Cancer Therapy: Preclinical

Zinc Finger Nucleases Targeting the Human Papillomavirus E7 Oncogene Induce E7 Disruption and a Transformed Phenotype in HPV16/18-Positive Cervical Cancer Cells

Wencheng Ding\textsuperscript{1, 2}, Zheng Hu\textsuperscript{1}, Da Zhu\textsuperscript{1}, Xiaohui Jiang\textsuperscript{1}, Lan Yu\textsuperscript{1}, Xiaoli Wang\textsuperscript{1}, Changlin Zhang\textsuperscript{1}, Liming Wang\textsuperscript{1}, Teng Ji\textsuperscript{1}, Kezhen Li\textsuperscript{1}, Dan He\textsuperscript{2}, Xi Xia\textsuperscript{3}, Dan Liu\textsuperscript{1}, Jianfeng Zhou\textsuperscript{4}, Ding Ma\textsuperscript{1}, and Hui Wang\textsuperscript{1}

Abstract

\textbf{Purpose:} Cervical cancer is mainly caused by infections of high-risk human papillomavirus (HR-HPV). Persistent expression of HR-HPV oncogenes E6 and E7 is implicated in malignant transformation. The aim was to provide proof-of-concept data to support use of zinc finger nucleases (ZFN) targeting HPV E7 to treat HPV-related cervical cancer.

\textbf{Experimental Design:} We designed and constructed ZFNs that could specifically recognize and cleave HPV16/18 E7 DNA. We tested the cleavage efficiency of selected ZFN16-E7-S2 and ZFN18-E7-S2 by using single-strand annealing (SSA) assay. Cell viability and colony formation assays were used to estimate the inhibition of cell growth that received treatments of ZFNs. Gene disruption of HPV E7 and downstream genes were examined by Western blotting. Cell apoptosis assay was used to test the specificity and efficiency of induction of HPV type-specific apoptosis. We also introduced xenograft formation assays to estimate the potential of inhibition of HPV-related disease.

\textbf{Results:} We found ZFN16-E7-S2 and ZFN18-E7-S2 disrupted HPV E7 oncogenes in HPV16/18-positive cervical cancer cells. Both ZFNs effectively led to inhibition of type-specific cervical cancer cell growth, and specifically induced apoptosis of corresponding HPV16- and HPV18-positive cervical cancer cell lines. ZFN16-E7-S2 and ZFN18-E7-S2 also repressed xenograft formation \textit{in vivo}.

\textbf{Conclusion:} ZFNs targeting HPV16/18 E7 could effectively induce disruption of E7 oncogenes and lead to type-specific and efficient growth inhibition and apoptosis of HPV-positive cells. ZFNs targeting HPV16/18 E7 oncogenes could be used as novel therapeutic agents for the treatment of HPV-related cervical cancer. Clin Cancer Res; 20(24); 6495–503. ©2014 AACR.

Introduction

Cervical cancer is the third most commonly diagnosed cancer in women worldwide \textsuperscript{(1)}. Persistent infection with high-risk human papillomavirus (HR-HPV) is considered the main etiologic factor for cervical cancers \textsuperscript{(2)}. The malignant transformation abilities of HR-HPVs, especially HPV16 and HPV18, are mainly dependent on sustained expression of the viral E6 and E7 oncogenes. E6 protein binds and degrades tumor suppressor p53, thus inhibiting p53-dependent growth arrest and apoptosis in aberrant proliferative host cells. E7 interacts with tumor suppressor RB1, and frees transcription factor E2F to trans-activate its cellular targets, thereby promoting host cell-cycle progression \textsuperscript{(3)}. Interestingly, previous studies also demonstrated that E7 alone, but not E6, is sufficient to immortalize human keratinocytes \textit{in vitro} \textsuperscript{(4)} and induce high-grade cervical dysplasia in a transgenic mouse model \textsuperscript{(5)}. Together, these data suggested that viral oncogene E7 may serve as an ideal target for molecular gene therapy of cervical cancer.

Zinc finger nuclease (ZFN) is an artificial endonuclease which is engineered by fusing DNA-binding zinc finger proteins to the FokI DNA-cleavage domain \textsuperscript{(6)}. Cleavage is induced when two custom-designed ZFNs dimerize upon binding DNA to form an active FokI nuclease. By cleaving specific target DNA, ZFNs create double-strand breaks (DSB) that are then repaired by the nonhomologous end joining pathway or, if codelivered with a donor template, repaired by the homology directed repair pathway. As a
Cervical cancer is mainly caused by infections of high-risk human papillomavirus (HR-HPV). Persistent expression of HR-HPV oncoproteins E6 and E7 is implicated in malignant transformation. In the present study, we designed and constructed zinc finger nucleases (ZFN) that could specifically recognize and cleave HPV16/18 E7 DNA. ZFN-mediated disruption of HPV16/18 E7 DNA directly decreased the expression of E7, induced type-specific apoptosis in HPV16/18-positive cervical cancer cells, and inhibited cervical cancer cell growth in vitro. However, no effects were observed in ZFN-treated HPV-negative cells. Finally, ZFNs targeting HPV16/18 E7 were confirmed to have tumor suppression activity in a xenograft model of cervical cancer. Thus, this study is the first to report that HPV-DNA targeting ZFNs could be used as novel therapeutic agents for the treatment of HPV-related cervical cancer.

**Translational Relevance**

Cervical cancer is mainly caused by infections of high-risk human papillomavirus (HR-HPV). Persistent expression of HR-HPV oncoproteins E6 and E7 is implicated in malignant transformation. In the present study, we designed and constructed zinc finger nucleases (ZFN) that could specifically recognize and cleave HPV16/18 E7 DNA. ZFN-mediated disruption of HPV16/18 E7 DNA directly decreased the expression of E7, induced type-specific apoptosis in HPV16/18-positive cervical cancer cells, and inhibited cervical cancer cell growth in vitro. However, no effects were observed in ZFN-treated HPV-negative cells. Finally, ZFNs targeting HPV16/18 E7 were confirmed to have tumor suppression activity in a xenograft model of cervical cancer. Thus, this study is the first to report that HPV-DNA targeting ZFNs could be used as novel therapeutic agents for the treatment of HPV-related cervical cancer.

Materials and Methods

**Plasmids and ZFN assembly**

Plasmids encoding different zinc finger modules were obtained from Addgene (Zinc Finger Consortium Expression Vector Kit v1.0). The potential ZFN target sites of HPV16 and HPV18 E7 were identified using ZiFiT software (http://bindr.gdcb.iastate.edu/ZiFiT/), and the multi-finger arrays were assembled by restriction digest-based context-dependent assembly (CoDA) or modular assembly as described previously (16, 17). The FokI endonuclease domain was digested from the pPGK-FokI expression vector. Other heterodimeric variants were mutated from wild-type FokI. These FokIs were cloned into the pcDNA3.1 plasmid (Invitrogen), and the sequences of the plasmids were confirmed using DNA sequencing. The sequences of FokI variants were shown in Supplemental Note S1.

**Cell culture and transfection**

Cervical cancer cell lines SiHa (HPV16 positive), HeLa (HPV18 positive), C33A (HPV negative), and CaSki (HPV16 positive) and human embryonic kidney cell line HEK293 (HPV negative) were purchased from ATCC and passaged in our laboratory. All the cell lines were authenticated at Shanghai Paternity Genetic Testing Center in June 2012, using short tandem repeat DNA profiling (ABI 3130d Genetic Analyzer, Life Technologies). All the cells were maintained in DMEM supplemented with 10% FBS (Gibco) and 100 U/mL penicillin and streptomycin (Invitrogen) at 37°C in a humidified incubator with 5% CO2. The S12 cell line is an immortalized human cervical keratinocyte cell line that contains integrated HPV16 genomes, which was a generous gift from Pro. Kenneth Raj (Health Protection Agency). The acquisition of the cell line was permitted by the original owner Pro. Margaret Stanley (18, 19). S12 was cultured in a 1:3 mixture of DMEM and Ham F-12 medium supplemented with 5% FBS, 8.4 ng/mL cholera toxin, 5 μg/mL insulin, 24.3 μg/mL adenine, 0.5 μg/mL hydrocortisone, and 10 ng/mL EGF. All the cells were transfected using X-tremeGENE HP DNA Transfection Reagent (Roche) according to the manufacturer’s instructions. The ratio of the reagent to DNA in each cell line was optimized in preliminary experiments. The experiments were performed three times in duplicate.

**Single-strand annealing recombination reporter assay**

The construction of the SSA luciferase reporter plasmid, pSSA Rep3-1, has been described previously (20). The pSSA Rep-3, GZF3-L3, and GZF1-R3 ZFN plasmids were a kind gift from Prof. David Segal (University of California, Davis, CA). Briefly, 25 ng of pSSA reporter plasmid with inserted ZFN target sequences, 100 ng of each ZFN plasmid, and 25 ng of pRL-TK-Renilla luciferase (Promega) were cotransfected into HEK293 cells at 80% confluence in a 24-well plate. At 48 hours posttransfection, the cells were harvested and lysed according to the protocol of the Dual-Luciferase Reporter Assay System (Promega). The firefly and Renilla luciferase activity were determined using a microplate luminometer (BioTek). All the experiments were performed three times in duplicate on different days.

**T7E1 assay**

The T7E1 assay was performed as previously described (21). Briefly, the genomic DNA was extracted from cells using the DNeasy Blood & Tissue Kit (QIAGEN) according
to the manufacturer’s handbook. The genomic region encompassing the ZFN target site was amplified, purified, melted, and annealed to form heteroduplex DNA. The annealed DNA (200 ng) was treated with 10 units of T7 endonuclease I (New England BioLabs) for 15 minutes at 37°C and then analyzed on a 10% TBE polyacrylamide gel.

**Western blot analysis**

Cells were lysed on ice for 30 minutes with a lysis buffer containing 150 mmol/L NaCl, 50 mmol/L Tris (pH = 7.4), 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, and protease inhibitor cocktail. The antibodies rabbit anti-β-actin (60008-1-lg, Proteintech Group), mouse anti-HPV18 E7 (sc-1590, Santa Cruz Biotechnology), mouse anti-HPV16 E7 (sc-6981, Santa Cruz Biotechnology), and rabbit anti-RB1 (10048-2-lg, Proteintech Group) were used for Western blotting.

**Cell proliferation assay**

Cell viability and cell proliferation were determined by the Cell Counting Kit-8 (CCK-8, Dojindo) according to the manufacture’s manual. Briefly, 2 × 10^3 cells per well were seeded in 96-well plates and cultured overnight. For cell viability, cells were transfected with various concentrations of ZFNs for 24 and 48 hours. For cell proliferation, cells were transfected with the IC_{50} values of ZFN16-E7-S2 and ZFN18-E7-S2 (1,022 ng/mL and 1,230 ng/mL, respectively) for the times as indicated. Then 10 μL of CCK-8 dye was added to each well and the cells were incubated at 37°C for 3 hours, the absorbance was determined at 450 nm using a microplate reader.

**Apoptosis assay**

After infection, cells were trypsinized and double stained with FITC-conjugated annexin V (annexin V-FITC) and propidium iodide (PI) using an Annexin V-FITC Apoptosis detection kit (KeyGEN BioTECH) according to the manufacturer’s instructions. Then, cells were analyzed using FACScalibur (BD Bioscience). A total of 20,000 events were acquired for each sample, and the data were analyzed using BD CellQuest software.

**Colony forming assay**

A total of 200 cells were plated in triplicate in complete growth medium in 12-well plates and incubated for 2 weeks. Colonies of more than 50 μm in diameter were counted using an Omnicom 3600 image analysis system. The colonies were stained with 0.04% crystal violet and photographed.

**Animal experiments**

Four-week-old specific pathogen-free Balb/c-nu nude mice were purchased from BEIJING HKF BIOSCIENCE and housed at the Experimental Animal Center, Tongji Medical College, Huazhong University of Science and Technology (HUST, Wuhan, China). Six mice were randomly assigned

---

**Figure 1.** ZFNs targeting HPV16/18 E7 induced cleavage of viral DNA in HPV16/18-positive cells. A, cleavage activity and toxicity of ZFNs targeting HPV16 E7 and HPV18 E7 (Supplementary Table S1) were determined by SSA assays. Top, firefly RLU represented the cleavage activity of ZFNs; bottom, Renilla RLU represented the toxicity of the ZFNs. White columns, signal in pSSA reporter with corresponding target sequence; black columns, signal in pSSA reporters cotransfected with ZFNs. B, activity of ZFN16-E7 containing wild-type FokI (wt) or heterodimeric variants of FokI (S0, S1, S2, and S3) was determined by SSA assays, neg, pSSA-16E7 reporter only. C, activity of ZFN18-E7 containing wild-type FokI (wt) or heterodimeric variants of FokI (S0, S1, S2, and S3) was determined by SSA assays, neg, pSSA-18E7 reporter only, pos, GZF3-L3 + GZF1-R3 + pSSA Rep3-1. **P < 0.05;** **P < 0.01, compared with the control reporter by ANOVA testing. D, T7 endonuclease 1 (T7E1) assay of ZFNs cleavage in SiHa and HeLa cells. Cells were untreated (Mock) or transfected with 1 μg/mL of ZFN empty vector (neg) or different concentrations of ZFN16-E7-S2 plasmids or ZFN18-E7-S2 plasmids for 48 hours, the genomic DNA of cells was extracted, PCR was performed with the primers encompassing the target sites, and then the PCR products were purified, annealed, digested with T7E1, and analyzed by gel electrophoresis. The expected cleaved DNA bands are indicated by blank arrows.
to each group and were injected subcutaneously in the right flanks with $5 \times 10^6$ HeLa or SiHa cells. When xenografts reached about 100 to 150 mm$^3$, we started to complex the formed using one-way ANOVA with equal variances. Statistical analyses were performed with GraphPad Prism 5.

GraphPad Prism 5.

Statistical analysis
The data were expressed as the mean ± SD and analyzed using Student $t$ test. The experiments were performed three times in duplicate. Statistical analysis for Fig. 5 was performed using one-way ANOVA with $P = 0.05$ and assuming equal variances. Statistical analyses were performed with GraphPad Prism 5.

Results

**ZFNs targeting HPV16/18 E7 induce cleavage of viral DNA in HPV16/18-positive cells**

To design zinc finger motifs that specifically target the $E7$ sequences, we analyzed the $E7$ sequences of HPV16 and HPV18 to identify possible binding sites for ZFN pairs, all these target sequences were rechecked by BLAST (Basic Local Alignment Search Tool). Five pairs of ZFNs target HPV16 $E7$ and three pairs target HPV18 $E7$ were designed and constructed by Modular Assembly or CoDA approach (Supplementary Table S1). All of these ZFNs were tested by SSA assay, ZFN-603 (ZFN16 $E7$, constructed by modular assembly) which targets HPV16 $E7$ and ZFN-758 (ZFN18 $E7$, constructed by CoDA) which targets HPV18 $E7$ were selected for the further study because of the best cleavage activity without any toxicity (Fig. 1A and Supplementary Table S1). To enhance the cleavage activity of ZFNs, we introduced a codon-optimized $FokI$ domain in addition to the wild-type DNA cleavage domain of the type II restriction enzyme $FokI$. Sharkey (S418P, K441E; ref. 23) and others reported heterodimeric variants of $FokI$ nuclease containing variable numbers of mutations, which were named S0 (KK: E490K, I538K/EL: Q486E, 1499 L; ref. 24), S1 (Sharkey + KK/Sharkey + EL), S2 (KKR: KK+H537R/ELD: EL+N496D), and S3 (Sharkey + KKR/Sharkey + ELD; ref. 15), were also applied. The complete sequences of all $FokI$ variants are provided in the Supplementary Note S1.

Next, to choose the best $FokI$ domain variants and assess the cleavage activities of the candidate ZFNs, we used a mammalian cell-based SSA assay, in which a ZFN target sequence and a stop codon were inserted between two direct repeat halves of the *firefly* luciferase gene pSSA Rep3-1 (20). A ZFN-induced DSB between the segments allows efficient SSA homologous recombination using the two halves as templates, resulting in an active luciferase gene. The *Renilla* luciferase plasmid was used as an internal transfection control and a marker for ZFN toxicity.

Figure 2. ZFN-mediated HPV16/18 E7 gene disruption. A, the representative images of γH2AX foci (red) in ZFN16–E7–S2- and si16–E7-treated SiHa cells and in ZFN18–E7–S2- and si18–E7–treated HeLa cells. Cell nuclei were indicated by DAPI staining (blue). Etoposide (0.25 μM/L) was used as positive control. B, quantification of γH2AX foci in SiHa and HeLa. Three independent cell fields of 30 cells each were counted as average number of γH2AX foci per nucleus. *, $P < 0.05$. C, the protein levels of HPV $E7$ and $RB1$ in ZFN16–E7–S2–treated SiHa and ZFN18–E7–S2–treated HeLa cells. Cells were transfected with 1 μg/mL ZFNs or 50 nmol/L of siRNA for 48 hours. The protein levels were detected by Western blotting. β-actin was used as a reference control. D, quantitative analysis of the protein levels shown in C. *, $P < 0.05$, compared with parent HeLa cells (n = 3 replications).
Compared with other FokI variants, ZFN16-E7-S2 and ZFN18-E7-S2 showed the best cleavage activity in the pSSA reporter system (Fig. 1B and C). Measurement of the cotransfected Renilla luciferase expression did not reveal any significant decrease in signal (Fig. 1B and C), which means low genotoxicity caused by off-target nuclease activity. Therefore, ZFN18-E7-S2 and ZFN16-E7-S2 were selected as candidates for further investigation. Moreover, to explore the effects of ZFN16-E7-S2 and ZFN18-E7-S2 on viral gene disruption, we examined the cleavage activity of E7 DNA based on the in vitro T7E1 assay. As shown in Fig. 1D, ZFN16-E7-S2 and ZFN18-E7-S2 could induce significant cleavage of the E7 genes. These results indicate that ZFN16-E7-S2 and ZFN18-E7-S2, which target HPV16 E7 and HPV18 E7, respectively, have efficient viral DNA cleavage activity.

**HPV16/18 E7 gene disruption by ZFN16/18-E7-S2**

To verify the cleavage effects resulted from genome editing of HPV E6/E7, we performed immunofluorescence (IF) staining of γH2AX, which is a sensitive marker for detecting DSBs. After treatment with ZFN16-E7-S2, the number of γH2AX foci in SiHa were significantly increased to 1.53 ± 0.10 per nucleus ($P < 0.05$, compared with Vector). Similarly, the number of γH2AX foci in HeLa increased to 20.34 ± 0.71 per nucleus after treatment with ZFNs ($P < 0.05$, compared with Vector).

However, both si16-E7 and si18-E7 did not affect the expression of γH2AX (Fig. 2A and B). It was reported that SiHa cells contain one to two copies of HPV16 and HeLa cells contain 40 to 50 copies of HPV18 (25). The number of γH2AX foci in ZFNs-treated SiHa and HeLa were no more than HPV copy numbers, respectively, indicating the specificity of ZFNs.

Our previous study showed that RNAi against HPV16 E7 led to downregulation of E7 and upregulation of RB1 (26). To investigate ZFN16/18-E7-S2–mediated HPV16/18 E7 gene disruption activity, SiHa cells were transfected with 1 µg/ml ZFN16-E7-S2 or 50 nmol/L of siRNA16-E7, and HeLa cells were transfected with 1 µg/ml ZFN18-E7-S2 or 50 nmol/L of siRNA18-E7. HPV E7, RB1 protein expression was detected by Western blotting 48 hours after transfection. In this assay, ZFN16/18-E7-S2 remarkably decreased HPV16/18 E7 expression in ZFN16/18-E7-S2–treated cells. E7 repression resulted in increased expression of RB1. Similar results were obtained in siRNA16/18-E7–treated cells (Fig. 2C and D). These results are consistent with those of the previous report (26).

Altogether, these results suggest that ZFN16/18-E7-S2 could effectively disrupt the HPV E7 oncogene in HPV16/18-positive cervical cancer cells, leading to repression of the endogenous E7 oncogene and restoration of the RB1 tumor suppressor pathway.
Knockdown of HPV E7 inhibits cervical cancer cell growth

It has been reported that stable suppression of E6/E7 oncogenes expression using specific siRNA induces inhibition of tumor growth both in vitro and in vivo (27, 28). To evaluate the effect of repression of E7 by ZFNs on tumor cell growth, SiHa and HeLa cells were transfected with different concentrations of ZFN16-E7-S2 and ZFN18-E7-S2, respectively, for 24 to 48 hours. Afterward, the cell viability was tested using a CCK8 assay. Significantly reduced viability of ZFN16/18-E7-S2–treated cells could be observed at concentrations of more than 500 ng/mL of ZFN16/18-E7-S2 (Fig. 3A). The IC50 values of ZFN16-E7-S2 and ZFN18-E7-S2 at 48 hours were calculated to be 1,022 ng/mL and 1,230 ng/mL, respectively. As shown in Fig. 3B, both SiHa cells treated with 1,000 ng/mL of ZFN16-E7-S2 and HeLa cells treated with 1,200 ng/mL of ZFN16-E7-S2 for 5 days exhibited significant growth inhibition compared with control groups. Similar results were achieved in siRNA16/18-E7–treated cells.

Moreover, colony-forming assays were used to determine ZFN16/18-E7-S2–mediated tumor suppression activity. SiHa and HeLa cells were treated with 1 μg/mL ZFN16-E7-S2 and ZFN18-E7-S2 for 24 hours, respectively. Similar to the results of the siRNA groups, knockdown of E7 by ZFN16-E7-S2 and ZFN18-E7-S2 caused a drastic drop in the number of clonogenic cells after 2 weeks (Fig. 3C and D). These observations suggest that knockdown of HPV E7 by specific ZNFs could effectively lead to inhibition of cervical cancer cell growth.

ZFN16/18-E7-S2 targeting E7 induces apoptosis of type-specific HPV-positive cells

To study the potential effects of apoptosis induction by ZFN16/18-E7-S2 in human HPV-positive cells, SiHa and HeLa cells were treated with ZFN16-E7-S2 and ZFN18-E7-S2, respectively. Cell apoptosis was quantified 48 hours later using Annexin V and PI staining. As shown in Fig. 4A, SiHa cells incubated with different concentrations (500, 1,000, and 1,500 ng/mL) of ZFN16-E7-S2 had an increased apoptotic rate. Similar results were obtained with HeLa cells incubated with different concentrations (600, 1,200, and 1,800 ng/mL) of ZFN18-E7-S2.

To further explore the efficacy of ZFN16/18-E7-S2 in the induction of apoptosis in other cell lines, we cross-transfected SiHa cells with ZFN16-E7-S2 and HeLa cells with ZFN18-E7-S2. The results showed that neither SiHa cells treated with ZFN18-E7-S2 nor HeLa cells treated with ZFN16-E7-S2 had an increased apoptotic rate (Fig. 4B). To further confirm the specificity of ZFN16/18-E7-S2, CaSki (HPV16 and HPV18 positive, HPV16 predominant), C33A (HPV-negative), and S12 (an immortalized cervical epithelial cell line that contains integrated HPV16 DNA) cells were treated with ZFN16-E7-S2 or ZFN18-E7-S2. Interestingly, as shown in Fig. 4C, treatment of ZFN16-E7-S2, but not ZFN18-E7-S2, induced substantial apoptosis of CaSki and S12 cells. This treatment had no effect on C33A cells (Fig. 4D). Finally, we also used the normal human embryonic kidney cell line HEK293 to detect the toxicity of ZFNs. We found that neither ZFN16-E7 nor ZFN18-E7 has a role in cell apoptosis in this cell
These results indicate that ZFN16-E7-S2 and ZFN18-E7-S2 could only specifically induce apoptosis of corresponding HPV16- and HPV18-positive cervical cancer cell lines.

In vivo assay of tumorigenicity in nude mice

To evaluate the potential effects of ZFN16-E7-S2- and ZFN18-E7-S2–mediated suppression of tumorigenicity in vivo, we used SiHa and HeLa cell xenograft models in Balb/c nude mice. Nude mice were inoculated subcutaneously with cells to form xenografts, then ZFN16-E7-S2 and ZFN18-E7-S2 were administrated intratumorally using in vivo transfection reagent. Mice bearing ZFN16-E7-S2–treated SiHa and ZFN18-E7-S2–treated HeLa showed slower growth of xenograft formation (Fig. 5A) and smaller tumor size compared with untreated groups (P < 0.05; Fig. 5B). Then hematoxylin and eosin (H&E) staining and IHC stainings of PCNA, caspase-3, and CD31 were performed to investigate the proliferation, apoptosis, and angiogenesis in the xenograft tumor sections. Both H&E and IHC stainings showed that E7-targeted ZFNs resulted in necrosis areas of various sizes in the central of xenograft tissues (Fig. 5C and D). In addition, compared with control groups, we found downregulation of proliferation marker PCNA and upregulation of caspase-3 in the margins of necrosis areas of the ZFNs-treated SiHa and HeLa groups, but not in the non-necrosis areas of these groups (Supplementary Fig. S1). We did not observe any effects of ZFN on angiogenesis marker CD31 in the xenograft tumor sections (Fig. 5C and D and Supplementary Fig. S1). Taken together, our results further confirmed the therapeutic effects of ZFN in vivo, but also suggested that the efficacy of E7-targeted ZFN could be improved by in vivo delivery methods, for example, viral packaging system.

Discussion

HR-HPV infections are the major cause of cervical cancers. The malignant transformation abilities of HR-HPV largely stem from consistent expression of viral oncogenes E6 and E7, making them ideal targets for cancer gene therapy. In this study, ZFNs targeting HPV16/18 E7 DNA decreased the expression of E7 and increased expression of RB1 and p53. E7 repression resulted in apoptosis and
growth inhibition in HPV16/18-positive cervical cancer cells.

Several research groups have demonstrated that siRNA targeting E6/E7 can effectively silence viral oncogene mRNA and induce cell apoptosis (27, 29–31). Although the siRNA approach has several advantages over traditional drugs in treating multiple cancers, there are several challenges that must be overcome (32). In the present study, we attempted to disrupt HPV E7 DNA using ZFNs instead of targeting viral mRNA. First, to engineer maximally active ZFNs, we compared the efficiencies of FokI variants. We found that the ZFNs containing S2 (KKR: ELD) variants could achieve more effective cleavage than any other mutations, including S3 (Sharkey + KKR: Sharkey + ELD) mutations. These results are not consistent with a previous report that showed that a combination of Sharkey and KKR: ELD mutations resulted in an additive effect of ZFN activity (15). It is possible that the Sharkey mutation not only enhances the FokI cleavage efficiency, but also increases nonspecific toxicity to target cells. Further experiments are currently ongoing to investigate whether extending the recognition sequence of ZFNs (increasing the number of zinc fingers) could reduce the toxicity of Sharkey mutations. ZFNs with S2 FokI variants with low genotoxicity were selected for further experiments.

We then introduced ZFNs into HPV-positive cells and found that ZFN16/18-E7-S2 could induce significant cleavage of E7 DNA. The disruption to viral DNA directly led to downregulation of E7 expression at the protein levels and restoration of the tumor suppressor genes RB1, resulting in apoptosis and growth inhibition of ZFN-treated HPV16/18-positive cervical cancer cells. We also compared the effects of ZFNs and siRNA targeting HPV E7. We found that the therapeutic effects of ZFNs on HPV-infected cells were comparable with those of siRNA. Although several therapeutic siRNAs are already being evaluated in clinical trials, concerns about their efficacy and safety have been raised (33). For instance, the effects of siRNAs are transient, and human cells can develop resistance to long-term application of siRNA. However, ZFNs may solve this problem by targeting the DNA such that disruption of the oncogene E7 by ZFNs is permanent and can be passed to the next generation if the virus replicates itself.

Specificity is one of the most important concerns for the development of new drugs. Our data showed that ZFN16-E7-S2, but not ZFN18-E7-S2, induced apoptosis of HPV16-positive SiHa, CaSki, and human cervical keratinocyte S12 cells. Moreover, HPV-negative cervical cancer C33A cells and normal HEK293 cells were insensitive to both ZFN16-E7-S2 and ZFN18-E7-S2. These findings confirmed that the effects of ZFNs are highly specific to HPV subtype. In the near future, it may be possible that patients could first be tested for HPV infection subtype and then administered the corresponding subtype-specific ZFNs. Although the results showed that ZFN16-E7-S2 and ZFN18-E7-S2 mediated suppression of tumorigenicity in a xenograft model of cervical cancer, further in vivo studies on ZFN delivery methods are warranted.

In conclusion, this study shows that ZFNs targeting HPV E7 could efficiently downregulate the expression of the E7 oncogene, resulting in apoptosis and growth inhibition in corresponding HPV-positive malignant cells. ZFNs targeting HPV DNA can be a novel effective strategy for gene therapy of cervical cancer.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: W. Ding, Z. Hu, D. Zhu, J. Zhou
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): L. Yu, X. Wang, C. Zhang, L. Wang, T. Ji, K. Li, D. He, D. Lau, X. Xia
Analysis and interpretation of data (e.g., statistical analysis, bioinformatics, computational analysis): W. Ding, Z. Hu, D. Zhu, X. Jiang, X. Xia, J. Zhou
Writing, review, and/or revision of the manuscript: W. Ding, Z. Hu, D. Zhu, X. Xia, H. Wang
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): X. Jiang, X. Xia
Study supervision: J. Zhou, M. Ding, H. Wang

Acknowledgments
The authors thank Prof. David J. Segal (University of California, Davis, CA) for the gift of the pSSA Rep3-1, GZF3-L3, and GZF1-R3 ZFN plasmids and Prof. Kenneth Raj (Health Protection Agency, Didcot, United Kingdom) for providing the S12 cell line, which was permitted by the primary owner Prof. Margaret Stanley (Division of Virology, National Institute for Medical Research, London, United Kingdom).

Grant Support
This work was supported by funds from the National Development Program (973) for Key Basic Research of China (No. 2015CB553093 and 2009CB521800 to D. Ma, No. 2013CB911304 to H. Wang, and No. 2009CB521806 to J. Zhou) and the National Natural Science Funding of China (NSFC, 81372805 to H. Wang, 81090414 to J. Zhou, 81402158 to Z. Hu, 81471508 and 81101971 to X. Xia, 81402159 to T. Ji, 81301126 to D. He, and 81302266 to K. Li).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received February 15, 2014; revised September 24, 2014; accepted October 1, 2014; published OnlineFirst October 21, 2014.

References


Clinical Cancer Research

Zinc Finger Nucleases Targeting the Human Papillomavirus E7 Oncogene Induce E7 Disruption and a Transformed Phenotype in HPV16/18-Positive Cervical Cancer Cells

Wencheng Ding, Zheng Hu, Da Zhu, et al.


Updated version
Access the most recent version of this article at:
doi: 10.1158/1078-0432.CCR-14-0250

Supplementary Material
Access the most recent supplemental material at:
http://clincancerres.aacrjournals.org/content/suppl/2014/10/21/1078-0432.CCR-14-0250.DC1

Cited articles
This article cites 33 articles, 10 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/20/24/6495.full#ref-list-1

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.