowest incidence of cell proliferation was observed in the pSh-E6-P53 treatment group.

TUNEL assays showed that pSi-E6, pP53, and pSi-E6-P53 transfected cells had undergone massive apoptosis, compared with Mock or pSh-Scramble transfected cells. The apoptosis index was higher in the pSh-E6-P53 group than in either the pSi-E6 or pP53 treatment groups.

Alternatively, apoptotic cell death was assessed using flow cytometry by measuring the fluorescence intensity after tumor cells were double-stained with Annexin V and PI. These percentage of apoptotic cells were, respectively, $10.82 \pm 2.24\%$, $19.65 \pm 1.68\%$ and $26.79 \pm 2.47\%$ for the treatment groups pSi-E6, pP53, and pSi-E6-P53 in which were much higher than seen with the Mock or pSi-Scramble groups. The pSi-E6-P53 group showed the highest level of apoptosis compared with the other groups (Fig. 2A), which was statistically significant ($P < 0.05$).

3.2.2. Detection of cell viability by CCK-8 assay—SiHa cells were cultured in 96-well plates. and 10 μ l CCK-8 reagent was added to each well incubated at 37 °C for 1–4 h, and shaken for 10 min; finally, the absorbance was measured at 450 nm. Each test group was assessed in three wells, with the final result being the average of these 3 wells. The

results showed that pSi-E6, pP53, and pSi-E6-P53 could all inhibit cell proliferation; the 48 h survival rates of SiHa cells were $75.41 \pm 0.74\%$, $69.98 \pm 3.12\%$, and $58.10 \pm 1.13\%$, respectively, compared with the Mock or pSi-Scramble groups. Transfection with pSi-E6, pP53, and pSi-E6-P53 resulted in significant inhibition of SiHa cell proliferation at 24 and 48 h. The highest inhibition of cell growth occurred in the presence of pSi-E6-P53 (Fig. 2B).

3.2.3. The expression of apoptosis-related proteins in SiHa cells—To determine the potential mechanism of cell growth inhibition in vitro, apoptosis-associated protein expression was examined using western blots. Cytochrome-c, cleaved-Caspase9, Caspase8, cleaved-Caspase3, and Bax protein expression was significantly increased in pSi-E6, pP53, and pSi-E6-P53 treatment groups, compared to the controls-Mock and pSi-Scramble groups. In contrast, Bcl-2 protein expression was decreased in the pSi-E6, pP53, and pSi-E6-P53 groups relative to the Mock or pSi-Scramble groups (Fig. 2C and D).

3.3. Effects of co-expression plasmid pSi-E6-P53 on the wound-induced migration of SiHa cells

To examine the impact of pSi-E6-P53 on cell motility in vitro, a wound-scratch healing model was used. A scratch was introduced into confluent monolayers expressing different treatment plasmids, and the time-dependent movement of cells into the injured area was monitored microscopically. Cells in the Mock and pSh-Scramble groups began migrating 8 h after scratching. There was no significant change between 8 and 12 h. After 48 h, cells in the pSi-E6 group, the pP53 group and the pSi-E6-P53 group migrated significantly less than those in the Mock and the pSi-Scramble groups. Compared to the pSi-E6 or the pP53 groups, the cells in the pSi-E6-P53 group significantly reduced in migration (Fig. 3A and B). We further examined the expression levels of MMP2 and MMP9, which are associated with cell migration and invasion, using western blotting. Compared to the Mock and pSi-Scramble groups, significantly reduced expression of MMP2 and MMP9 was observed in the pSi-E6, the pP53, and the pSi-E6-P53 groups. Furthermore, the group transfected with pSh-E6-P53 showed the most reduced expression ($P < 0.05$, as shown in Fig. 3D and E). To determine the mechanism of cell cycle arrest, we examined the expression of CDK-inhibitor p21 by western blotting. Consistent with the growth patterns, p21 expression was significantly increased in the pSh-E6, the pP53, and the pSi-E6-P53 groups. The pSi-E6-P53 group showed maximally enhanced expression compared to either the pSi-E6 or pP53 groups ($P < 0.05$, as shown in Fig. 3C).

3.4. pSi-E6-P53 strongly suppresses the growth of cervical tumor xenografts

To evaluate the effects of the recombinant treatment plasmids on cervical cancer growth in vivo, we used a nude mouse tumor xenograft model. SiHa cells (2×10^6) were inoculated s.c. into the right flank of female BALB/c nude mice, and palpable tumors were established. By day 12, when palpable tumors had developed at the sites of injection, tumor bearing animals were divided into 6 groups of 5 mice each and injected intravenously with either buffer alone (untransfected) or attenuated *S. Typhimurium* vector carrying the different recombinant treatment plasmids. Animals were sacrificed on day 36, and tumor sizes were determined.

Tumor volumes (Fig. 4A and B) and the weights (Fig. 4C) of mice treated with bacteria carrying pSi-E6, pP53 and pSi-E6-P53 were significantly diminished, when compared to those that received the Mock, PQ vector alone or pSi-Scramble treatments. Furthermore, the group treated with pSi-E6-P53 had marked tumor growth suppression as compared with the pSi-E6 or pP53 ($P < 0.05$).

The weights of the mice, of the tumors, and the volumes of the tumors are shown in Table 1. There was no statistical difference in the average weight for all groups ($P > 0.05$). Compared with the Mock group, the PQ group and the pSi-Scramble group, tumor volumes in the pSi-E6, the pP53 and the pSi-E6-P53 groups were significantly decreased ($P < 0.05$). Tumor volume in mice treated with pSi-E6-P53 declined most drastically relative to the other groups in this experiment ($P < 0.05$).

3.5. Tumor growth inhibition occurred due to tumor cell death in vivo

To determine the mechanisms underlying the in vivo anti-tumor effects of Si-E6 and p53, tumors were excised and analyzed with H&E staining, immunohistochemistry and TUNEL assays (Fig. 5A). Proliferating cell nuclear antigen (PCNA), a nuclear protein that is expressed in G1-M phases of the cell cycle, was assessed. PCNA levels were remarkably reduced by pSi-E6, pP53, and pSi-E6-P53 treatments compared to the Mock and pSh-Scramble groups. Furthermore, tumor sections from mice treated with pSi-E6, pP53, and pSi-E6-P53 showed a pronounced increase in apoptosis compared to the Mock and pSi-Scramble groups, as observed by TUNEL assay. The proliferation index was dramatically reduced, and the apoptosis index greatly increased, in the pSi-E6-P53 group relative to the pSi-E6 and pP53 groups.

There was a significantly increased proportion of apoptotic cells among the tumors of each group. Quantitative analysis with flow cytometry showed that apoptotic death increased significantly in the pSh-E6($23.78 \pm 2.45\%$) and pP53($25.40 \pm 1.63\%$) groups compared with that of Mock or pSh-Scramble groups. However, the highest level of apoptosis ($38.00 \pm 1.97\%$) among all groups was found in the pSi-E6-P53 group (Fig. 5B).

3.6. The effects of pSi-E6-P53 on apoptosis and anti-angiogenesis in grafted tumor tissues

To elucidate the potential mechanism of xenograft tumor growth inhibition in vivo, apoptotic protein expression was detected using western blotting. Cytochrome-c, cleaved-Caspase9, Caspase8, cleaved-Caspase3, and Bax protein expression in the pSi-E6, pP53, and pSi-E6-P53 treatment groups were significantly elevated as compared to Mock, PQ, or pSi-Scramble groups. Bcl-2 protein expression was strongly decreased in the pSh-E6, pP53, and pSi-E6-P53 groups compared to the Mock, PQ, or pSi-Scramble group (Fig. 6A–E). Lastly, to demonstrate anti-angiogenic effects, we examined the expression of VEGF using western blot analysis. Compared with Mock, PQ, or pSh-Scramble groups, VEGF protein expression was strongly and significantly decreased in the pSi-E6, pP53, and pSi-E6-P53 groups ($P < 0.05$) (Fig. 6F and G).

4. Discussion

High-risk HPVs encode three transforming proteins, E5, E6, and E7. The E6 and E7 oncoproteins are consistently expressed in cervical cancers [7,27–32]. The E6 and E7 are low molecular-weight cysteine-rich metal binding proteins which lack intrinsic enzymatic activities and specific DNA-binding properties. While the rate of invasive cervical cancer has decreased in areas with aggressive cervical cancer screening, the incidence of cervical cancer in developing nations and low socio-economic groups, however, remains high. Epidemiological studies have shown that virtually all cervical cancers are caused by persistent infection with one of approximately 15 high-risk HPV types [28,33]. HPV types 16 and 18 are the two most abundant high-risk HPVs and are present in approximately 50% of high-grade cervical intraepithelial neoplasias (CIN) and approximately 70% of cervical cancers [29,34]. HPV16 is most frequently detected in squamous cell carcinomas, whereas HPV18 appears to be more frequently associated with adenocarcinomas [35]. Prophylactic vaccines have shown nearly 100% efficacy in preventing precancerous lesions of the cervix

due to HPV types 16 and 18 among previously uninfected individuals. In this regard, consequently, gene therapy aimed at HPV may offer an efficient therapeutic approach for patients with cervical cancers.

Tumor suppressor p53 is a powerful anti-tumor molecule that is often inactivated by over expression of its negative mediators. In a normal cell, unscheduled DNA synthesis or DNA damage led to the activation of the p53 tumor suppressor protein and subsequent growth arrest or cell death, depending on the cell type and damage. HPV E6 decreases p53 through interaction with the E6-associated protein (E6-AP), an E3 ubiquitin ligase, which causes proteasome-dependent degradation of p53 (and E6-AP), allowing for increased cell proliferation [4,36]. In the current study, transfection of the pSh-E6-P53 plasmid strongly decreased and increased E6 and p53, respectively (Fig. 1B and D). It is well-known that p53 promotes cell death via transcriptional activation of genes encoding proteins involved in modulating or promoting cell death, such as caspases, death receptors and ligands, iNOS, and the Bcl-2 family. In our study, we showed that the expression of Bax, Cytochrome-c, cleaved-Caspase9, Caspase8 and cleaved-Caspase3 was greatly increased in vitro (Fig. 2C) and in vivo (Fig. 6E) following treatment with pSi-E6, pP53, and pSi-E6-P53. In contrast, the expression of Bcl-2 was diminished under these treatment conditions. Consistent with these observations, pSi-E6-P53 caused robust apoptosis and tumor growth suppression ($P < 0.05$) compared to controls or to pSi-E6 or pP53 treatments alone.

MMP-2 can degrade type IV collagen, one of the extracellular matrix components and an integral part of the basement membrane, and is assumed to play a key role in the metastatic process. MMP-2 and MMP-9 both possesses gelatinase activity and play important roles in cancer cell invasion and metastasis [37]. We found that pSi-E6-P53 also decreased the expression of MMP-2 and MMP-9.

p53 is also known to cause cell cycle arrest by stimulating the expression of p21 which inhibits the action of G (1) cyclin-dependent kinases. p21 induces cell cycle arrest in response to DNA damage both in the presence and in the absence of functional p53 [10]. We also showed that restoration of p53 resulted in an up-regulation of p21 (Fig. 3C).

HPV oncoproteins have also been reported to promote angiogenesis [38] and induce several hallmarks of cancer-associated epithelial to mesenchymal transition, a process that is important in invasion and metastasis [39]. We found that compared to the Mock, PQ, or pSh-Scramble groups, VEGF expression was strongly ($P < 0.05$) decreased for the pSi-E6, pP53, and pSi-E6-P53 treatment groups (Fig. 6F and G). Mice treated with pSi-E6-P53 had marked tumor growth suppression compared with either the pSi-E6 or pP53 treatment ($P < 0.05$).

Salmonella are facultative anaerobes that grow in tumors, in which hypoxic microenvironments exist. As a model delivery system for mice, we have utilized the PhoP/PhoQ deficient-attenuated *S. Typhimurium* carrying a plasmid designed to synthesize siRNA and a tumor suppression protein under the control of eukaryotic gene promoters. Regardless of the route of administration, Salmonella-delivered gene therapeutics caused significant antitumor effects with no detectable toxicity to mice [26,40–44]. In the present study, we have established, for the first time, that a Salmonella-delivered plasmid containing both E6-shRNA and wt p53 (pSi-E6-P53) is synergistically effective for the treatment of cervical cancer cells.

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Fig. 1. SiRNA-mediated knockdown of HPV16 E6 expression and P53 expression in SiHa cells transfected with various plasmids. (A) Constructions of various expression plasmids. (B) mRNA levels of E6 and P53 as determined by RT-PCR analyses. (C) Quantification of E6 and P53 mRNA. (D) Protein expression levels of E6 and P53 as determined using western blot assays. (E) Quantification of E6 and P53 protein levels. The data are presented as means $\pm$ SD of three separate experiments. a, $P < 0.05$ versus Mock or pSi-Scramble group. b, $P < 0.05$ versus pSi-E6 group. c, $P < 0.05$ versus pP53 group.

Fig. 2. pSi-E6-P53 plasmid induces SiHa cells apoptosis. (A) Analysis of apoptosis after transfection with various plasmids in each group using a bright field microscope, Rhodamine B, TUNEL assay (magnification of 100× with TUNEL-positive cells green) and Flow cytometry analysis of apoptosis cells with Annexin V-FITC and propidium iodide staining at 48 h after transfection. (B) Growth inhibitory effects were evaluated with CCK8 assays for the SiHa cells transfected with different plasmids, and the data were presented as the ratio to control (Mock). (C) Bax, Bcl-2, Cyto-c, cleaved-Caspase9, Caspase8, and cleaved-Caspase3 protein expression was determined by western blot assay. The data represent the means ± SD of three separate experiments. a, $P < 0.05$ versus Mock or pSi-

Scramble group. b, $P < 0.05$ versus pSi-E6 group. c, $P < 0.05$ versus pP53 group. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 3. Analysis of cell proliferation and migration after transfected with various plasmids. (A) Migration abilities as determined by wound assays. (B) The wound gaps measured at six reference points along the wound, and the results are expressed as the average wound gap. (C) P21 protein expression as determined by western blot assay. (D) MMP-2 and MMP-9 protein expression as determined by western blot assay. (E) Quantification of MMP-2 and MMP-9 protein levels. The data are presented as the mean $\pm$ SD of triplicate experiments. a, $P < 0.05$ versus Mock or pSi-Scramble group. b, $P < 0.05$ versus pSi-E6 group. c, $P < 0.05$ versus pP53 group.

Fig. 4.

Inhibition of tumor growth in vivo by treatments with various plasmids. (A) Images of mice with different plasmid treatments collected after sacrifice at day 36. (B) Tumor growth curves over the 36-day period was established based on the tumor sizes measured every fourth day. (C) Tumor wet weights measured after sacrifice at day 36. The data were presented as the mean $\pm$ SD of triplicate experiments. a, $P < 0.05$ versus Mock, PQ, or pSi-Scramble group. b, $P < 0.05$ versus pSi-E6 group. c, $P < 0.05$ versus pP53 group.

Fig. 5.

Effects of various plasmid treatments on the induction of apoptosis and inhibition of proliferation in the tumor xenografts. (A) Tumor H&E staining (magnification, 200×), immunohistochemical staining for PCNA (magnification, 400×) expression were performed for the tumor cells transfected with different plasmids in tumor-bearing mice. (B) The raw data of FCM with Annexin V and PI staining to detect cell death in tumor tissue. a, $P < 0.05$ versus Mock, PQ, or pSi-Scramble group. b, $P < 0.05$ versus pSi-E6 group. c, $P < 0.05$ versus pP53 group.

Fig. 6. Effects on apoptosis and anti-angiogenesis in grafted tumor tissues using western blot analysis. (A) Bax and Bcl-2 protein expression was determined by western blot assay. (B) Quantification of Bax and Bcl-2 protein levels. (C) Cyto-c, cleaved-Caspase9, Caspase8, and cleaved-Caspase3 protein expression was determined by western blot assay. (D and E) Quantification of Cyto-c, cleaved-Caspase9, Caspase8, and cleaved-Caspase3 protein levels. (F) VEGF protein expression was determined by western blot assay. (G) Quantification of VEGF protein levels. The data were presented as the mean $\pm$ SD of triplicate experiments. a, $P < 0.05$ versus Mock, PQ, or pSi-Scramble group. b, $P < 0.05$ versus pSi-E6 group. c, $P < 0.05$ versus pP53 group.

Table 1

Mouse and tumor weights and tumor volume after treatments.

Group	Mean mouse weight (g)	Mean tumor weight (g)	Mean tumor volume (mm ³)
Mock	22.17 ± 2.00	2.21 ± 0.41	1525.46 ± 285.97
PQ	23.23 ± 1.21	1.78 ± 0.36	1356.28 ± 276.53
pSi-Scramble	21.87 ± 1.43	1.96 ± 0.27	1411.81 ± 193.93
pSi-E6	23.37 ± 1.79	1.07 ± 0.29 ^a	784.50 ± 190.96 ^a
pP53	22.61 ± 0.86	0.76 ± 0.11 ^a	594.30 ± 83.40 ^a
pSi-E6-P53	23.76 ± 1.48	0.37 ± 0.09 ^{a,b,c}	296.07 ± 74.22 ^{a,b,c}

The data are presented as means ± SD of triplicate experiments.

^a $P < 0.05$ versus Mock, PQ, or pSi-Scramble group.

^b $P < 0.05$ versus pSi-E6 group.

^c $P < 0.05$ versus pP53 group.