The efficacy of a DNA vaccine containing inserted and replicated regions of the E7 gene for treatment of HPV-16 induced tumors

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Abstract

A majority of cervical cancers are associated with Human Papillomavirus (HPV)-16. A DNA vaccine (E7IR) was designed for prophylactic and therapeutic treatment of HPV-16+ tumors containing two repeats of the E7 gene to inactivate transformation and duplicate available epitopes. Mice were vaccinated then tumor challenged, or challenged and then immunized and monitored for tumor volume and survival. Splenocytes were utilized for in vivo CTL assays. The E7IR vaccine demonstrated decreased tumor volume and enhanced survival in prophylactic and therapeutic experiments and improved CTL-mediated lysis. The E7IR vaccine shows promise in prevention of tumor formation and elimination of established tumors.

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1. Introduction

Cancer of the uterine cervix is the second most common cause of cancer-related deaths in women worldwide. Approximately 510,000 new cases and 288,000 deaths are reported annually [1]. The development of cervical cancer is associated with infection with high risk types of Human Papillomaviruses (HPV). HPV is detected in 99.7% of cervical carcinomas [2], with high risks types HPV-16 and 18 accounting for nearly 70% of cervical cancer cases [3]. In contrast to many other cancers, the fact that cervical cancer is highly associated with a viral infection creates a unique opportunity for therapeutic vaccination against HPV to either prevent cervical cancer once an individual is infected or after the virus has caused cervical cancer. Integration of the viral genome into the host cell genome, a necessary step in cellular transformation, leads to the loss of some genes necessary for a productive viral life cycle and to deregulated or over-expression of the E6 and E7 oncoproteins. Because E7 has been shown to be consistently expressed in HPV-16 positive tumors, it is a logical target in HPV-16 vaccination strategies.

Recent efforts in the field of HPV tumor immunotherapy have focused on the delivery of naked DNA vaccines. DNA based vaccines are attractive candidates because of simplicity of delivery, stability, and overall safety. DNA vaccines based on the E6 and or E7 oncoproteins can either stably integrate into the genome or are maintained in an episomal form allowing for extended expression of HPV antigens. Of course, in this instance mutations in the p53 and pRB binding sites are essential in order to prevent vaccination induced cellular transformation. Furthermore, administration of DNA based vaccines has been shown to induce CTL to HPV antigens [4].

Preclinical studies using DNA based vaccine approaches tend to focus on enhancing presentation of HPV antigens via the MHC class I or MHC class II pathways [5]. Several different approaches have been examined including the attachment
of ubiquitin to DNA constructs [6, 7], targeting of tumor antigens to centrosomal compartments to enhance MHC class I presentation [8], the attachment to constructs of calreticulin to target HPV antigens to the MHC I pathway [9], optimization of codon usage for enhanced expression and presentation of HPV oncoproteins [6, 10, 11], utilization of modified genes to decrease the transformation capacity [12–14] or enhancement of intercellular spreading in order to increase the number of cells expressing antigen [15]. All of these approaches have demonstrated increased antigen presentation and superior tumor eradication in mouse models.

The current study was undertaken in order to assess whether insertional replication of the HPV-16 E7 gene could enhance immune responses compared to vaccination with the wild type gene, by increasing the overall number of T cell epitopes while simultaneously providing a safe construct. In this study, this approach did in fact enhance immunity compared to vaccination with the wild type construct as evidenced by the ability to prevent tumor formation and therapeutically treat existing tumors in C57BL/6 mice and furthermore this effect appears to be CTL-mediated as evidenced by increased killing seen in vivo CTL assays. Taken together these results suggest that insertional replication of the E7 gene provides a unique method to ablate the transforming activity of the E7 gene and simultaneously enhance its immunogenicity. This strategy provides an important basic DNA construct for superior vaccination against HPV-16 E7 especially when coupled with current techniques for enhancing vaccine potency.

2. Materials and methods

2.1. Mouse strains and cell lines

Specific pathogen free 6–10 week old female C57BL/6 mice were purchased from Taconic Farms (Germantown, NY, USA) and housed under SPF conditions, conforming to institutional animal use guidelines at the animal facilities at the University of Southern California. Tumor challenge studies were performed utilizing the HPV-16 E6/E7 expressing TC-1 tumor cell line and cultured as described [16]. Phoenix cells (Orbigen Inc., San Diego, CA) were cultured in DMEM (Invitrogen) containing 10% heat inactivated fetal bovine serum (Omega), 100 U/ml Penicillin, 100 U/ml Streptomycin (Invitrogen), and 2 mM l-glutamine (Invitrogen).

2.2. Construction of the E7IR vaccine

The native 98 amino acid HPV-16 E7 protein contains three regions that were arbitrarily designated as a, b, or c. The E7IR DNA construct was designed containing two repeats of the first, second, and third regions of the E7 gene. The nucleotide sequence was synthesized by Entelechon (Entelechon GmbH, St. Veit-Weg 2, 93051 Regensburg) and was cloned into a PCRII topo vector and sub-cloned into the APL023 expression vector [7]. In the a region, amino acids 21, 24, and 26 were modified from DLYCYEQ to GLGYGQQ to reduce transformation activity and enhance antigenicity [17, 18]. In the b region, a split was introduced between two cystine residues at position 31 and 32 to reduce transformation activity. In the c region two zinc finger binding motifs were disrupted by a split between amino acids 60 and 61 and modifications of C61 to G61 and C94 to G94. All three regions, a, b, c were replicated to enhance expression, and H-2Db and HLA-A*0201 epitopes were added downstream of the aabbcc construct with Ala-Ala-Tyr (AAY) as an optimal spacer for proteosomal cleavage [7]. In addition to further enhancing expression, addition of these epitopes provided unique primer sites at the 3’ end of the construct that were necessary for cloning. The addition of a Kozak sequence on the 5’ end of the construct, also served to create a unique primer site as well as enhance translation of the construct in eukaryotic cells [19]. Lastly, the codons in the E7IR construct were optimized for expression in mice. The construct was cloned into the multiple cloning site of the APL023 expression vector [7] utilizing Bam HI and Not I restriction enzymes.

2.3. Retroviral expression of the E7IR gene

PCR of the E7IR gene was performed utilizing the following primers sets 5’ CGT GGA TCC GCC GCC ACC ATG CAT GGA GAC ACA CCA ACC CTG 3’ and 5’ GCT CAG CAC GCC GGC GCT ATT CTA TCT CGA TCT CTA GGG 3’, 5’ CGT GGA TCC GCC ACC ATG TAC CCA TAC GAT GTT CCA GAT TAC GCT ATG CAT GGA GAC ACA CCA ACC CTG 3’ containing a Bam HI restriction site in the forward primer and a Not I restriction site in the reverse primer. The last primer listed was utilized as the forward primer in a separate reaction to generate a HA-tag encoding sequence for use in Western blotting analysis. The resultant PCR products were cloned into the LZRSrBMN-Z retroviral expression vector (a kind gift of Dr. Gary Nolan, Stanford University, Palo Alto, CA). Retroviral infection of Phoenix cells with LZRSrBMN-Z constructs was conducted utilizing the reagents and protocol from Orbigen Inc., San Diego, CA, USA. Briefly, Phoenix cells were transfected by the CaCl2 precipitation method and after 48 h, 3 ml of harvested retroviral supernatants were utilized to infect 5 × 10⁵ NIH 3T3 cells. Forty-eight hours later, NIH 3T3 cells were screened for retroviral infectivity by x-gal staining and lac z negative cells were selected by adding 2 μg/ml puromycin (EMD Biosciences Inc., San Diego, CA).

2.4. Western blots

Cellular protein extracts from transfected NIH 3T3 cells were obtained using the M-Per Mammalian Protein Extraction Kit (Pierce, Rockford, IL). Thirty micrograms of protein extract was separated by SDS-PAGE using a 12% Bis-Tris gel (Invitrogen) and transferred onto a PVDF membrane.
(Bio-Rad, Hercules, CA) using a semi dry blotting system (Invitrogen). HA-tagged E7IR was detected with a HA-specific polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and the WT E7 and the E7IR proteins were detected with an HPV-16 E7 specific mouse monoclonal antibody (Zymed clone 8C9, Invitrogen). The GAPDH-specific antibody (Chemicon International, Temecula, CA) was used to verify that equal amounts of protein were loaded. Bands were visualized by enhanced chemiluminescence for HRP detection (Pierce).

2.5. Soft agar assay

Anchorage-independent growth capability was determined by assessing the colony formation efficiency of cells suspended in soft agar.Transformed and control cells were plated in triplicate dilutions (1 × 10^4, 2 × 10^4, and 4 × 10^4) in 2 ml of 0.3% overlay agar and added to 6 well plates coated with 3 ml of 0.6% underlay agar. Plates were allowed to dry and were incubated at 37 °C. Two weeks after the cells were plated, colonies were stained with p-iodonitrotetrazolium violet (Sigma) and counted. The mean transformation efficiency of the E7IR was calculated based on the triplicate dilution series and compared to the mean foci numbers of the E7 wild type.

2.6. In vivo CTL assay

On day 1 of the assay, spleens were harvested from naïve C57BL/6 mice and single cell suspensions were prepared for use as peptide loaded targets. Red blood cells were then lysed by treatment with RBC lysis buffer (Sigma) for 3 min and washed twice with IMDM media. Cells were then counted and split into two tubes; one containing the E7 (49–57) H-2Db specific peptide RAHYNIVTF [20] and the other containing prostate specific antigen 5 (PSA 5), VTVGAAAPL, a prostate specific antigen based peptide that binds to Kb and Db [32] as a non-specific peptide at a concentration of 0.5 μg/ml. Peptide labeled cells were then incubated at 37 °C for 90 min. After incubation, the cells were washed three times in IMDM media, and once in 0.1% BSA in PBS before labeling with CFSE (Molecular Probes, Eugene, OR). Cells were labeled at a concentration of 5 μM/ml for the relevant peptide and 0.25 μM/ml for the irrelevant peptide for 10 min at 37 °C. The reaction was stopped by adding 20 ml ice cold IMDM (Cambrex, Walkersville, MD). Labeled cells were then washed twice in PBS, resuspended at a concentration of 50 × 10^6 cells/ml and relevant and irrelevant peptides were mixed in a 1:1 ratio and the peptide coated cells were injected i.v. into vaccinated or control mice. Eighteen hours later, vaccinated and control mice were sacrificed and spleens were harvested. Vaccinated and control spleens were resuspended to 3 × 10^8 total cells per sample and run on a Beckman Coulter Cytomics FC 500 Flow Cytometer (Beckman Coulter, Miami, FL). Data were analyzed using CXP software (Beckman Coulter) and percent lysis was calculated based on the equation: (Q − R/Q) × 100 where Q equals the CFSE high labeled relevant peptide and R equals the CFSE low labeled irrelevant peptide.

2.7. DNA-gold bullet preparation and gene gun-mediated delivery

The Helios gene gun system (Bio-Rad, Hercules, CA) was used for intradermal delivery of vaccine and control genes as previously described [7]. Bullets containing 2 μg of DNA/shot were generated according the manufacturer’s protocols. Briefly, 100 μg of DNA was precipitated on 25 mg of 1-mm gold particles in the presence of 100 μl of 0.05 M spermidine (Sigma, St. Louis, MO) using 100 μl of 1 M CaCl_2 preparation. The gold was washed three times with 1 ml of 100% 200 proof ethanol (Sigma) and was resuspended in 3 ml of 0.1 mg/ml polyvinylpyrrolidone in 100% ethanol. The gold was then loaded into the tubing using the tubing prep station (Bio-Rad), and the gold loaded tubing was cut into 0.5-in. pieces to load into the cartridges. The bullet containing cartridges were loaded into the gene gun and delivered into the mouse epidermis at a helium pressure of 450 psi.

2.8. Immunization schemes

2.8.1. Immune monitoring of the E7IR vaccine

Mice were anesthetized by i.p. injection of 80/kg of ketamine (Abbott Laboratories, Chicago, IL) and 10 mg/kg of xylazine (Sigma) mixed in 80 μl of PBS. The abdominal area was shaved, and the DNA was delivered into the epidermis. Vaccination was administered at days 0 and 30. Ten days following the last vaccination, mice were sacrificed and spleens were harvested in order to conduct in vivo CTL assays.

2.8.2. Tumor protection experiments

Vaccination was administered at days 0 and 14. At day 21 mice were challenged with 6 × 10^4 TC-1 tumor cells in 100 μl of HBSS (Sigma). Individual mice were monitored two or three times a week for 57 days for tumor growth and survival. TC-1 cells were derived from a large batch of TC-1 tumor cells of the same passage, tested for tumor formation, and frozen in liquid nitrogen. For every tumor injection a vial from this batch was thawed and grown according to a standard procedure for 10 days to obtain the required number of tumor cells. On the day of injection, 80% confluent cell cultures were trypsinized (Invitrogen) and washed three times with HBSS (Sigma). Cells were concentrated at 6 × 10^5 cells/ml, and 100 μl was injected s.c. in the left flank of the mice.

2.8.3. Tumor therapy

Mice in therapy experiments received a tumor s.c. injection with 2 × 10^5 cells/ml of TC-1 cells in 100 μl of HBSS (Sigma) 10 days before DNA introduction. By day 10, all mice had palpable tumors defined as the presence of a small
3.2. Anti-tumor efficacy of the E7IR vaccine in a prophylactic setting

Groups of five C57BL/6 mice were vaccinated with 4 μg of DNA constructs containing the E7IR gene, the E7WT gene, or the APL023 vector alone twice at 2 week intervals and challenged with $6 \times 10^4$ TC-1 tumor cells 7 days later. The mice were then monitored and measured for 57 days for tumor growth and survival. By day 33 all naïve and vector vaccinated mice were tumor bearing, 3/5 mice vaccinated with the WT construct were tumor bearing and none of the animals vaccinated with the E7IR construct were tumor bearing. As a result, vaccination with the E7IR construct promoted 100% survival in these mice to 57 days (Fig. 4).

3.3. Efficacy of the E7IR vaccine to treat established tumors

Given the promising results of the prophylactic tumor studies, a therapeutic study was performed to test the ability of the E7IR vaccine to treat existing tumors. Groups of five C57BL/6 mice received $2 \times 10^4$ TC-1 tumor cells. After 10 days, all mice had palpable tumors defined as the presence of a small mass bearing an approximate volume of 0.125 mm$^3$, and were immunized with either E7WT, vector alone or the E7IR DNA vaccine. Therapeutic treatment was initiated at day 10 and repeated 7 days later. Fig. 5 demonstrates that vaccination with the E7IR construct resulted in a decreased tumor growth compared to control groups on day 34, the last time point before mice had to be sacrificed due to overt tumor induced pathology. A repeat of this experiment, combined with the data shown in Fig. 5 produced a composite p-value ($p = 0.01$) that indicates that vaccination with the E7IR construct showed a significant decrease in tumor volume compared to vaccination with the E7WT construct.

Fig. 1. E7IR vaccine construction. (A) Design of the E7IR vaccine construct. The native E7 protein was split into three arbitrary regions designated as a, b, or c. In the a region amino acids 21, 24, and 26 were modified from DLYCYEQ to GLYGYGQ to reduce transformation activity. In the b region a split was introduced between two cystine residues at position 31 and 32 to reduce transformation activity. In the c region two zinc finger binding motifs were disrupted by a split between amino acids 60 and 61 and modifications of C61 to G61 and C94 to G94 (both in bold). All three regions, a, b, and c were replicated to enhance expression and H-2Db and HLA-A*0201 epitopes were added downstream of the aabbcc construct with AAY as a spacer. Start (M) and stop (OCH) codons were included on the 5′ and 3′ end respectively. (B) The amino acid sequence of the vaccine used for vaccination with the human HLA-A*0201 and the H2Db epitopes underlined and with the AAY spacers in bold.

mass bearing an approximate volume of 0.125 mm$^3$; DNA administration was initiated on day 10 and repeated 7 days later. During this time the tumor sizes in the mice were recorded two or three times a week.

2.9. Statistical analysis

Graphs were generated and one-tailed T-tests were performed using GraphPad Prism Version 4.00 for Windows (GraphPad Software, San Diego, CA, USA, www.graphpad.com). Composite one-tailed p-values for tumor challenge studies were generated from two independent experiments with equal variance by combining z values generated from the mean and standard deviation in a Fischer’s method meta-analysis using the program Quick Calses Graphpad Prism Software (http://www.graphpad.com/quickcals).

3. Results

3.1. Analysis, protein expression and transformation capacity of the E7IR vaccine construct

The construction of the E7IR DNA construct utilized for vaccination studies is represented in Fig. 1. Protein expression of the E7IR construct in the APL023 mammalian expression vector was confirmed and compared to expression of the HPV-16 E7 WT by Western blot analysis using anti-HA antibodies to show the E7IR construct with an HA tag (Fig. 2A) and the E7WT and E7IR protein with an HPV-16 E7 antibody (Fig. 2B). Retroviral infected NIH 3T3 cells confirmed the background level transformation efficiency of the E7IR construct compared to the wild type in a soft agar transformation assay (Fig. 3).
3.4. Cellular immune responses induced by vaccination with E7IR DNA construct

Immune responses generated by vaccination with the E7IR construct were evaluated in a cellular immune assay. Mice were vaccinated twice with the E7WT, vector alone or E7IR DNA vaccine. Splenocytes from vaccinated and naive animals were then tested for MHC class I-restricted T-cell responses to the RAHYNIVTF H-2Db peptide by in vivo CTL. The in vivo CTL assay measured the specific CTL-mediated killing of naïve splenocyte target cells loaded with the RAHYNIVTF peptide and a PSA5 irrelevant control peptide. Fig. 7 demonstrates the percent lysis of loaded targets in the different vaccinated groups based on the remaining ratio of relevant to irrelevant peptide. Mice that were immunized with the E7IR construct showed significantly increased survival in prophylactically vaccinated mice. Survival was monitored for 57 days in mice prophylactically vaccinated with the E7IR construct. Groups of five mice were vaccinated twice at 2 week intervals and challenged with TC-1 cells 7 days later. At 57 days post TC-1 challenge, 100% of E7IR mice survived compared to 40% of mice vaccinated with the E7WT construct or 0% of naïve or vector alone treated animals. Data are representative of two experiments.
Fig. 5. Mean tumor volume in therapeutic E7IR vaccine treatment. In therapeutic experiments with the E7IR vaccine, $2 \times 10^4$ TC-1 cells per mouse were injected into 5 mice per group on day 0. After all mice in all groups had a palpable tumor (day 10), the first gene gun vaccination was given, followed by a second vaccination 7 days later. Numbers equal the number of tumor bearing mice in each group at day 34, the last day that all mice were living. Data are representative of two experiments. A composite $p$-value generated from these two experiments indicates that vaccination with the E7IR construct showed a significant decrease in tumor volume compared to vaccination with the E7WT construct ($p = 0.01$).

Fig. 6. Percent survival in therapeutically treated mice. Survival was monitored for 103 days in groups of five mice therapeutically treated with the E7IR construct. At 103 days post TC-1 challenge, 60% of E7IR mice survived compared to 0% of naïve or E7WT vaccinated mice. Data are representative of two experiments.

Fig. 7. In vivo CTL. Five mice per group were vaccinated with the E7IR vaccine twice in a 30 day interval, CFSE-labeled target cells loaded with E7 (49–57) peptide or control peptide were injected into vaccinated mice. After 18 h, splenocytes from vaccinated mice were harvested and flow cytometry was utilized to determine percentages of CFSE$^{\text{low}}$ and CFSE$^{\text{high}}$ target populations and were used to perform an in vivo cytotoxicity assay. Data were normalized compared to background lysis seen in naïve, non-tumor challenged mice. Administration of the E7IR vaccine caused significantly more lysis of target cells than administration of the wild type E7 gene ($p = 0.0001$).

4. Discussion

High risk infections with HPV are highly prevalent but generally transient and are believed to be controlled by the cell mediated immune response since a majority of HPV-induced lesions spontaneously regress [21]. The essential factors necessary for HPV-induced malignant progression are largely unknown but may be related to suboptimal or inappropriate T cell responses to HPV antigens during a natural infection [21]. The human HPV E6 and E7 proteins are selectively expressed in cervical cancer cells, making them excellent targets for T cell mediated immunotherapy [22]. Therefore, the goal of immunization against HPV-induced carcinomas is to activate cellular components of the immune system to recognize and attack cells infected with HPV. Numerous vaccination strategies against HPV-induced tumors have yielded encouraging results in mice. Among them, DNA vaccination has emerged as an attractive strategy because of low production costs, overall stability and ease of storage [14]. Additionally, naked DNA vaccines lack unwanted immune responses directed against other components of the vaccine that is commonly observed in the case of familiar vectors in vector based prime-boost regimens [14,23]. Lastly, it has been shown that delivery of gold coated DNA particles into the skin will activate dendritic cells and induce migration to the draining lymph nodes to initiate an immune response [14].

The E7IR DNA vaccine candidate presented in the current study is based on the E7 oncogene of HPV-16. HPV-16 is the most common type among cervical cancer cases worldwide with about 54% of cervical carcinomas attributed to the HPV-16 genotype [24]. As the HPV-16 E7 oncoprotein is constitutively expressed in cervical cancers, it is considered a good target for immune therapy of existing lesions and several E7-specific therapeutic vaccines are currently under development in preclinical models [25]. Additionally, the E7 oncoprotein contains several identified MHC I CTL epitopes [26]. However, although DNA vaccination appears to be a safe means to deliver HPV antigens, DNA vaccination with oncoproteins still harbors the risk of transformation for the cells that receive and express the oncoproteins [27,28]. Thus, E7 based vaccine strategies seek to simultaneously eliminate or reduce the transforming ability and enhance the immunogenic potential of the E7 oncoprotein.

The E7IR vaccine design attempts to resolve these challenges first by utilizing well characterized mutations that interfere with binding to the host cell Rb protein and by creating a gene coding for an artificial E7 protein that is unlikely to keep its original function through inserted and replicated sequences thus reducing the transformation capacity of the construct [17]. Another commonly utilized method to reduce the transforming capacity of the E7 oncoprotein has
been to create epitope based vaccines typically containing a few common human epitopes [12]. Comparatively, an advantage offered by the E7IR vaccine strategy is that the entire coding region is intact and thus there are no HLA restrictions that require consideration in wide-spread vaccine application. In addition, duplication of the gene sequence should cause duplication of all inherent CTL epitopes and the E7IR construct was designed with codon modifications that favor translation in mice, a strategy that has proved effective in enhancing translation of the E7 gene [10,29]. Mechanistically, enhanced expression of CTL epitopes is probably accomplished by increased protein production as the Western blot in Fig. 2B would suggest. In the experiments presented here, the immunological effects of the vaccine are likely due to the H-2Db RAHYNIVTF (49–57) epitope, the only E7 CTL epitope described for the C57BL/6 mouse. The in vivo CTL assay shown in Fig. 7, in which target cells are coated with the RAHYNIVTF peptide, is showing roughly two times higher lysis in the mice vaccinated with the E7IR than those vaccinated with the wild type. Additionally, H-2Db and HLA-A*0201 epitopes were added downstream of the construct using AAY spacers, which has been shown to increase epitope processing resulting in higher CTL precursors [7]. One limitation to this approach in which it was necessary to attach unique sequences onto the 3’ end for the purposes of cloning, is that in its current form we cannot definitively decipher the contribution that additional H-2Db and HLA-A*0201 epitopes are making to the immune response. However, we felt that in contrast to attaching an irrelevant epitope, attachment of these two well characterized CTL epitopes would serve to maximize vaccine potency. The results indicate that indeed this construct did enhance the immune response to E7. Therefore, the E7IR may provide a good and safe backbone representation of mutated E7, especially when combined with other well characterized modifications such as targeting antigens to endosomal/lysosomal compartments [30]. One last advantage of the E7IR vaccine strategy is that it could easily be incorporated into vector based approaches, such as Venezuelan Equine Encephalitis virus (VEE) replicons, for which there is no pre-existing immunity in a majority of the human population and which specifically target dendritic cells in order to initiate an immune response [25]. Other possibilities include combining sophisticated and elegant methods of targeting antigens to the MHC I and II pathways [31] with the E7IR gene as a backbone. Ultimately the pairing of some other well characterized modifications such as targeting antigens to endosomal/lysosomal compartments [30].

In the present study, vaccination with the modified E7IR construct was able to significantly reduce tumor volume and enhance survival in both prophylactic and therapeutic experiments in mice compared to the WT E7 gene. Additionally, vaccination with E7IR lead to a significant increase in CTL-mediated lysis of peptide loaded target cells compared to vaccination with the WT construct. Taken together these results indicate that this design may represent an innovative way to reduce the transforming capacity and simultaneously enhance the immunogenicity of the HPV-16 E7 gene for use in therapeutic vaccination regimens to treat cervical cancer.

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