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Original Article

Deletion of HPV18 *E6* and *E7* genes using dual sgRNA-directed CRISPR/Cas9 inhibits growth of cervical cancer cells

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Abstract: Human papillomavirus (HPVs) type 18 is a major cause of cervical adenocarcinoma. *E6* and *E7* viral genes play significant roles in the pathogenesis of cervical carcinoma. Single guide RNA (sgRNA)-directed clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated 9 (Cas9) system is a highly efficient genome engineering tool. Here, we used dual sgRNA-directed CRISPR/Cas9 to delete the HPV18 *E6* and *E7* genes simultaneously. We deleted the *E6* and *E7* genes with a high mutation rate of 81.8% in HeLa cells. Deletion of the *E6* and *E7* genes restored the p53 and Rb protein levels, induced apoptosis and inhibited the proliferation of HeLa cells. Apoptosis and proliferation of the control-SiHa cell line were not affected. Therefore, dual sgRNA-guided CRISPR is a highly specific method for reversing the malignant phenotype of cervical cancer cells. Deletion of the *E6* and *E7* genes using CRISPR provides us a new therapy to treat HPV18 infection.

Keywords: CRISPR/Cas9 system, HPV18 E6, HPV18 E7, HeLa, apoptosis, proliferation

Introduction

HPV18 is a major cause of cervical adenocarcinoma [1]. Tumorigenesis mainly results from disruption of the tumor suppressor proteins p53 and Rb upon HPV invasion of the normal cervical epithelium. Upon HPV infection, viral oncoprotein E6 disrupts p53 protein to inhibit the p53-dependent growth arrest and apoptosis of host cells [2]. The oncoprotein E7 interacts with Rb protein to cause aberrant cell cycle progression [3, 4].

CRISPR is a newly discovered genome engineering technique to defend against phages in bacteria and archaea [5, 6]. The type II CRISPR system is composed of a Cas9 endonuclease and a sgRNA sequence [7]. sgRNA recognizes a specific domain, directs Cas9 endonuclease to cause DNA double-strand breaks (DSBs). DSBs then activate genome repair pathway-non-homologous end joining (NHEJ). Because NHEJ is error-prone, insertions or deletions may occur, leading to functional ablation of targeted genes [8]. The recognition process mediated by sgRNA also requires a 5'-NGG-3' motif, termed the protospacer adjacent motif (PAM) [7]. Previous studies have modified several organisms, including *Caenorhabditis elegans*, zebrafish, mice, and viruses using the CRISPR/Cas9 system [9-14]. Because HPV18 is the major cause of cervical adenocarcinoma and the incidence of cervical adenocarcinoma is increasing, treatments for HPV18-related cervical carcinoma are urgently needed [15].

In this study, we used paired sgRNAs to delete the HPV18 *E6* and *E7* genes simultaneously. Deletion of the E6 and E7 proteins increased apoptosis, inhibited proliferation in HeLa cells. Our results showed that deletion of the HPV18 genome using CRISPR is highly efficient in inhibiting growth of HPV18-related cervical carcinoma. Hence the CRISPR/Cas9 system may serve as a new therapy to treat HPV18-related malignancies.

Table 1. sgRNAs sequences and corresponding PAM sequences

Name	sgRNA sequence (5'-3')	PAM sequence (5'-3')	Strand	Target gene
sgR-H1	ttcgtgctgcaaccgagcac	CCA	Template	HPV18-E6
sgR-H2	gcagctgtttctgaacaccc	CCA	Template	HPV18-E7

Material and methods

CRISPR design and plasmids

CRISPR sgRNAs targeting the *E6* and *E7* ORFs of the HPV18 genome integrated in HeLa cell line were designed following the protocol published by Mali, *et al.* [7]. sgRNA plasmids were constructed by replacing the sgRNA targeting site of gRNA_GFP-T2 vector (Addgene: 41820) with HPV18 E6 and E7 sgRNAs. The Cas9 plasmid was obtained from Addgene (Addgene: 41815). Sequences of the sgRNAs used in this paper are shown in **Table 1**.

Cell culture and transfection

HeLa and SiHa cells were purchased from American Type Culture Collection (ATCC). The cells were cultured in Dulbecco's modified Eagel's medium (DMEM) with 10% Fetal Bovine Serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin in a humidified chamber with 5% CO_2 at 37°C. The cells were plated onto 6-well plates. When cellular confluence reached 80%, cells were transfected with 1.6 μg Cas9 plasmid and 400 ng sgRNA plasmid using X-tremeGENE HP DNA Transfection Reagent (Roche). Culturing medium was replaced at 24 h post-transfection.

DNA extraction and PCR amplification

Genomic DNA was extracted at 48 h post-transfection using a Tissue DNA kit (Omega Bio-tek) following the manufacturer's instructions and PCR-amplified using the following procedure: 95°C for 5 min, 33 amplification cycles (95°C for 20 s, 55°C for 30 s and 68°C for 30 s), 68°C for 7 min and hold at 4°C. The PCR products were purified with a Cycle Pure Kit (Omega Bio-tek). The primers used are described in **Table 2**.

T7 Endonuclease I assay (T7E1) and detection of mutational spectra

200 ng purified PCR products were denatured and reannealed to form DNA heteroduplexes. The products were treated with 1 μl T7E1 endonuclease (NEB) for 20 min at 37°C following the protocol which was described previously [16]. Finally, 2 μl of 0.25 M EDTA was added to stop the reaction. The results were analyzed on a 10% Tris-Borate-EDTA (TBE) gel (Invitrogen). DSBs efficiency were quantified with ImageJ software using the following formula, $100\times(1-(1-(b+c)/(a+b+c))^{1/2})$, where a represents the intensity of the undigested PCR product, b and c represent intensity of the cleaved PCR product. To better determine the mutational spectra, PCR products were cloned into a Zero Blunt® PCR cloning kit (ThermoFisher Scientific) following the manufacturer's directions. Individual clones were picked and sequenced using Sanger sequencing. The CRISPR-induced gene modification ratio was calculated by counting the ratio of mutated clones to totally picked clones.

Western blotting

HeLa and SiHa cells were plated and transfected as previously mentioned. At 48 h post-transfection, cellular proteins were extracted using RIPA Lysis Buffer (Beyotime). 40 μg protein was run on SDS-PAGE and transferred to a polyvinylidene fluoride (PVDF) membrane. The membrane was blocked with 5% skim milk and incubated at room temperature for 1 h. The final results were detected using a Super-Signal™ West Pico Chemiluminescent Substrate (ThermoFisher Scientific). The antibodies used were anti-p53 (10442-1-AP, Proteintech), anti-Rb (17218-1-AP, Proteintech), anti-HPV18 E6 (G-7) (sc-365089, Santa Cruz Biotechnology), anti-HPV18 E7 (N-19) (sc-1590, Santa Cruz Biotechnology), anti-HPV16 E6 (orb108-37, Biorbyt), anti-HPV16 E7 (orb10573, Biorbyt) and anti-β-actin (60008-1-Ig, Proteintech). β-actin was used as the internal control.

Apoptosis

At 48 h post-transfection, HeLa and SiHa cells were trypsinized and washed with PBS. The cells were then double stained with fluorescein isothiocyanate (FITC)-conjugated annexin V (annexin V-FITC) and propidium iodide (PI) using an Annexin V-FITC apoptosis detection kit (KeyGen Biotech) following the manufacturer's

Table 2. Primers used for PCR

Name	Forward primer (5'-3')	Reverse primer (5'-3')	Use	PCR product size (bp)
sgR-H1+H2-primer	cgggaccgaaaacggtgtat	tgcatcccagcagtaagcaa	T7E1	858

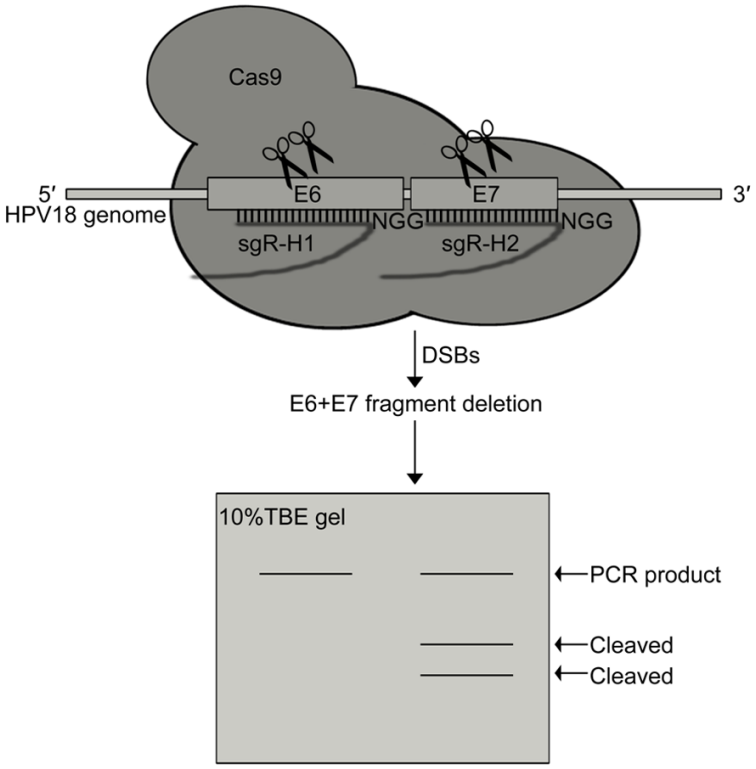

Figure 1. Schematic overview of HPV18 E6 and E7 dual-sgRNA-directed deletion of the *E6*, *E7* genes and the T7E1 assay. sgR-H1 targeting *E6* ORF and sgR-H2 targeting *E7* ORF were used. Upon deletion of *E6* and *E7* ORFs using CRISPR, genomic DNA was purified and PCR-amplified. The results were run on a 10% TBE gel to show PCR products and the cleaved fragments. NGG represents the PAM sequence.

protocol. The apoptosis effect was analyzed using FACSCalibur™ (BD Bioscience).

Colony formation assay

At 12 h post-transfection, HeLa and SiHa cells were trypsinized and plated onto 12-well plates, with 100 cells per well. After culturing for 14 days, the cells were stained with 0.04% crystal violet and photographed. Colonies larger than 50 μm in diameter were calculated.

Statistical analysis

The results are presented as mean ± standard deviation (SD). All the experiments were repeated for three times independently. Data were conducted with Student's t test. The level of significant statistical difference was set to *P<0.05 and **P<0.01.

Results

Deletion of E6 and E7 genes using dual sgRNA-directed CRISPR/Cas9

Two sgRNAs were designed to delete the HPV18 *E6* and *E7* genes. sgR-H1 was designed targeting the HPV18 E6 open reading frame (ORF); sgR-H2 targeting the HPV18 E7 ORF (**Figure 1**). sgR-H1 and sgR-H2 were cotransfected to delete HPV18 *E6* and *E7* genes in HeLa cells. At 48 h post-transfection, cellular DNA was extracted, PCR-amplified, and subjected to a T7E1 mutation detection assay. The results were electrophoresed on a 10% TBE gel. Untreated HeLa cells and HeLa cells transfected with only Cas9 plasmid were used as controls. No DSB events were detected in the control groups. The mutation rate detected using the T7E1 assay was 43.4% (**Figure 2A**). To further quantify the mutation rate, the PCR products were subcloned into a Zero Blunt® PCR cloning kit (ThermoFisher Scientific) and Sanger sequenced. The mutation rate revealed by Sanger sequencing was 81.8% for the sgR-H1+H2 group with the largest deleted fragment of 338 bp among the 22 sequenced clones (**Figure 2B**). In summary, fifteen deletions and three indels were detected smong the 22 sequenced clones. Representative sequencing chromatographs are shown in **Figure 2C**. The sgRNA sequences and corresponding PAM sequences are shown in **Table 1**. PCR primers used to amplify the targeting regions are shown in **Table 2**.

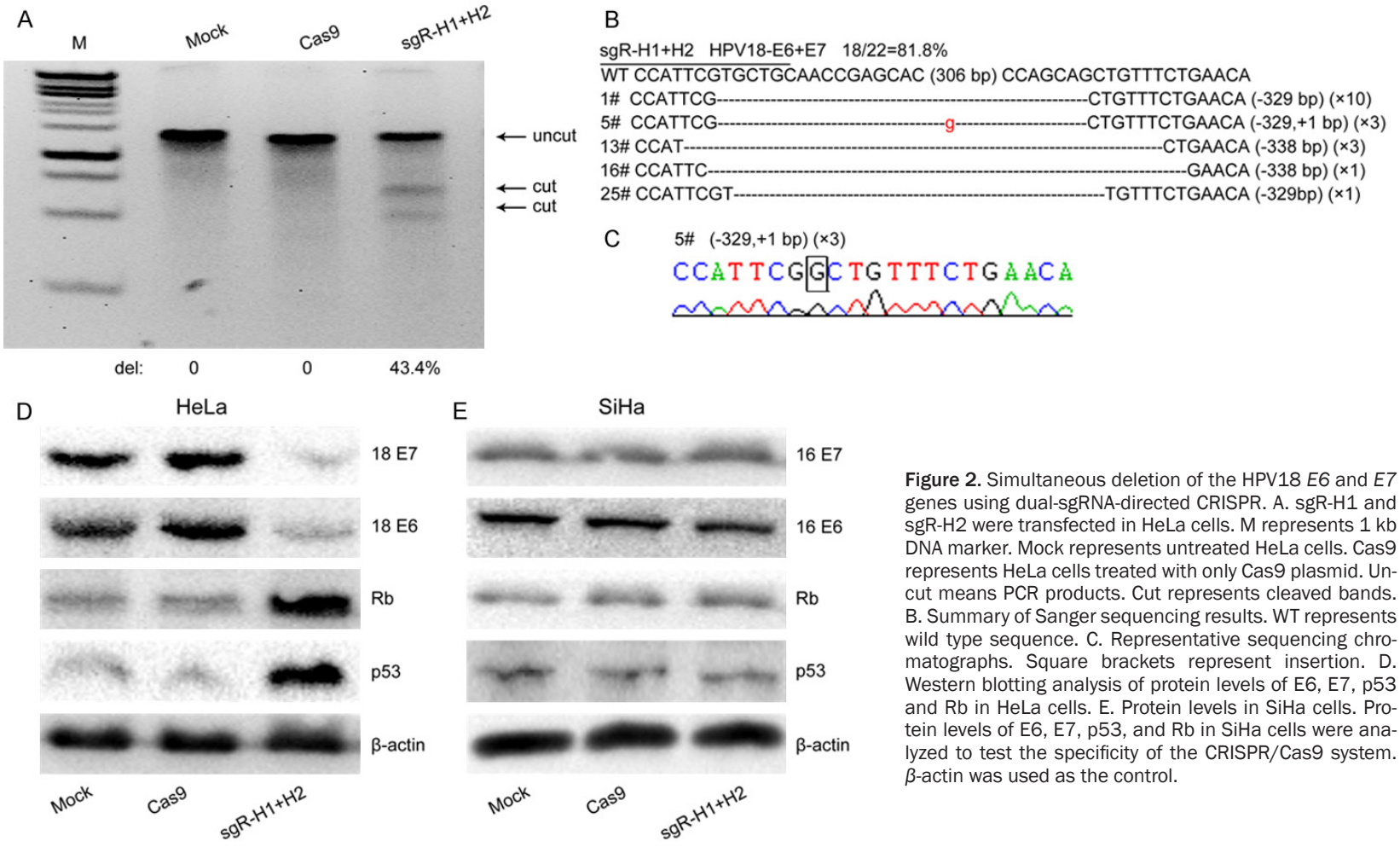

Figure 2. Simultaneous deletion of the HPV18 *E6* and *E7* genes using dual-sgRNA-directed CRISPR. A. sgR-H1 and sgR-H2 were transfected in HeLa cells. M represents 1 kb DNA marker. Mock represents untreated HeLa cells. Cas9 represents HeLa cells treated with only Cas9 plasmid. Uncut means PCR products. Cut represents cleaved bands. B. Summary of Sanger sequencing results. WT represents wild type sequence. C. Representative sequencing chromatographs. Square brackets represent insertion. D. Western blotting analysis of protein levels of E6, E7, p53 and Rb in HeLa cells. E. Protein levels in SiHa cells. Protein levels of E6, E7, p53, and Rb in SiHa cells were analyzed to test the specificity of the CRISPR/Cas9 system. *β*-actin was used as the control.

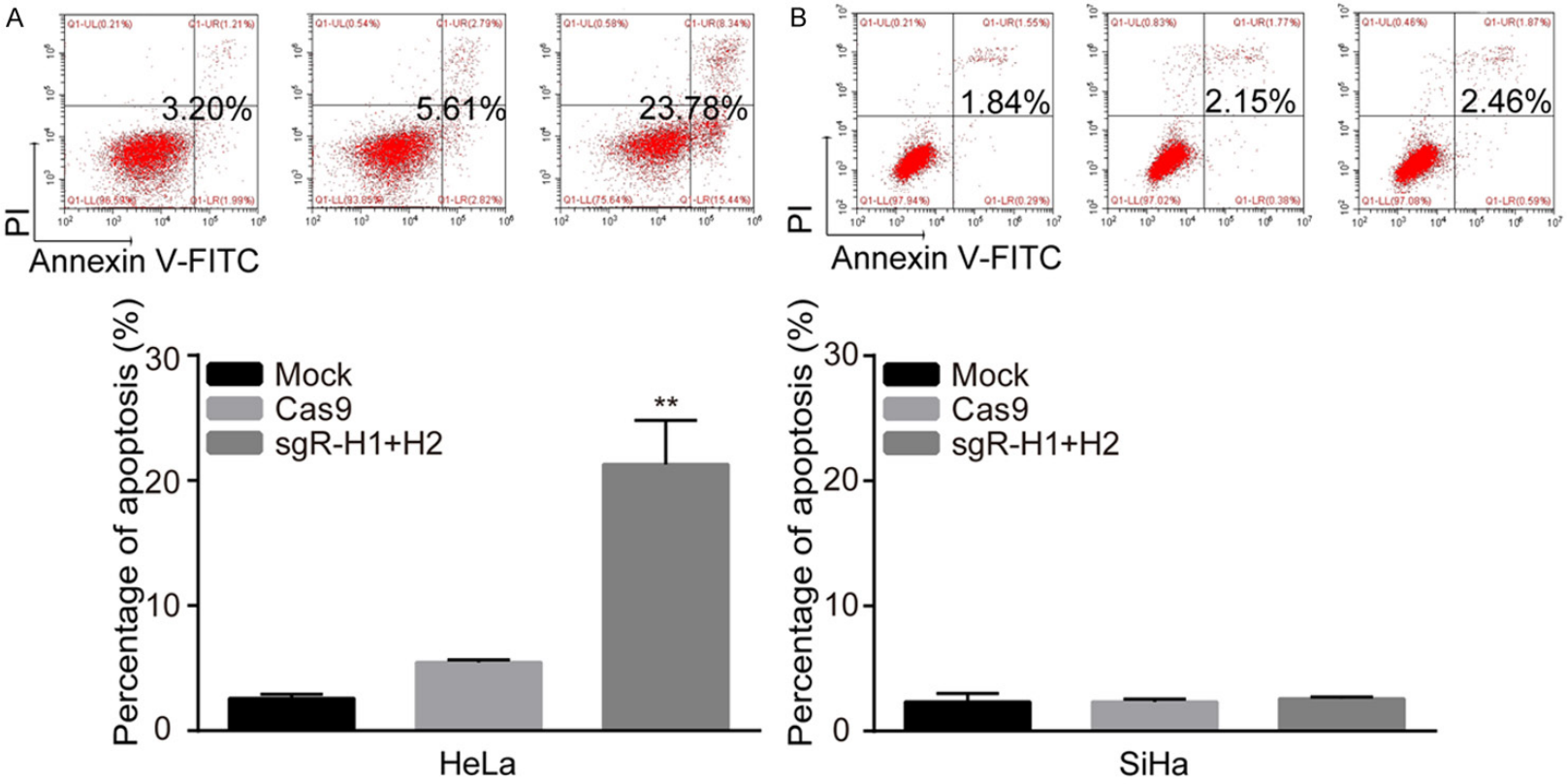

Figure 3. Apoptosis induced by simultaneous deletion of HPV18 E6 and E7 proteins. A. HeLa cells were double stained with annexin V-FITC and PI at 48 h post-transfection. Apoptosis was analyzed using FACS. B. Apoptosis in SiHa cells. Data were analyzed using Student's t test. Data represent the mean ± sd. The experiments were conducted for three independent times. (**P<0.01 compared to Mock group, n=3, per Student's t-test).

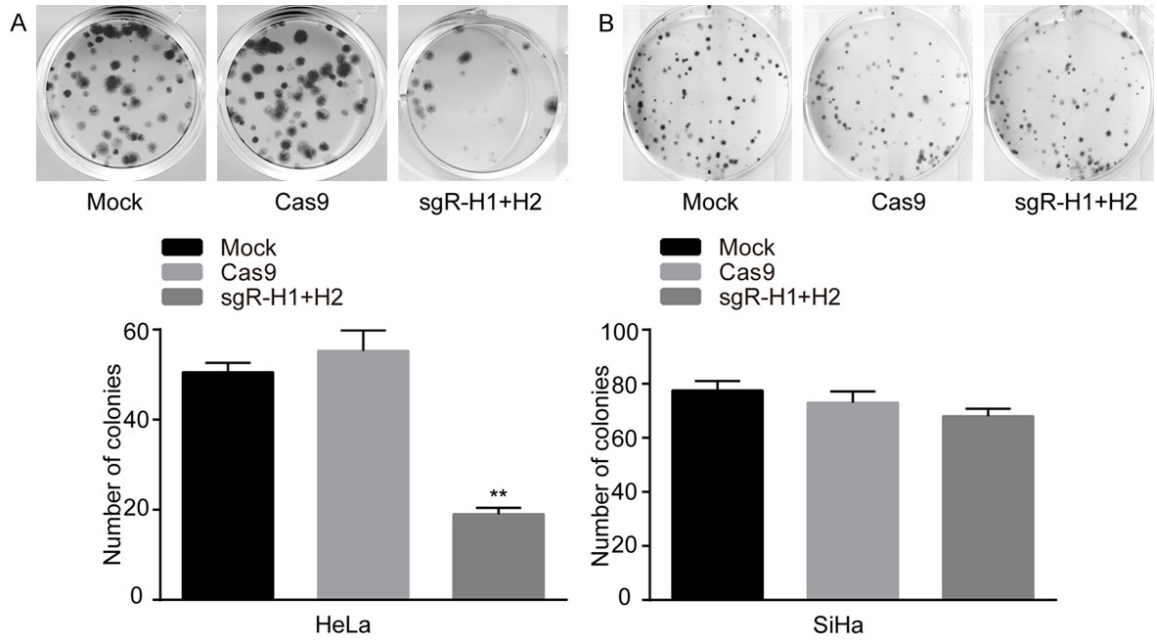

Figure 4. Proliferation inhibition caused by deletion of HPV18 E6 and E7 proteins. A. HeLa cell proliferation detected using colony formation assay. B. SiHa cell proliferation detected using colony formation assay. Data represent the mean ± sd. The experiments were repeated for three times independently. (**P<0.01 compared to Mock group, n=3, per Student's t-test).

Upregulation of p53 and Rb proteins upon E6 and E7 deletion

The *E6* and *E7* genes promote malignant transformation by interacting with the p53 and Rb proteins. We therefore reasoned that deletion of *E6* and *E7* might restore p53 and Rb protein levels. Western blotting was performed to detect E6, E7, p53 and Rb protein levels. SiHa cell line was used as the control. Western blotting results showed that the HPV18 E6 and E7 proteins were downregulated in HeLa cells in the sgR-H1+H2 group (**Figure 2D**). In contrast, HPV16 E6 and E7 protein levels were not affected in SiHa cells (**Figure 2E**). Deletion of E6 and E7 increased the protein levels of p53 and Rb in HeLa cells, but not in SiHa cells.

Deletion of E6 and E7 genes induced apoptosis and growth inhibition in HeLa cells

The HPV18 E6 and E7 proteins maintain the malignant phenotype in HeLa cells. A previous study showed that loss of the HPV16 E6 and E7 proteins induced cellular apoptosis and growth inhibition in cervical cancer cells [3, 14]. *E6* and *E7* genes of both HPV16 and HPV18 contribute to the pathogenesis of cervical carcinoma. Therefore, cell apoptosis and growth inhibition may be observed after HPV18 E6 and E7 deletion using the CRISPR/Cas9 system.

Apoptosis was detected at 48 h post-transfection in HeLa and SiHa cells. The apoptosis rate was 23.78% for the sgR-H1+H2 group in HeLa cells, which was significantly different compared to mock group HeLa cells (**Figure 3A**). To characterize the specificity of HPV18 E6 and E7 sgRNAs, SiHa cells were also transfected. The apoptosis rates of the mock, Cas9 control group and the sgR-H1+H2 group were all below 5% in SiHa cells (**Figure 3B**).

Colony formation assay was performed to determine whether deletion of the E6 and E7 proteins resulted in growth inhibition of HeLa cells. SiHa cells were also used as controls. Compared to the number of colonies in the mock and Cas9 groups, the sgR-H1+H2 group showed decreased colony numbers in HeLa cells (**Figure 4A**). Colony numbers of the sgR-H1+H2 group were not significantly different in SiHa cells compared to the control group (**Figure 4B**).

Discussion

HPVs are major risk factors for cervical carcinoma. Among the different subtypes, HPV16

and HPV18 are high-risk HPVs. Several experiments examining disruption of the HPV genome have been conducted [2, 13, 14, 16, 17]. Previous studies used siRNA, shRNA, zinc finger nucleases (ZFNs), or transcription activator-like effector nucleases (TALENs) to disrupt the HPV *E6* and *E7* genes. Disruption of HPV16 E6 and E7 proteins restored p53 and Rb protein levels, prevented uncontrolled proliferation, and reversed the malignant phenotype of cervical cancer cells [13, 14, 16-19]. Therefore, the *E6* and *E7* genes are potential targets of HPV-related cervical malignancy. Previous studies of our lab suggested that deletion of the HPV16 genome using ZFNs disrupted the *E7* gene and restored the Rb protein *in vitro* [16]. Growth inhibition of tumor xenografts in mice was also observed [16]. TALENs were also applied to modulate the *E6* and *E7* genes [17]. Apoptosis induction and growth inhibition were observed in HPV-positive cervical cancer cells. HPV16 E7-targeting TALENs reversed the malignant phenotype of a K14-HPV16 transgenic mouse model [17]. Previous results from our lab showed that CRISPR/Cas9 disrupted the HPV16 genome [14]. HPV16 *E6*-specific CRISPRs increased apoptosis and inhibited growth in HPV16-positive SiHa and Caski cells, whereas HPV16-negative C33A and HEK293 cells were unaffected suggesting high specificity of the CRISPR/Cas9 system [14].

In this study, we used dual sgRNAs-guided CRISPR/Cas9 system to delete the HPV18 *E6* and *E7* genes simultaneously. We obtained a high genome editing rate of 81.8% using Sanger sequencing. Because specificity of the CRISPR/Cas9 system is only determined by the short sgRNA and PAM sequence, off-target effects are major obstacles of the system. To rule out off-target effects, two methods were applied. First, we NCBI-blasted specificity of the two sgRNAs and found no perfect matches to the human genome. Second, SiHa cells were used as controls. We did not observe apoptosis induction or proliferation inhibition in SiHa cells. Therefore, deletion of the HPV18 *E6* and *E7* genes using CRISPR/Cas9 is highly specific. However, off-target effects can't be completely ruled out. Therefore, further optimization of the CRISPR/Cas9 system is required. As reported by Ran *et al*., off-target effects can be alleviated by transforming the Cas9 nuclease into a nickase. The Cas9 endonuclease contains two nuclease domains, the RuvC domain and HNH domain [20]. By mutating one of the nuclease domains to create a D10A site mutation or H840A mutation, DNA nickases can be created [20]. Because nickases only induce single-strand nicks on one DNA strand, not DSBs, paired nickases should be used to produce DSBs. Combining two sgRNAs to create DSBs reduces off-target effects. A catalytically inactive Cas9 fused to a FokI domain can also be used to minimize off target effects [21]. Under this circumstance, only two monomers dimerize can DNA cleavage be initiated, largely extending the recognition sequences.

In summary, we deleted the *E6* and *E7* genes of the HPV18 genome using the CRISPR/Cas9 system in HeLa cells. Growth inhibition was observed by deletion of E6 and E7 proteins in HeLa cells. The results show the possibility of using the CRISPR/Cas9 system to reverse the malignancy of HPV18-infected cervical cancer cells. Thus, deletion of the *E6* and *E7* genes using CRISPR provides us a new therapy to treat HPV18 infectious diseases.

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Disclosure of conflict of interest

None.

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