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The Application of CRISPR/Cas9 System in Cervical Precancerous Lesions

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Abstract

Background: The clustered regularly interspaced short palindromic repeat (CRISPR)/Cas9 system is becoming a promising gene therapy method. Herein, we evaluated the therapeutic effect of CRISPR/Cas9 system in cervical carcinogenesis, especially cervical precancerous lesions.

Methods: In cervical cancer/pre-cancer cell lines, we transfected the CRISPR/Cas9, transcription activator–like effector nuclease (TALEN), and zinc finger nuclease (ZFN) plasmids, respectively. We used the cell apoptosis, cell viability, and colony formation assays to examine the efficiency and specificity of inhibition of cell apoptosis and growth between the different gene editing tools. Western blotting was used to estimate the related protein expression. We used xenograft formation assays to examine the ability of inhibition of cell growth in vivo. In the K14-HPV16 transgenic mice model of HPV-driven cervical carcinogenesis, we investigated the therapeutic effect by vaginal administration.

Results: Compared to ZFN and TALEN, CRISPR/Cas9 has shown comparable efficiency and specificity of inhibition of cell apoptosis and growth in cervical cancer cell lines, which seem to be more pronounced in the S12 cell line derived from the low-grade cervical lesion. In xenograft formation assays, CRISPR/Cas9 could inhibit tumor formation in vivo and affects the expression of the corresponding protein. In the K14-HPV16 transgenic mice, CRISPR/Cas9 treatment caused mutations of the E7 gene and restored the expression of RB, E2F1, and CDK2, thereby reversing the cervical carcinogenesis phenotype.

Conclusion: In this study, we have demonstrated that CRISPR/Cas9 targeting HPV16 E7 could effectively reduce the expression of E7 protein in vitro. Additionally, it could revert the HPV-related cervical carcinogenesis in K14-HPV16 transgenic mice, which has shown great potential in clinical treatment.

Key words: Cervical precancerous lesions; HPV; CRISPR/Cas9; Gene therapy

Background

Cervical cancer is the fourth most common malignancy in women worldwide [1]. Human papillomavirus 16 (HPV16) is the most predominant high-risk HPV type with the highest risk of progression to cervical malignancy [2, 3]. More than 80% of women who have at least one opposite sex partner will acquire HPV infection in their lifetime [4]. High-risk HPV persistent infection has been considered to be a leading cause of cervical carcinogenesis [5]. Researchers also found that the HPV viruses could integrate their genes into the human genome, and it seemed to be a critical event in the progression of carcinogenesis [6]. The integration of HPV leads to persistent expression of the HPV oncogene, making it difficult to eliminate. At present, there is no effective treatment for patients with persistent HPV infection or the integration of HPV genes [7].

The gene editing tools mainly include zinc finger nuclease (ZFN) [8], transcription activator–like effector nuclease (TALEN). and clustered regularly interspaced short palindromic repeat (CRISPR/Cas9). All these gene editing tools could induce targeted DNA double-strand breaks (DSBs) and edit targeted genes by stimulating the DNA repair mechanisms [9]. With the improvements of these gene-editing tools, gene therapy is becoming more precise and effective. In previous studies, our team has demonstrated that these gene editing techniques designed for HPV oncoprotein genes could effectively

influence targeted cells [10, 11]. However, the comparison of the efficacy of gene therapies in HPV infection disease is not identified yet.

For the CRISPR/Cas9 system, researchers only need to design the gRNA complementary to the target DNA sequence, without any other component [8]. The CRISPR/Cas9 system might be an ideal alternative to ZFN and TALEN for inducing targeted gene editing because it is rapid and easy to design. It could cause DSB at the specific site which could be fixed by the cells' self-repairing system in the form of NHEJ (non-homologous end-joining) or HDR (homologous-dependent repair), resulting in gene deletion, reversion, and insertion [9]. Some previous reports have suggested that the CRISPR/Cas9 system targeted HPV oncogene might have a therapeutic effect on HPV-related cervical cancer [12]. However, there has been a scarcity of dynamic observation of the treatment process in a suitable animal model until now.

The high-risk HPV oncogenes E6 and E7 play a key role in the development of carcinogenesis through the interaction with tumor suppressor genes—E6 for TP53 and RB for E7 [13, 14]. HPV 16 E6 targeting TP53 could induce infected cells ceased apoptosis and the transformed cells were able to continually replicate E7 oncoprotein binds to the retinoblastoma family members for degradation, resulting in the release of E2F transcription factors which indirectly promote the replicate of transformed cells [15, 16]. A recent study showed that during the infectious period, the HPV16 genome shared more amino acid-changing variants, while E7 was genetically strictly conserved [17]. This meant

that the gene editing tool targeting the HPV16 E7 oncogene had more clinical significance in the therapy of cervical carcinogenesis.

In this study, we conducted CRISPR/Cas9 targeting HPV16 E7 in vitro and in vivo experiments and compared its efficiency with ZFN and TALEN. The knockout of the E7 oncogene induced cell apoptosis and reduced cell proliferation. It was also found to efficiently revert the HPV-related cervical carcinogenesis in K14-HPV16 transgenic mice. It has shown great potential in clinical treatment.

Materials and Methods

Cell Culture and Transfection

The cervical cancer cell line SiHa was purchased from the American Type Culture Collection (ATCC) and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Gibco) and 100U/ml of penicillin and streptomycin (Invitrogen) at 37℃ in a humidified incubator with 5% CO_2. The S12 cell line was a gift from Pro. Kenneth Raj (Health Protection Agency), and it was permitted by the original owner, Pro. Margaret Stanley. The S12 cells which contained the integrated HPV16 genome was an immortalized human cervical keratinocyte cell line and was cultured in a mixture of DMEM and Ham F12 medium at a ratio of 1:3, which was supplemented with 5% FBS, 8.4 ng/mL of cholera toxin, 5 μg/mL of insulin, 24,3 μg/mL of adenine, 0.5 μg/mL of hydrocortisone and 10 ng/mL of EGF. All the cells were transfected by X-tremeGENE HP DNA Transfection Reagent (Roche) according to the

manufacturer's instructions. The ratio of reagent to DNA was optimized in preliminary experiments. Each experiment was repeated 3 times.

Plasmid

The gRNA targeting HPV16 E7 was designed using the online tool (http://crispor.tefor.net/crispor.py) according to the protocol of Mali et al. [18] in our lab and synthesized by the Genewiz Company [19]. The Cas9 plasmid was obtained from Addgene. The sgRNA sequence targeting HPV16 E7 were provided from our previous study [20]. The sequence of gRNA-HPV16 E7-1 was 5'- GCTGGACAAGCAGAACCGGA-3', and the sequence of gRNA-HPV16 E7-2 was 5'- GAGACAACTGATCTCTACTG-3'. The ZFN (MA13 and MA14) and TALEN (T512) plasmids were from our own laboratory, which was used in primary experiments [10, 11]. We cloned the sgRNAs into the pSpCas9(BB)-2A-GFP (#48138) obtained from Addgene.

T7E1 Assay

After transfection for 48 h, the DNA of cells was extracted using the QIAamp DNA Mini kit. The primers used for amplification are F: tgtcaaaagccactgtgtcc, R: taaaatctaccaaatcttcacctgt, 200ng purified polymerase chain reaction (PCR) products containing the sgRNA targets were denatured and reannealed. 2 units T7E1 enzyme (NEB) were added to the tube and incubated at 37℃ for 30 min. The digested products were viewed in 2% agarose gels, and the gene editing rate was calculated as follows: (1-(1-cleaved bands)1/2)*100.

Western Blot Analysis

After 48 h of transfection of the CRISPR plasmid, the protein of the cells was extracted and quantified. Forty micrograms of total proteins were used in the 1% SDS-PAGE electrophoresis. The primary antibodies used were rabbit anti-HPV16-E7 (1:200, orb10573, Biorbyt), rabbit anti-RB (1:1,000,10048-2-Ig, Proteintech), rabbit anti-CDK2 (1:200,10122-1-AP, Proteintech), rabbit anti-E2F1 (1:200, 12171-1-AP, Proteintech), rabbit anti-GAPDH (1:5,000, 60004-1-Ig, Proteintech).

Immunohistochemistry (IHC) and Immunofluorescence Staining

The xenografts in nude mice and the uterine cervixes and vaginas of the transgenic mice were isolated and fixed by 4% paraformaldehyde. Paraffin-embedded sections (5 μm) were subjected to IHC staining according to the Proteintech protocol (http://www.ptgcn.com/support/protocols/). After antigen retrieval for 30 min and blocking with 3% hydrogen peroxide for 20 min, the paraffin sections were incubated overnight at 4℃ by the following primary antibodies: rabbit anti-HPV16E7 (1:100, orb10573, Biorbyt), rabbit anti-RB (1:200,10048-2-Ig, Proteintech), rabbit anti-CDK2 (1:200, ab6538, Abcam), rabbit anti-E2F1 (1:200, 12171-1-AP, Proteintech), rabbit anti-Ki67 (1:200, ab16667, Abcam), rabbit anti-p16 (1:100, A11337, Abclonal), rabbit anti-PCNA (1:100, 10205-2-AP, Proteintech), rabbit anti-CD31 (1:100, 11265-1-AP, Proteintech), rabbit anti-Caspase-3 (1:100, 19677-1-AP, Proteintech). Next, samples were incubated with proper secondary antibodies for 1 h at room temperature. A 3,3'-diaminobenzidine (DAB) kit was used to detect the

antibodies, and the slide was photographed at random site using the cellSens Dimension (version 1.8.1, Olympus). The staining intensity was measured by ImagePro Plus.

Cell Proliferation Assay

Cell proliferation was determined using Cell Counting Kit-8 (CCK8) according to the manufacturer's instructions. After 24 h of transfection of CRISPR/Cas9 plasmids, cell lines, including S12, SiHa, C33A, and HeLa, were seeded in a 96-well plate with 2 x 10^3 per well. 10 μl CCK8 dye and 90 μl fresh DMEM was added into each well, and the cells were incubated for 3 h. The absorbance at 450 nm was read by a microplate reader.

Apoptosis Assay

After 48 h transfection of CRISPR/Cas9 plasmids, SiHa, C33A, HeLa, and S12 cells were collected and washed 3 times in PBS. Annexin V-FITC apoptosis kits (KenGen Biotech) were used following the manufacturer's protocol, and samples were detected on the FACSCalibur™ (BD Bioscience). The experiments were repeated 3 times.

Colony-Forming Assay

Twelve hours post-transfection, SiHa and S12 cells were digested and washed with PBS, and 200 cells per group were plated into the 6-well plate. After 14-day culture, the cells were stained with 4% crystal violet and scanned. The colony numbers were counted using ImageJ.

Animal Experiments

All animal experiments were approved by the Ethical Committee of Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology. Four-week-old BALB/c-nu mice were purchased from Beijing HFK Bio-technology Co., Ltd. and kept at the Experimental Animal Center, Tongji Medical College, HUST. 5 x 10^6 SiHa cells were resuspended in 100 μl of 1 x PBS and injected subcutaneously in the right flank. When the xenografts reached approximately 100 mm^3, the mice were randomly assigned to 4 groups. A mixture of 10 μg of plasmid and TurboFect in vivo Transfection Reagent (#R0541, Thermo Fisher Scientific) were injected intratumorally every 3 days according to the manufacturer's protocol. The volume of the xenografts was measured and recorded using a digital Vernier caliper every 3 days. Mice were sacrificed 5 weeks after SiHa cell injection, and the tumors were surgically isolated and weighed.

FVB.Cg-Tg(KRT-HPV16)wt1Dh (K14-HPV16) transgenic mice were provided by the National Cancer Institute (NCI) Mouse Repository (Frederick, Maryland, USA). The mice were also housed and bred at the Experimental Animal Center, Tongji Medical College, HUST. The genotyping of the offspring has been described elsewhere in detail. The female K14-HPV16 mice were randomly assigned to different groups when they were 6-8 weeks old. After anesthetization, plasmids complexed with TurboFect were piped into the vagina of the mice, which was washed 3 times with saline. The mice were kept on the electric heating blanket for at least 30 min dorsally. The vagina and other organs

were dissected and fixed for HE or IHC.

Statistical Analysis

All statistical analysis was performed on SPSS 21.0 (SPSS Inc., Chicago, IL, USA) and GraphPad Prism 8 (GraphPad Software, USA). The significance of different groups was determined by a 2-tail student's t test. The results are expressed by mean (±) standard deviation (SD). *p*-values below 0.05 were considered statistically significant.

Results

1：CRISPR/Cas9 system efficiently mediates cleavage of the HPV16 E7 gene in HPV16-positive cells compared to ZFN and TELAN

To compare the ability of specifically induced DNA cleavage among CRISPR/Ca9, ZFN, and TELAN in HPV16 positive cell lines， we used the immunofluorescence (IF) staining of γ-H2AX to detect DSBs. We separately expressed the CRISPR/Cas9, TALEN, and ZFN plasmids in HPV16-positive cancer cell line SiHa and HPV16-positive immortalized cervical epithelial cell line S12. Previous research reported that there were 1-2 copy numbers and 1-3 copy numbers of HPV16 among the SiHa cells and S12 cells, respectively [10]. After treatment, the number of γ-H2AX foci were from 0.1±0.1 (Vector) to 1.67±0.03 (CRISPR/Ca9), 1.59±0.04 (ZFN), and 1.32±0.02 (TALEN) per nucleus in SiHa, and from 0.08±0.07 (Vector) to 1.58±0.04 (CRISPR/Ca9), 1.67±0.06 (ZFN), and 1.46±0.03 (TALEN) per nucleus in S12 ($p < 0.05$, compared with Vector) (Figure 1, a-d).

We used a mismatch sensitive T7 endonuclease I (T7EI) assay to validate the targeted DNA disruption (Figure 1, e-h), and the results of T7EI digestion proved that there were corresponding indel mutations in the HPV16 E7 gene region of cell lines. In the S12 cell line, the frequency of the CRISPR/Cas9-induced indel mutations had the highest efficiency among the 3 tools. In the SiHa cell line, the CRISPR/Cas9 seemed to be more effective than TALEN in mediating DNA disruption, which might show differences in 3 gene editing techniques among the different cancer cell lines. The T7E1 assay could only detect the mismatch sequences of genes. However, it did not have access to validate identical mutant sequences. Hence, the real efficiency of gene editing techniques inducing DNA mutation may be underestimated.

2: Knockdown of HPV16 E7 induced apoptosis of specific HPV-positive cells

To investigate the cell apoptosis induced by gene editing techniques, each 1 *10^6 cells (SiHa, S12, HeLa, and C33A) were transfected with the corresponding plasmid. We found that increased apoptotic fractions by CRISPR/Cas9 among HPV16-positive cell lines SiHa and S12. However, in HeLa and C33A cell lines, the marginal effects of apoptosis were observed. A similar phenomenon of cell apoptosis was also found in ZFN and TALEN conducted HPV16-positive cell lines (Figure 2, a-d).

3: CRISPR/Cas9 mediates specific inhibition of cell growth and colony formation of HPV16 positive cells compared to ZFN and TELAN

To explore whether the decrease of E7 expression could affect the growth of HPV16-positive cells, we transfected CRISPR/Cas9-E7 plasmid into HPV16-positive cell lines SiHa and S12, and used HPV18-positive cell line HeLa and HPV-negative cell line C33A as the control to detect the specific effect of CRISPR/Cas9 on cell growth by CCK8 assay (Figure 2, e-h). The growth of SiHa and S12 cell lines was significantly inhibited for 4 days. However, there was no significant difference between CRISPR/Cas9-treated groups and control groups among the C33A and HeLa cell lines shown in Figure 2 (c-d).

Furthermore, we used the colony-forming assay to investigate the possible negative effect of CRISPR/Cas9 to the specific HPV-positive cells (Figure 2, i-j). After transfecting CRISPR/Cas9 plasmid, we found that the colony formation number of S12 and SiHa cells significantly decreased after 2 weeks, and similar results were obtained in the TALEN- and ZFN-treated groups.

4: CRISPR/Cas9 reduces the expression of HPV16 E7 and recovers the expression of the related protein in HPV16-positive cell lines.

The results of the T7E1 assay and γ-H2AX confirmed that the CRISPR/Cas9 system could successfully induce HPV16 E7 gene cleavage. To compare the ability of specifically induced decreased expression of HPV16 E7 oncoprotein, we transfected CRISPR/Ca9, ZFN, and TALEN in HPV16-positive cell lines. shRNA HPV16 E7 was used as a positive control, and gRNA-GFP+Cas9 was used as a negative control. After 48 h, as observed by Western blotting, the CRISPR/Cas9 system could efficiently reduce the HPV16 E7 expression in

SiHa and S12 cells (Figure 2, k-l). In the S12 cell line, CRISPR/Cas9 was observed to more effectively decrease E7 expression than the other two gene editing tools. In addition, we found that the HPV16 E7 related protein expressions have also be influenced. The expression of RB protein was increased with the decrease of HPV16 E7 expression.

5: CRISPR/Cas9 inhibits tumor formation in vivo and affects the expression of the corresponding protein

To further explore the effect of CRISPR/Cas9-introduced inhibition of tumorigenicity in vivo, we inoculated S12 cells in Balb/c nude mice subcutaneously to form xenografts models. We injected the CRISPR/Cas9 plasmid into the tumor using transfection reagent and measured the size of xenografts every 6 days, and we used the gRNA-GFP+Cas9-treated group as the vector group. During 24 days, we found that the size of the tumors of the 2 CRISPR/Cas9-treated groups (gRNA-E7-1+Cas9/ gRNA-E7-2+Cas9) were significantly smaller, and tumors that formed grew more slowly compared to the blank group and vector group (Figure 3, a-c). There was a statistical difference in the tumor size between the gRNA-E7-1 group and vector group. Next, we performed hematoxylin and eosin (H&E) staining and immunohistochemistry staining on the xenograft tumor sections with HPV16 E7, Caspase-3, CD31, and PCNA antibodies. Compared with blank and vector groups, the 2 CRISPR-treated groups had higher expression of Caspase-3 and lower expression of HPV E7, CD31, and PCNA (Figure 3d-e).

6: Vaginal application of CRISPR/Cas9 induces E7 mutation and the reversal of cervical malignancy in K14-HPV16 transgenic mice

We introduced the HPV16 integrated mice model to further explore the efficacy and dynamic changes in the treatment of the CRISPR/Cas9 system in vivo. The K14-HPV16 mice could spontaneously exhibit different degrees of squamous epithelial hyperplasia in the cervical cervix and vagina, which could be an ideal animal model for evaluating the therapeutic efficacy. We evaluated mRFP plasmid to prove the successful expression of the plasmid in mouse vagina. This showed that the mRFP fluorescence can last at least 6 days. Six days after transfection of mRFP plasmids, the vagina of K14-HPV16 mice was extracted, and the red fluorescence was observed in the frozen section (Figure 4a). To explore the most rational ratio, we tested different plasmid to polymer ratios in mice and decided the highest transfection efficiency ratio by comparing the mRFP expression in exfoliated cervical cells after transfection (Figure 4b).

To observe the dynamic changes during the therapy, we applied the vaginal transfection of gRNA-E7-1+Cas9 in mice every 3 days, and sacrificed mice at the different time points. After 12 days, we found that the expression of HPV16 E7 protein was decreased in cervical epithelia in IHC results. This trend became increasingly obvious over time, and HPV16 E7 expression was almost invisible on the 24th day. At the same time, we also observed the decreased expression of P16 protein in cervical epithelial cells. The H&E results showed that in gRNA-E7-1+Cas9 treated mice the CIN of mice was gradually returned to normal-like

cervical epithelial with the increase of treatment time (Figure 4c).

We assigned the female K14-HPV16 positive mice randomly into 2 groups. The experimental group was treated with gRNA E7-1+Cas9, while the control group was treated with gRNA-GFP+Cas9. FVB background K14-HPV16 negative mice of the same age were kept at the same time. We observed the HPV16 E7 DNA mutation in CRISPR/Cas9 treated mice after 24 days. DNA sequencing of the E7 gene showed deletions and point mutations after receiving treatment of CRISPR/Cas9, which displayed gene editing in this progression and was not observed in the control group. The examples above illustrated that the CRISPR/Cas9 system could induce DNA DSB and be repaired through NHEJ repair way (Figure 4d). After that, we continued to explore the pathological change of these 2 groups. HE staining showed that the gRNA E7-1+Cas9-treated group could reverse the malignant phenotype of cervical epithelia in a gradual way, with normal nuclear and well-differentiated epithelia. IHC staining of HPV16 E7 decreased, with the RB expression restored, which indicated that CRISPR/Cas9 system could inhibit the proliferation of HPV16 expression cells in vivo through the RB signaling pathway. The downstream expression of CDK2 and E2F1 also showed that the CRISPR/Cas9 system could induce cell cycle arrest, which was also illustrated by the downregulated expression of Ki67 (Figure 5a). Quantifications of the protein expression of HPV16 E7, RB, Ki67, E2F1, and CDK2 in these groups were exhibited in Figure 5b. To evaluate potential systemic side effects in

treated mice, we got their organs other than the cervix for immunohistochemical staining and H&E staining at the endpoint. The IHC staining showed that there was no obvious Cas9 protein expression in these organs, and H&E staining showed that the treatment did not induce any significant morphological change in experimental and control groups (Figure 6). In conclusion, the results showed that the CRISPR/Cas9 system could be a promising treatment method for cervical epithelial neoplasia in vivo.

Discussion

At present, biopsies and repeated screenings are generally adopted in clinical practice for patients with persistent HPV infection. Compared with cervical cancer, there are far more people with precancerous lesions, as only a small percentage of people with precancerous lesions develop invasive cancer [21, 22]. For cervical pre-cancer patients who are at a high risk for cervical cancer, surgery is usually recommended. Most previous studies on CRISPR/Cas9 have been focused on cervical cancer rather than precancerous lesions [12, 23, 24]. In our study, we used the S12 cell line derived from the low-grade cervical lesion and K14-HPV16 mice in vivo/in vitro [25, 26], which were representative cervical precancerous models.

For K14-HPV16 mice, previous studies have observed that the premalignant stages of squamous carcinogenesis in the K14-HPV16 mice is highly similar to that of humans [26], making it an ideal model for cervical precancerous lesions. Through in vivo experiments, Hu et al. proved that intravaginally regional

352 application of TALENs is an effective method of administration [10]. Following a
353 period of administration of the CRISPR/Cas9 plasmid in K14-HPV16 mice, we
354 found that the mouse cervical epithelium gradually reversed to histologically
355 normal compared with the control group. Furthermore, we used IHC staining to
356 evaluate the expression of the related proteins. In the CRISPR/Cas9-treated
357 group, we found that the cleavage of the E7 gene resulted in downregulation of
358 E7 protein expression, and the gradual restoration of expression of RB and its
359 downstream targets E2F1 and CDK2, thus reversing the malignant phenotype
360 of the cervix in vivo. Our research indicated that among cervical cancer-related
361 lesions, especially cervical pre-cancerous lesions, CRISPR/Cas9 has
362 promising clinical prospects.

363 CRISPR/Cas9 has been considered to have potential advantages for many
364 chronic pathogenic diseases caused by DNA viruses, which cannot be cured
365 using available drugs [27-29]. Compared with ZFN and TALEN, we observed
366 that CRISPR/Cas9 also has significant growth inhibitory and apoptotic effects
367 on cervical cancer and cervical pre-cancer cell lines and inhibited the tumor
368 formation of the S12 cell line in nude mice. In fact, the target sites of these 3
369 gene editing tools were not exactly the same point; thus, the comparison
370 between them also has its limits. However, the incomparably fast design
371 process, high scalability, and affordability make CRISPR/Cas9 an ideal gene
372 editing tool compared with ZFN and TALEN.

The off-target effect and safety of CRISPR/Cas9 has always been the focus of our concern. Through in vivo and in vitro experiments, we observed that CRISPR/Cas9 could cause significant suppression of colony formation and cell growth and apoptosis of HPV16-positive cell lines S12 and SiHa. In contrast, CRISPR/Cas9 did not affect the growth of HPV18-positive cell line HeLa and HPV-negative C33A cells, demonstrating the specificity of HPV16-E7-targeted CRISPR/Cas9. Also, in the past, the systemic injection was used in the application of CRISPR/Cas9, which might increase the risk of side effects. In our study, the administration of CRISPR/Cas9 was concentrated on the targeted site to ensure the therapeutic usefulness and lowest systemic side effects.

A recent study showed that during the infectious period, the HPV16 genome shared many amino acid-changing variants, while E7 protein was genetically conserved [17]. In addition, HPV16 E7 is considered to be a single oncoprotein that could cause cervical cancer in the animal model and immortalize human keratinocytes in vitro [30, 31]. This made HPV16 E7 an ideal target for the therapy of HPV16-induced cervical cancer, which means that our CRISPR/Cas9 system targeted for HPV16 E7 might have promising clinical applications. In addition, the HPV16 E6 gene is also a good candidate cleavage site for gene therapy, which has obtained promising results in vitro and in nude mouse models [8, 12]. We have also considered expanding the CRISPR/Cas9 cleavage sites of HPV16 in a follow-up study.

This study still has some limitations. Our goal was to apply the designed HPV16 E7-targeted CRISPR/Cas9 system to clinical patients, but many factors still need to be taken into consideration, such as the vaginal fluid and pH value, which need to be confirmed by further experiments. In addition, the comprehensive analysis of host immune response and assessment of long-term effects were insufficient in our study. Three kinds of gene editing tools target different HPV16 E7 cleavage sites, and further experiments are needed to confirm their therapeutic effects. In this experiment, we focused on the HPV16 E7 protein, which may not explore the impact of other cleavage sites of HPV16. In future studies, we also hope to continue to design CRISPR/Cas9 systems for other cutting sites of HPV and explore their effect.

In conclusion, we have demonstrated that CRISPR/Cas9 targeting HPV16 E7 can effectively reduce the expression of E7 protein in vivo/in vitro and have potential treatment effects on HPV-related cervical cancer and precancerous lesions.

Figure legend

Fig. 1 Efficacies comparison of ZFN, TALEN, and CRISPR/Cas9 on HPV16-positive cell lines

The representative images of γ-H2AX foci (green signals) in ZFN-, TALEN-, and CRISPR/Cas9-treated S12 cells (**a**) and SiHa cells (**b**). Etoposide (0.25 μM) was used as the positive control, and vector plasmid was used as the negative control. Quantification of γ-H2AX foci in S12 (**c**) and SiHa (**d**). T7 endonuclease

1 (T7E1) assay of ZFN-, TALEN-, and CRISPR/Cas9-induced cleavage at 48 hours in S12 cells (**e**) and SiHa cells (**f**). Quantification of DNA indel rate in S12 (**g**) and SiHa (**h**). ns, no significance; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$. (n = 3 replications) Scale bars: 50 μm.

Fig. 2 ZFN, TALEN, and CRISPR/Cas9 induced cell growth deficit and cell apoptosis in vitro

(**a**-**d**) Growth curves of ZFN, TALEN, and CRISPR/Cas9-treated S12 (a), SiHa (**b**), C33A (**c**), and HeLa (**d**) cells were constructed using the CCK-8 assay. (**e**-**h**) Apoptosis rate of S12 (**e**), SiHa (**f**), C33A (**g**), and HeLa (**h**) cells 48 h after treatment with ZFN, TALEN, and gRNA-E7-1+Cas9 plasmids. (**i**) The colony-forming assay of SiHa and S12 cells after treatment with ZFN, TALEN, and gRNA-E7-1+Cas9 plasmids. (**j**) Quantification of number of colonies in S12 and SiHa cells of different treatment groups. (**k**-**l**) HPV16 E7 and RB expression of S12 (**k**) and SiHa (**l**) cells 48 h after treatment with ZFN, TALEN, and gRNA-E7-1+Cas9 plasmids. ns, no significance; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$. (n = 3 replications)

Fig. 3 CRISPR/Cas9 inhibit S12 cell growth in vivo.

Balb/c-nu mice were injected subcutaneously in the right flanks with 5×10^6 of S12 cells. Then, the CRISPR/Cas9 plasmids complexed with in vivo transfection reagents were injected intratumorally when the xenografts reached approximately 50 mm^3. (**a**) The xenografts were measured every 6 days after treatment with gRNA-E7-1+Cas9, gRNA-E7-2+Cas9, gRNA-GFP+Cas9, and

PBS. (**b**) The photograph of S12 xenografts in different treatment groups. (**c**) The estimated tumor size of S12 xenografts in different treatment groups. (**d**) Representative pictures of HE staining and IHC staining of HPV16 E7, Caspase-3, CD31 and PCNA in gRNA-E7-1+Cas9, gRNA-E7-2+Cas9, gRNA-GFP+Cas9, and PBS treated S12 xenografts. Scale bars: 50 μm**.** (**e**) The average necrosis area and protein expression of HPV16 E7, Caspase-3, CD31, and PCNA in different groups. **, $p<0.01$. (n = 4 replications)

Fig. 4 Establishment and application of CRISPR/Cas9 system in K14-HPV16 transgenic mice.

(**a**)The expression of mRFP was localized in the cervical epithelia of transgenic mice. Scale bars: 50 μm. (**b**) Transfection efficiency was optimized at the DNA-to-polymer ratio of 10 μg:1.0 μl, 10 μg:1.2 μl and 10 μg:1.5 μl. The exfoliation of cervical cells was collected at 2, 4, and 6 days after vaginal transfection. (**c**) Representative HE staining and IHC staining of HPV16 E7 and p16 of gRNA-E7-1+Cas9 treated K14-HPV16 transgenic mice at days 0, , 18, and 24. N=3, Scale bars: 50 μm. (**d**) The cervical DNA sequencing of gRNA-E7-1 targeted region of HPV16 E7 gene in gRNA-E7-1+Cas9 treated K14-HPV16 transgenic mice.

Fig. 5 Histopathological and protein expression changes in cervical epithelia of K14-HPV16 transgenic mice treated with HPV16 E7 targeting CRISPR/Cas9.

(**a**) Representative images of the HE staining and IHC staining of HPV16 E7,

RB, Ki67, E2F1, and CDK2 in cervical epithelia of HPV-, gRNA-GFP+Cas9-, and gRNA-E7-1+Cas9-treated mice. (**b**) Quantification of the protein expression of HPV16 E7, RB, Ki67, E2F1, and CDK2 in these 3 groups. **, $p<0.01$; ***, $p<0.001$. (n = 3 replications) Scale bars: 50 μm.

Fig. 6 The conduction of regional plasmid transfection showed no influence on other organs.

The HE staining of different organs in gRNA-E7-1+Cas9 and gRNA-GFP+Cas9-treated K14-HPV16 transgenic mice. Scale bars: 50 μm.

Abbreviations

CRISPR/Cas9: the clustered regularly interspaced short palindromic repeat (CRISPR)/Cas9; TALEN: transcription activator–like effector nuclease; ZFN: zinc finger nuclease; HPV: human papilloma virus.

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Authors' contributions

*These authors contributed equally to this work.

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Availability of data and materials

All data analyzed in this study are included in this article.

Ethics approval and consent to participate

The animal experiments received approval of the Animal Research Ethics Committee of Tongji Hospital and were conducted in accordance with standard operation guidelines.

Consent for publication

Not applicable.

Competing interests

The authors declare no conflict of interest.

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References

1. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A: Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin 2018, 68(6):394-424.

2. Vintermyr OK, Andersland MS, Bjorge T, Skar R, Iversen OE, Nygard M, Haugland HK: Human papillomavirus type specific risk of progression and remission during long-term follow-up of equivocal and low-grade HPV-positive cervical smears. Int J Cancer 2018, 143(4):851-860.

3. Wang SS, Zuna RE, Wentzensen N, Dunn ST, Sherman ME, Gold MA, Schiffman M, Wacholder S, Allen RA, Block I et al: Human papillomavirus cofactors by disease progression and human papillomavirus types in the study to understand cervical cancer early endpoints and determinants. Cancer Epidemiol Biomarkers Prev 2009, 18(1):113-120.

4. Chesson HW, Dunne EF, Hariri S, Markowitz LE: The estimated lifetime probability of acquiring human papillomavirus in the United States. Sex Transm Dis 2014, 41(11):660-664.

5. Kjaer SK, Frederiksen K, Munk C, Iftner T: Long-term absolute risk of cervical intraepithelial neoplasia grade 3 or worse following human papillomavirus infection: role of persistence. J Natl Cancer Inst 2010, 102(19):1478-1488.

6. Kadaja M, Sumerina A, Verst T, Ojarand M, Ustav E, Ustav M: Genomic instability of the host cell induced by the human papillomavirus replication machinery. EMBO J 2007, 26(8):2180-2191.

7. Sudenga SL, Shrestha S: Key considerations and current perspectives of epidemiological studies on human papillomavirus persistence, the intermediate phenotype to cervical cancer. Int J Infect Dis 2013,

17(4):e216-220.

8. Chen T, Wu D, Chen H, Yan W, Yang D, Chen G, Ma K, Xu D, Yu H, Wang H et al: Clinical characteristics of 113 deceased patients with coronavirus disease 2019: retrospective study. BMJ 2020, 368:m1091.

9. Gaj T, Gersbach CA, Barbas CF, 3rd: ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering. Trends Biotechnol 2013, 31(7):397-405.

10. Hu Z, Ding W, Zhu D, Yu L, Jiang X, Wang X, Zhang C, Wang L, Ji T, Liu D et al: TALEN-mediated targeting of HPV oncogenes ameliorates HPV-related cervical malignancy. J Clin Invest 2015, 125(1):425-436.

11. Ding W, Hu Z, Zhu D, Jiang X, Yu L, Wang X, Zhang C, Wang L, Ji T, Li K et al: Zinc finger nucleases targeting the human papillomavirus E7 oncogene induce E7 disruption and a transformed phenotype in HPV16/18-positive cervical cancer cells. Clin Cancer Res 2014, 20(24):6495-6503.

12. Zhen S, Hua L, Takahashi Y, Narita S, Liu YH, Li Y: In vitro and in vivo growth suppression of human papillomavirus 16-positive cervical cancer cells by CRISPR/Cas9. Biochem Biophys Res Commun 2014, 450(4):1422-1426.

13. zur Hausen H: Papillomaviruses and cancer: from basic studies to clinical application. Nat Rev Cancer 2002, 2(5):342-350.

14. Narisawa-Saito M, Kiyono T: Basic mechanisms of high-risk human

papillomavirus-induced carcinogenesis: roles of E6 and E7 proteins. Cancer Sci 2007, 98(10):1505-1511.

15. Arroyo M, Bagchi S, Raychaudhuri P: Association of the human papillomavirus type 16 E7 protein with the S-phase-specific E2F-cyclin A complex. Mol Cell Biol 1993, 13(10):6537-6546.

16. Spangle JM, Munger K: The HPV16 E6 oncoprotein causes prolonged receptor protein tyrosine kinase signaling and enhances internalization of phosphorylated receptor species. PLoS Pathog 2013, 9(3):e1003237.

17. Mirabello L, Yeager M, Yu K, Clifford GM, Xiao Y, Zhu B, Cullen M, Boland JF, Wentzensen N, Nelson CW et al: HPV16 E7 Genetic Conservation Is Critical to Carcinogenesis. Cell 2017, 170(6):1164-1174 e1166.

18. Mali P, Yang L, Esvelt KM, Aach J, Guell M, DiCarlo JE, Norville JE, Church GM: RNA-guided human genome engineering via Cas9. Science 2013, 339(6121):823-826.

19. Zhu N, Zhang D, Wang W, Li X, Yang B, Song J, Zhao X, Huang B, Shi W, Lu R et al: A Novel Coronavirus from Patients with Pneumonia in China, 2019. N Engl J Med 2020, 382(8):727-733.

20. Hu Z, Yu L, Zhu D, Ding W, Wang X, Zhang C, Wang L, Jiang X, Shen H, He D et al: Disruption of HPV16-E7 by CRISPR/Cas system induces apoptosis and growth inhibition in HPV16 positive human cervical cancer cells. Biomed Res Int 2014, 2014:612823.

21. Kulasingam SL, Hughes JP, Kiviat NB, Mao C, Weiss NS, Kuypers JM, Koutsky LA: Evaluation of human papillomavirus testing in primary screening for cervical abnormalities: comparison of sensitivity, specificity, and frequency of referral. JAMA 2002, 288(14):1749-1757.

22. Dalstein V, Riethmuller D, Pretet JL, Le Bail Carval K, Sautiere JL, Carbillet JP, Kantelip B, Schaal JP, Mougin C: Persistence and load of high-risk HPV are predictors for development of high-grade cervical lesions: a longitudinal French cohort study. Int J Cancer 2003, 106(3):396-403.

23. Yoshiba T, Saga Y, Urabe M, Uchibori R, Matsubara S, Fujiwara H, Mizukami H: CRISPR/Cas9-mediated cervical cancer treatment targeting human papillomavirus E6. Oncol Lett 2019, 17(2):2197-2206.

24. Kennedy EM, Kornepati AV, Goldstein M, Bogerd HP, Poling BC, Whisnant AW, Kastan MB, Cullen BR: Inactivation of the human papillomavirus E6 or E7 gene in cervical carcinoma cells by using a bacterial CRISPR/Cas RNA-guided endonuclease. J Virol 2014, 88(20):11965-11972.

25. Arians N, Nicolay NH, Brons S, Koerber SA, Jaschke C, Vercruysse M, Daffinger S, Ruhle A, Debus J, Lindel K: Carbon-ion irradiation overcomes HPV-integration/E2 gene-disruption induced radioresistance of cervical keratinocytes. J Radiat Res 2019, 60(5):564-572.

26. Smith-McCune K, Zhu YH, Hanahan D, Arbeit J: Cross-species

593 comparison of angiogenesis during the premalignant stages of
594 squamous carcinogenesis in the human cervix and K14-HPV16
595 transgenic mice. Cancer Res 1997, 57(7):1294-1300.
596 27. Kennedy EM, Cullen BR: Gene Editing: A New Tool for Viral Disease.
597 Annu Rev Med 2017, 68:401-411.
598 28. Li H, Sheng C, Liu H, Wang S, Zhao J, Yang L, Jia L, Li P, Wang L, Xie
599 J et al: Inhibition of HBV Expression in HBV Transgenic Mice Using AAV-
600 Delivered CRISPR-SaCas9. Front Immunol 2018, 9:2080.
601 29. Lin SR, Yang HC, Kuo YT, Liu CJ, Yang TY, Sung KC, Lin YY, Wang HY,
602 Wang CC, Shen YC et al: The CRISPR/Cas9 System Facilitates
603 Clearance of the Intrahepatic HBV Templates In Vivo. Mol Ther Nucleic
604 Acids 2014, 3:e186.
605 30. Halbert CL, Demers GW, Galloway DA: The E7 gene of human
606 papillomavirus type 16 is sufficient for immortalization of human
607 epithelial cells. J Virol 1991, 65(1):473-478.
608 31. Arbeit JM, Howley PM, Hanahan D: Chronic estrogen-induced cervical
609 and vaginal squamous carcinogenesis in human papillomavirus type 16
610 transgenic mice. Proc Natl Acad Sci U S A 1996, 93(7):2930-2935.
611

Figures

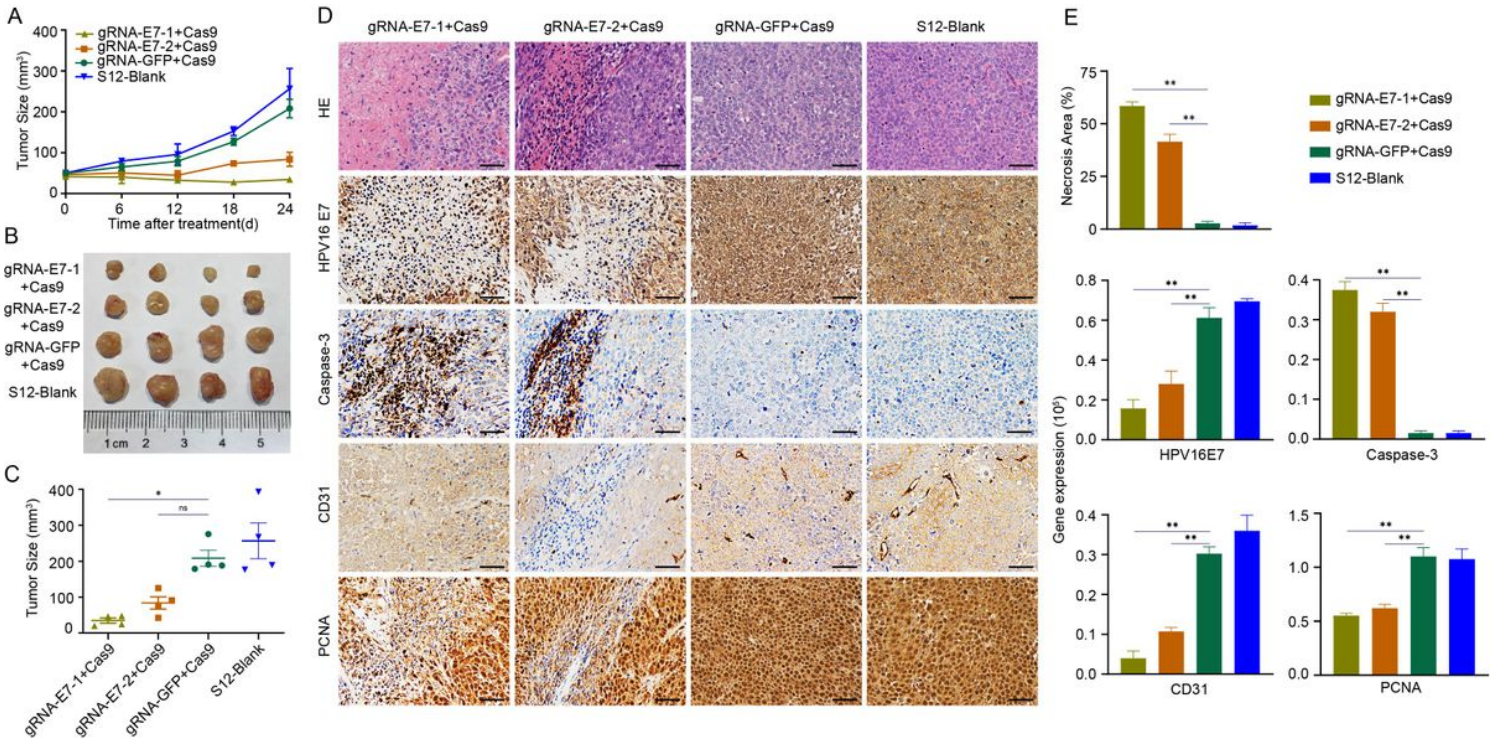


Figure 1

CRISPR/Cas9 inhibit S12 cell growth in vivo. Balb/c-nu mice were injected subcutaneously in the right flanks with 5×10^6 of S12 cells. Then, the CRISPR/Cas9 plasmids complexed with in vivo transfection reagents were injected intratumorally when the xenografts reached approximately 50 mm^3. (a) The xenografts were measured every 6 days after treatment with gRNA-E7-1+Cas9, gRNA-E7-2+Cas9, gRNA-GFP+Cas9, and PBS. (b) The photograph of S12 xenografts in different treatment groups. (c) The estimated tumor size of S12 xenografts in different treatment groups. (d) Representative pictures of HE staining and IHC staining of HPV16 E7, Caspase-3, CD31 and PCNA in gRNA-E7-1+Cas9, gRNA-E7-2+Cas9, gRNA- GFP+Cas9, and PBS treated S12 xenografts. Scale bars: 50 μm. (e) The average necrosis area and protein expression of HPV16 E7, Caspase-3, CD31, and PCNA in different groups. **, p<0.01. (n = 4 replications)

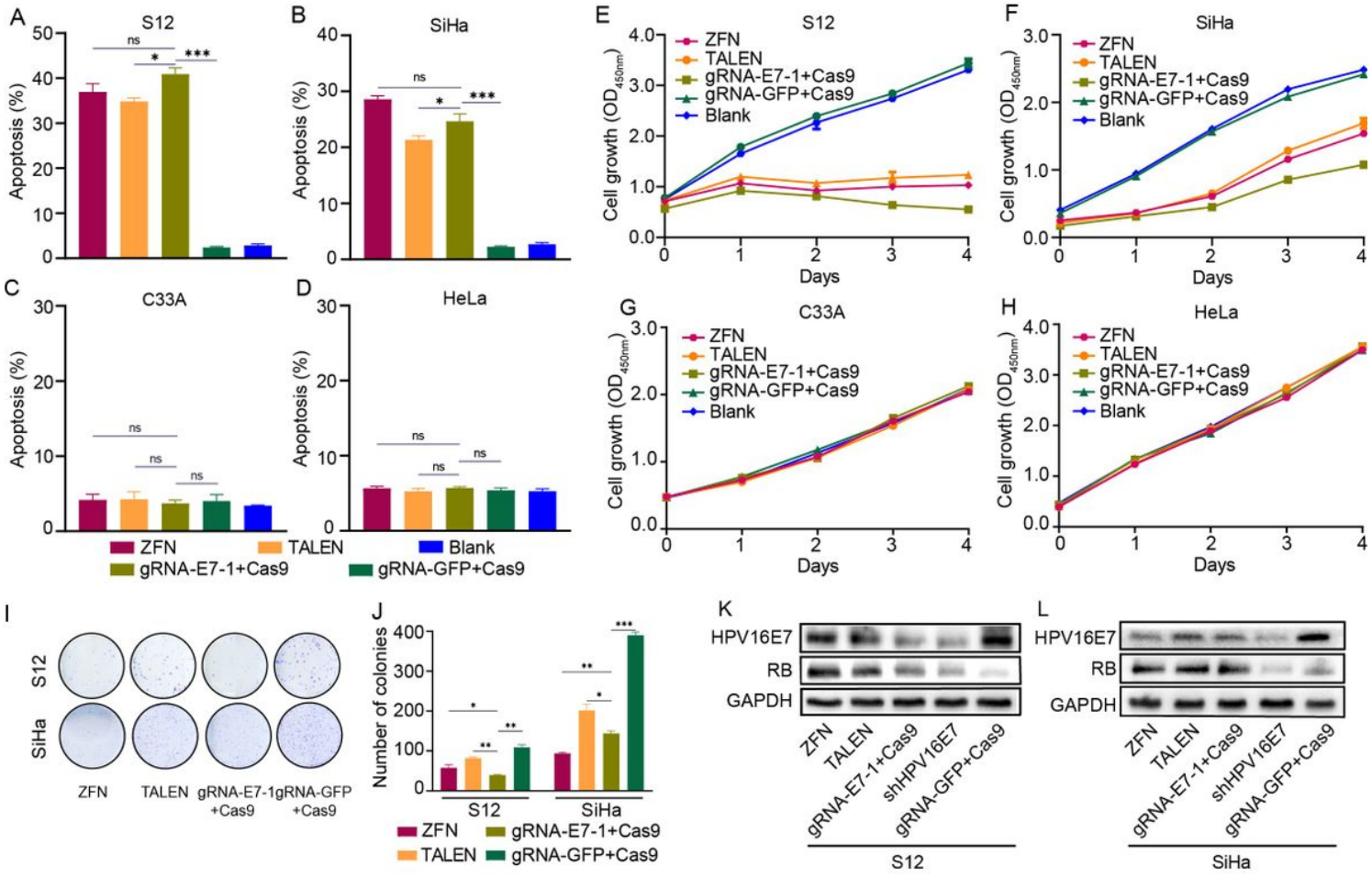
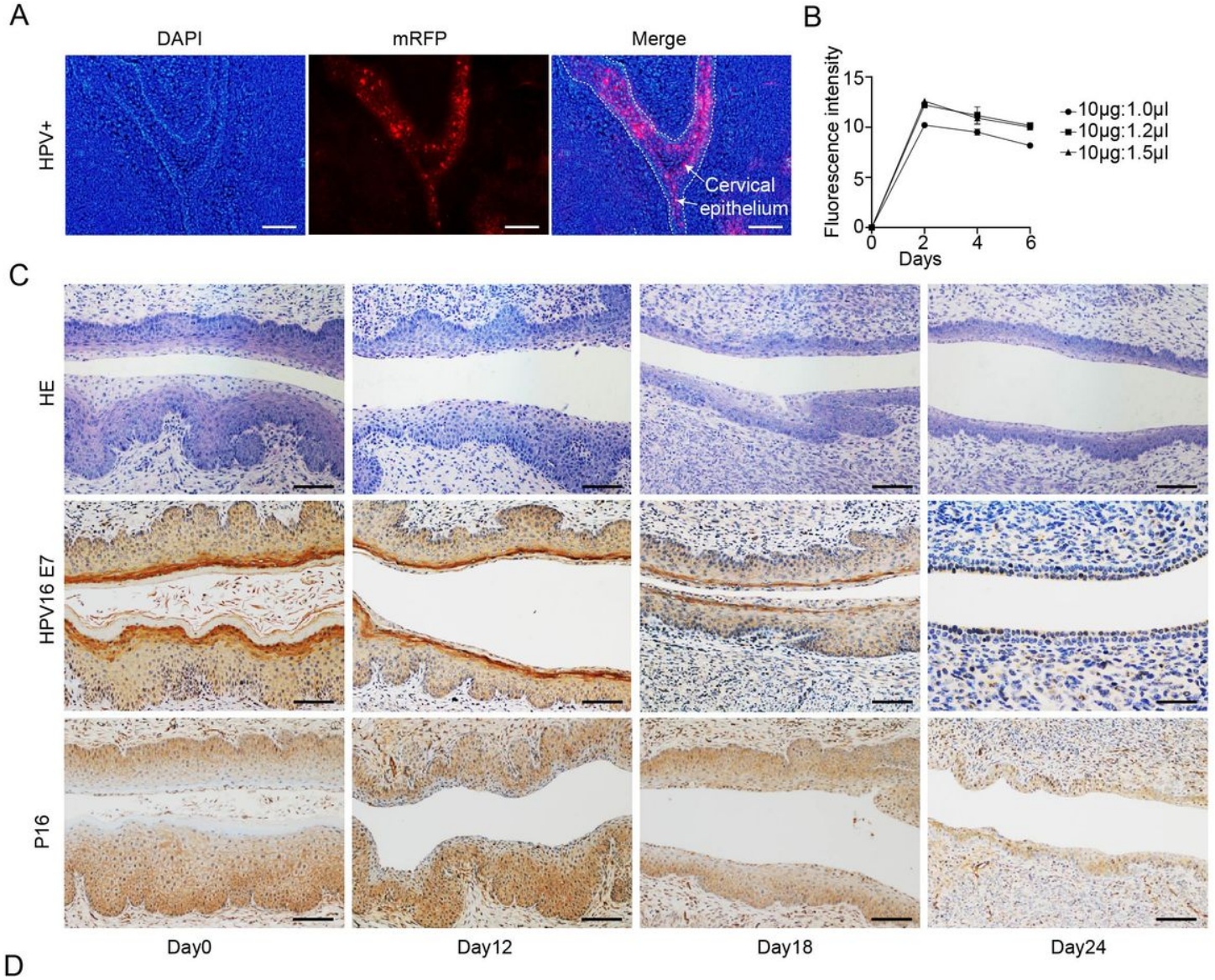


Figure 2

ZFN, TALEN, and CRISPR/Cas9 induced cell growth deficit and cell apoptosis in vitro (a-d) Growth curves of ZFN, TALEN, and CRISPR/Cas9-treated S12 (a), SiHa (b), C33A (c), and HeLa (d) cells were constructed using the CCK-8 assay. (e- h) Apoptosis rate of S12 (e), SiHa (f), C33A (g), and HeLa (h) cells 48 h after treatment with ZFN, TALEN, and gRNA-E7-1+Cas9 plasmids. (i) The colony- forming assay of SiHa and S12 cells after treatment with ZFN, TALEN, and gRNA-E7-1+Cas9 plasmids. (j) Quantification of number of colonies in S12 and SiHa cells of different treatment groups. (k-l) HPV16 E7 and RB expression of S12 (k) and SiHa (l) cells 48 h after treatment with ZFN, TALEN, and gRNA- E7-1+Cas9 plasmids. ns, no significance; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$. (n = 3 replications)



D

WT:

PAM gRNA-E7-1

GGTCCAGCTGGACAAGCAGAACCGGACAGAGCCCATTACAATATTGTAACCTTTTGTTGCAAGTGTGACTCTACGCTTCGGTTGTGC

Sequence of CRISPR/Cas9 treated mice:

GGTCCAGCTCGTAGAGTCACACTTGCAACAAAAGGTTACAATATTGTAATGGGCTCTGTCCGGTTCTGCTTGTCCCTTCGGTTGTGC
GGTCCAGCT---------- CTTCGGTTGTGC
GGTCCAGCT---------- TCGGTTGTGC
GGTCCAGCTCGCAGAGTCACACTTGCAACAAAAGGTTACAATATTGTAATGGGCTCTGTCCGGTTCTGCTTGTCCCTTCGGTTGTGC
GGTCCAGCTGGTAGAGTCACACTTGCAACAAAAGGTTACAATATTGTAATGGGCTCTGTCCGGTTCTGCTTGTCCCTTCGGTTGTGC
GGTCCAGCT---- TAGAGTCACACTTGCAACAAAAGGTTACAATATTGTAATGGGCTCTGTCCGGTTCTGCTTGTCCCTTCGGTTGTGC

Figure 3

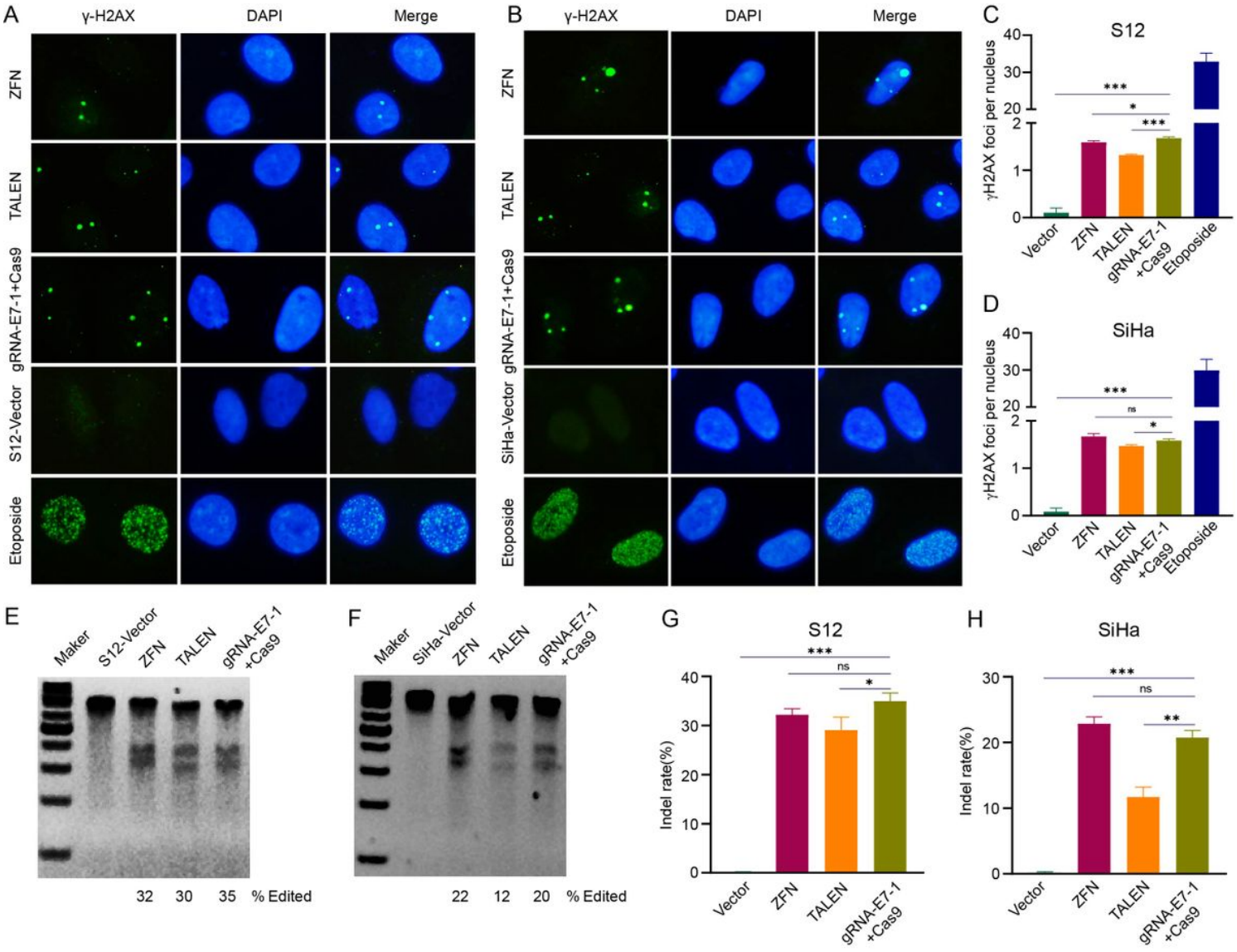


Figure 4

Efficacies comparison of ZFN, TALEN, and CRISPR/Cas9 on HPV16- positive cell lines The representative images of γ-H2AX foci (green signals) in ZFN-, TALEN-, and CRISPR/Cas9-treated S12 cells (a) and SiHa cells (b). Etoposide (0.25 μM) was used as the positive control, and vector plasmid was used as the negative control. Quantification of γ-H2AX foci in S12 (c) and SiHa (d). T7 endonuclease 1 (T7E1) assay of ZFN-, TALEN-, and CRISPR/Cas9-induced cleavage at 48 hours in S12 cells (e) and SiHa cells (f). Quantification of DNA indel rate in S12 (g) and SiHa (h). ns, no significance; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$. (n = 3 replications) Scale bars: 50 μm.

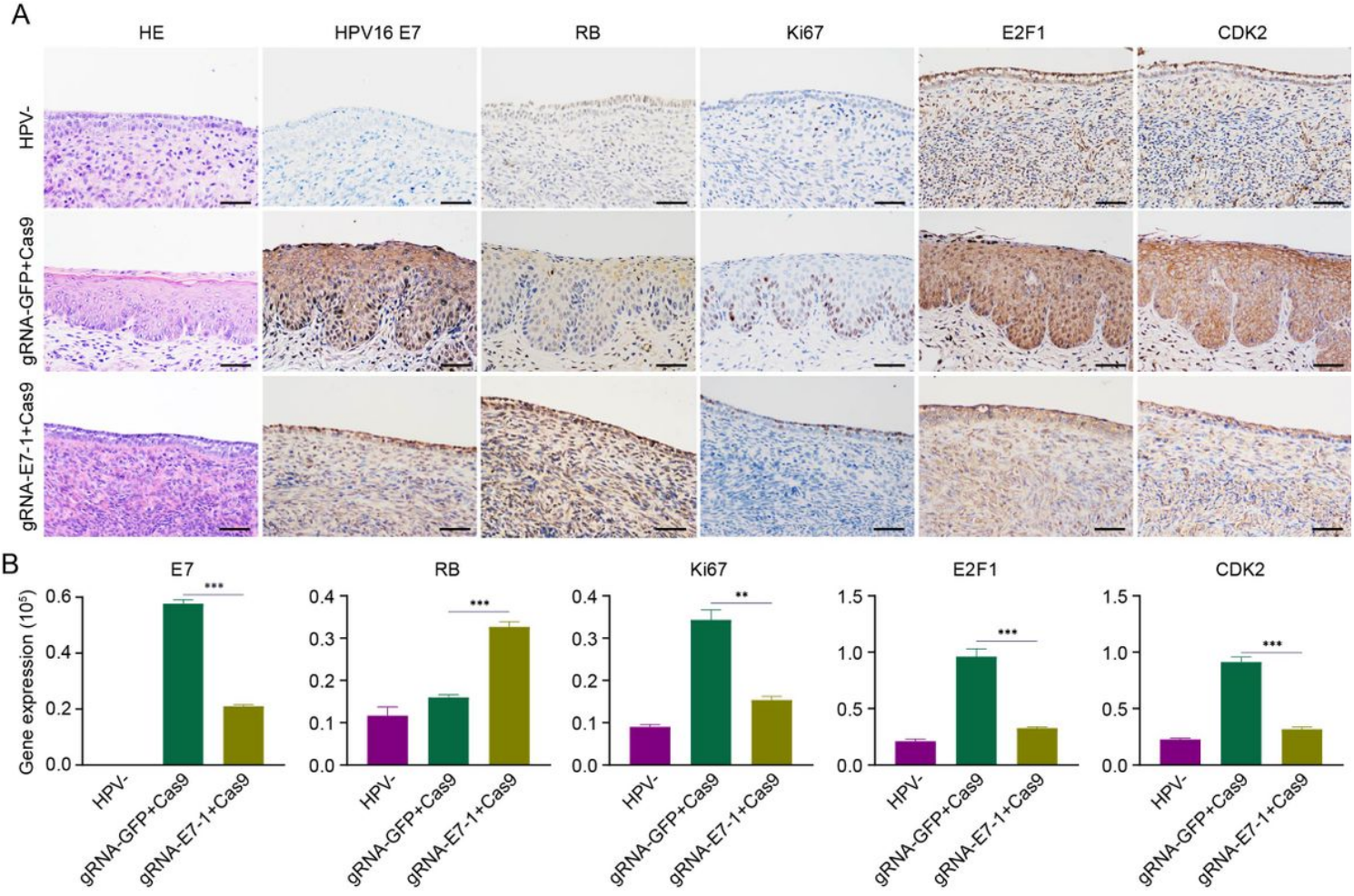

A
HE
HPV16 E7
RB
Ki67
E2F1
CDK2
HPV-
gRNA-GFP+Cas9
gRNA-E7-1+Cas9
B
E7
RB
Ki67
E2F1
CDK2
Gene expression (10⁵)
HPV-
gRNA-GFP+Cas9
gRNA-E7-1+Cas9

Figure 5

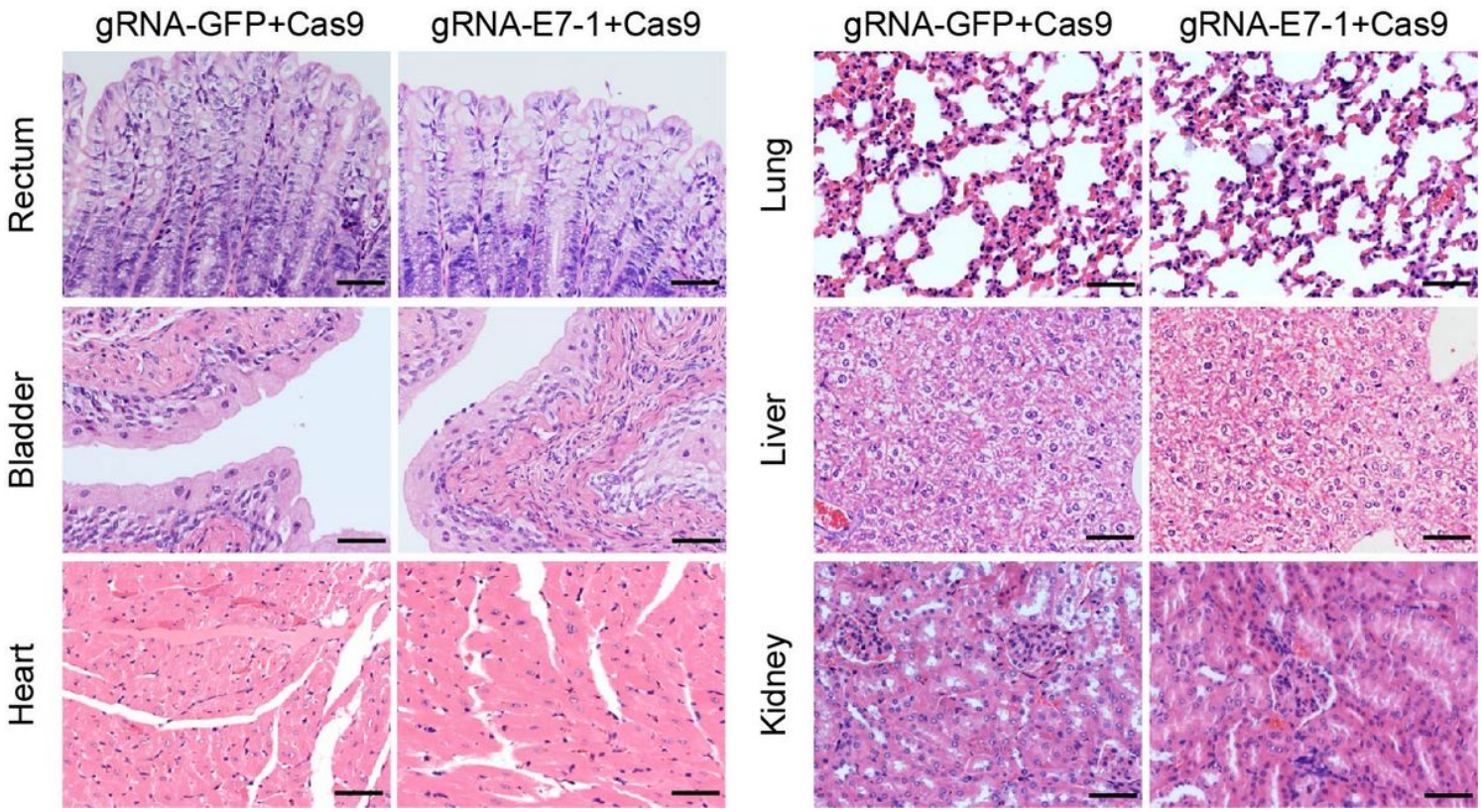

gRNA-GFP+Cas9
gRNA-E7-1+Cas9
Rectum
Bladder
Heart
Lung
Liver
Kidney

Figure 6

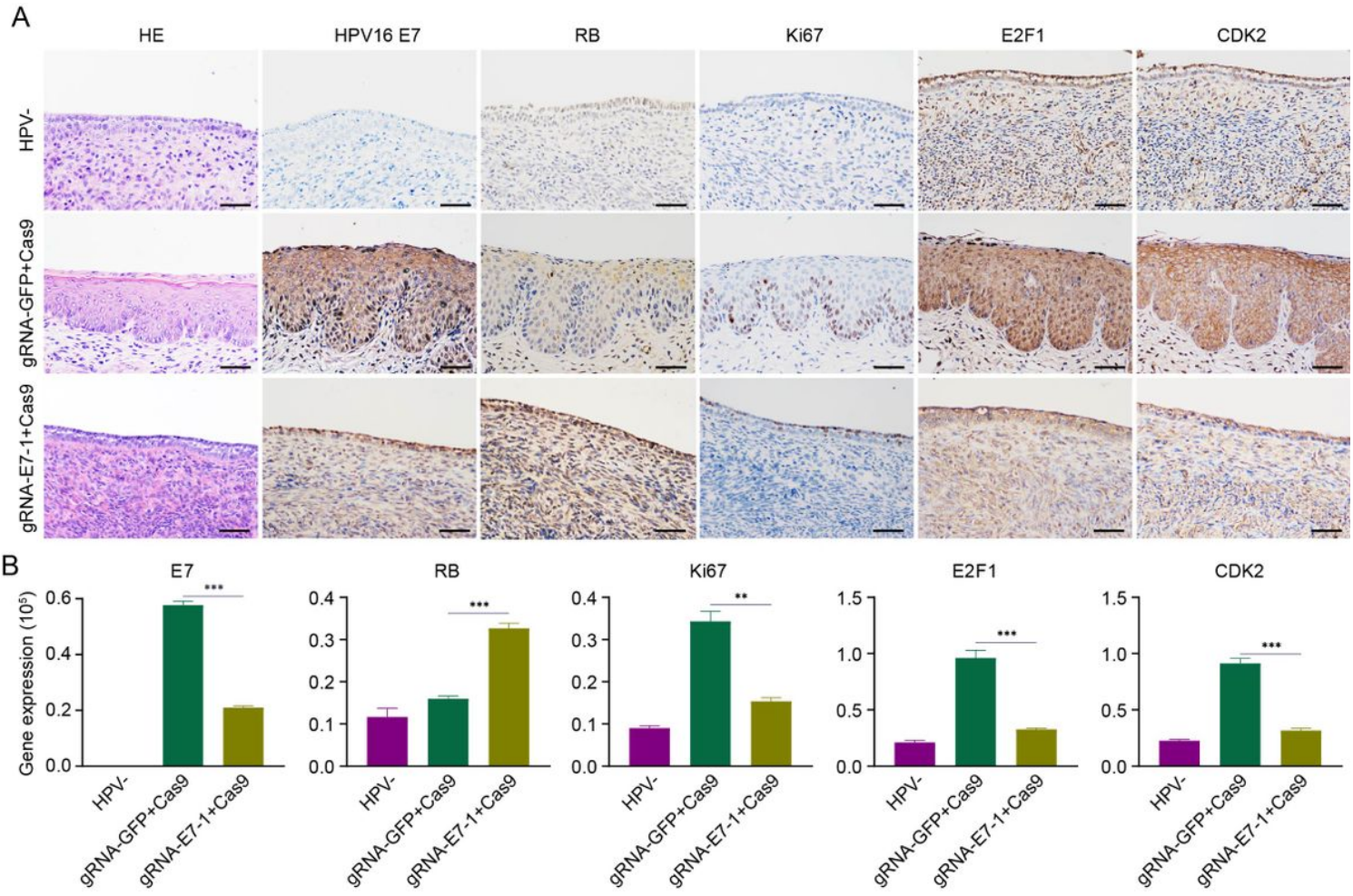


Figure 7

Histopathological and protein expression changes in cervical epithelia of K14-HPV16 transgenic mice treated with HPV16 E7 targeting CRISPR/Cas9. (a) Representative images of the HE staining and IHC staining of HPV16 E7, RB, Ki67, E2F1, and CDK2 in cervical epithelia of HPV-, gRNA-GFP+Cas9-, and gRNA-E7-1+Cas9-treated mice. (b) Quantification of the protein expression of HPV16 E7, RB, Ki67, E2F1, and CDK2 in these 3 groups. **, $p<0.01$; ***, $p<0.001$. (n = 3 replications) Scale bars: 50 μm.

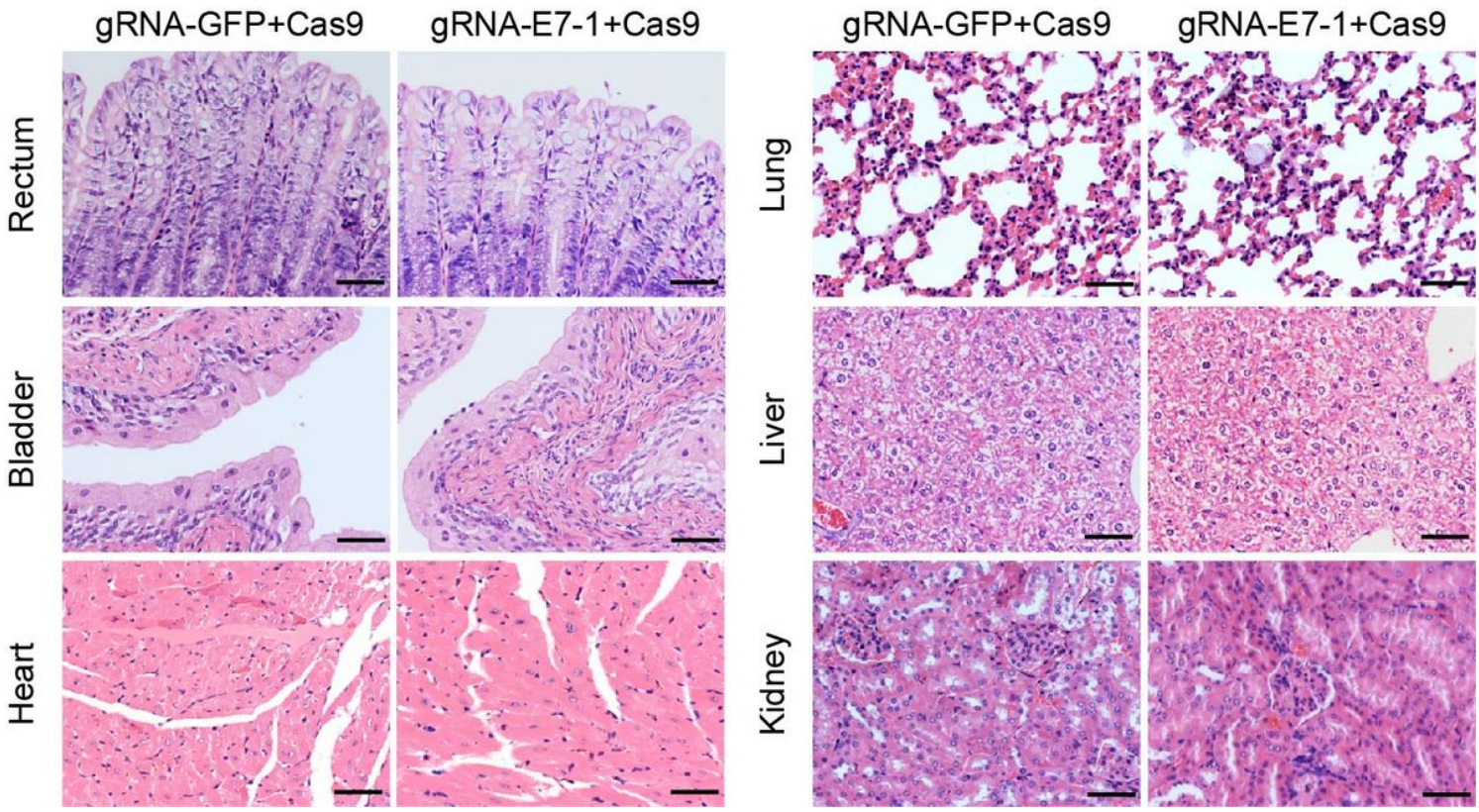


Figure 8

The conduction of regional plasmid transfection showed no influence on other organs. The HE staining of different organs in gRNA-E7-1+Cas9 and gRNA- GFP+Cas9-treated K14-HPV16 transgenic mice. Scale bars: 50 μm.