

DNA nanotherapy for pre-neoplastic cervical lesions

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HIGHLIGHTS

- ▶ HPV16 genomic sequences have potential for targeting therapeutic gene expression to cervical lesions.
- ▶ Thermal sensitive gels, e.g., Lutrol F127, can be used to target DNA-nanoparticle uptake to cervical pre-neoplastic cells.

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ABSTRACT

Objective. This study aims to test the hypothesis that targeted nanoparticle delivery of DNA encoding HPV16-regulated diphtheria toxin (DT-A) will result in the death of HPV16-infected cells.

Materials and methods. Plasmid constructs containing a HPV16 Long Control Region (LCR) DNA sequence upstream of DT-A or luciferase reporter (Luc) DNA sequences were used to formulate poly(β -amino ester) nanoparticles. The effect on tumor growth of HPV/DT-A-nanoparticle injection directly into HPV16⁺ CaSki human cervical cancer cell-derived xenografts in mice was determined. To evaluate the ability of the HPV16 LCR regulatory sequence to activate gene expression specifically in HPV16-infected cells, mice underwent bioluminescent optical imaging following intraperitoneal injection of HPV/Luc-nanoparticles. The use of Lutrol F127, a thermal-sensitive gel, to target delivery of nanoparticles and subsequent gene expression to cervical epithelial cells was evaluated in *ex vivo* cultures of mouse cervix and following intravaginal delivery of nanoparticle/gel in mice, as well as in *ex vivo* cultures of surgical LEEP samples.

Results. The selected HPV16 LCR regulatory sequence activates gene expression in both HPV16-infected cells and non-infected cells. However, in the cervix, it is specifically active in epithelial cells. Following exposure of cervical cells to HPV/DT-A-nanoparticles mixed with Lutrol F127 gel, DT-A is expressed and cells die.

Conclusions. An HPV16 DNA sequence that targets gene expression specifically to HPV16-infected cells remains to be discovered. Topical application of a Lutrol F127 thermal gel/nanoparticle mix is illustrative of how to restrict exposure of cells to therapeutic nanoparticles, thereby allowing for targeted DNA delivery to cervical pre-cancerous lesions.

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Introduction

Cervical cancer is the third most common malignancy affecting women worldwide and responsible for a quarter of a million deaths each year, primarily in developing countries [1]. While improved screening tests and vaccines have significantly reduced the incidence of cervical cancer in the United States, cervical dysplasia, often the precursor to invasive malignancy, remains a common clinical challenge, particularly in younger women.

Excisional surgical procedures including cold-knife conization and loop electrosurgical excision procedure (LEEP) are the standard treatments for high-grade precancerous lesions [cervical intraepithelial neoplasia 2 and 3 (CIN2 +)]. Up to 500,000 of these procedures are

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performed each year in the United States. These treatments usually have minimal short-term complications including cramping and bleeding. There are, however, more serious associated risks of reproductive complications including preterm delivery, increased rate of cesarean sections, premature rupture of membranes, and infection [2,3]. In addition, these procedures require trained practitioners with appropriate surgical skills, a requirement that restricts their use in developing countries where they are especially needed. Gynecological care would greatly benefit from an effective, simply-administered, noninvasive therapy for high-grade pre-neoplastic cervical lesions.

To address this need, we have considered the now well-established role that infection with any one of the 13 genotypes of the human papilloma virus (HPV) plays as the inciting event in nearly all cases of cervical cancer [4]. HPV16 infection causes over half the cases of cervical cancer worldwide (54.6%), while HPV18 infection causes 15.8%. The HPV circular genome of ~8 kb is functionally divided into 3 regions – the early, late, and long control regions. The early and late regions encode viral proteins (E1, E2, E8+E2, E4, E6, E7 and L1–L2), while the LCR, consisting of 850 base pairs between the L1 and E6 genes, contains binding sites for multiple transcription factors and other proteins that define enhancer and silencer sequences, as well as the early p97 promoter that regulates expression of the major transforming proteins, E6 and E7 [5]. Prototypical and variant control sequences, such as those within the HPV16 LCR, have evolved to efficiently support a complex program of early gene expression that supports the establishment of HPV infection and its persistence in epithelial cells [6].

In prior work, we have established a strong foundation for the use of gene promoters that are highly active in tumor cells to target diphtheria toxin (DT-A) expression to these cells [7–9]. In these studies, polymeric nanoparticle delivery of transcriptionally-regulated DT-A DNA to prostate and ovarian tumor cells resulted in targeted death of tumor cells and minimal non-specific toxicity. Now, in this study, we set out to target DT-A expression to HPV16-infected cells, but not to HPV16-non-infected cells, using a DNA sequence from the HPV16 LCR to regulate DT-A expression. The selected sequence failed to restrict gene expression to HPV16-infected cells, but it did target toxin production specifically to epithelial cells. We show in further studies that use of a copolymer with thermoreversible properties, Lutrol F127, in combination with HPV16/DT-A DNA-nanoparticles, allows for delivery of the nanoparticles to the tissue overlaid by the thermo-induced gel-nanoparticle mix, and consequently yields expression of the toxin in the underlying targeted cells.

Materials and methods

Plasmid constructions

pRG4X/Luc

pRG4X/CAT (6) was digested with *SacI* and *HindIII* to remove the CAT sequence. A DT-A fragment, obtained from pGL4/HE4/DT-A plasmid [7] by digestion with *BglII* and *XbaI*, was ligated to (*NheI* and *BglII*)-digested pGL4/Luc2.0. Following ligation, the new pGL4/DT-A construct was cut with *SacI* and *HindIII* in order to obtain the DT-A fragment. This 1300 bp fragment was then ligated with the RG4X to make RG4X/DT-A.

pRG4X/DT-A

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pRG4X/XX

pRG4X/CAT was digested with *HindIII* and *SacI* to remove the CAT sequence. Mung bean nuclease was used to blunt the cut ends that were then ligated to obtain pRG4X/XX (*i.e.*, no coding sequence).

pRG4X/GFP

pEGFP-1 was digested with *AflIII* (4.2 kb), cut ends were blunted, and then the 4.2 kb fragment was digested with *HindIII* to yield a 3.2 kb fragment and 1 kb EGFP fragment. pRG4X/XX was digested with *SacI*, cut ends were blunted, and then the 4.6 kb fragment was digested with *HindIII* to yield 1.6 kb and 3.0 kb fragments. The 3.0 kb fragment was ligated to the 1 kb EGFP fragment.

Cells

CaSki, SiHa, HeLa, and HepG2 cells were purchased from the American Type Culture Collection (ATCC). CaSki cells were grown in RPMI-1640 + 10% FBS; the other cell lines were grown in ATCC-formulated Eagle's Minimum Essential Medium + 10% FBS. Cells were transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturer's directions.

Nanoparticle formulation

Complexation of DNA with C32-117 poly(β -amino ester) polymer to generate nanoparticles was performed as previously described [10]. DNA was prepared using the Endofree Plasmid Kit (Qiagen).

Lutrol F127/nanoparticle mix

90 μ l 40% Lutrol F127 (Sigma) in OptiMEM (LifeTechnologies) was mixed well with chilled 120 μ l nanoparticles prepared with 100 μ g DNA. A pre-chilled syringe with a 18G 11/2 blunt needle was filled with the total volume of 210 μ l and used to inject the mix into the vagina of host mouse.

Mouse experiments

All experiments using mice were approved by the Lankenau Institutional Animal Care and Use Committee (IACUC).

Xenografts

To generate xenografts, eight-week old female athymic nude-*Foxn1^{tmu}/Foxn1⁺* (nude) mice (Harlan) were first anesthetized with Avertin (500 mg/kg, *i.p.*), and then injected subcutaneously in each flank with 2×10^6 HPV16⁺ CaSki cells suspended in 100 μ l PBS containing 20% Matrigel using 30G needles. When a tumor volume of ~150 mm³ was identified, tumors were injected with RG4X/DT-A or RG4X/XX nanoparticles (50 μ g DNA/injection in 60 μ l volume), or buffer only ($n = 5-8$ /treatment group). Mice were weighed and calipers were used to measure tumor dimensions prior to each injection. Tumor volume was calculated using the formula ($\text{length} \times \text{width}^2 \times 0.52$).

Intraperitoneal injection

120 μ l DNA nanoparticles (100 μ g) was injected intraperitoneally using a 28G 1/2 insulin needle. 6 h and 24 h post-injection with luciferase DNA/nanoparticles, mice were optically imaged using IVIS 100 series Bioluminescence Imaging System (Caliper Life Science), using 5 minute integration time as previously described [9]. After the 24 h whole body image was obtained, mice were euthanized and *ex vivo* imaging performed.

Terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL)

Apoptotic cells were identified by TUNEL assay using an In Situ Detection Kit (Roche Boehringer Mannheim) as previously described [8].

LEEP and hysterectomy specimens

Specimens for this study were obtained from consenting patients. The surgeon sterilely excised a 1–2 mm³ piece from LEEP specimens, being careful not to disturb the margins of the specimen. All procedures for specimen procurement were approved by the Main Line Hospitals Institutional Review Board.

HPV16 PCR genotyping

DNA was isolated from surgical specimens using a DNeasy Blood and Tissue Kit (Qiagen). HPV16 status was assayed by PCR using the following primers: 5'-AAGGCCAACTAAATGTCAC-3' (forward primer) and 5'-CTGCTTTTATACAACCGG-3' (reverse primer). PCR reaction mixes contained 100 ng genomic DNA, 1 × GoTaq buffer, 3 mM MgCl₂, 200 μM dNTP, 0.4 μM of each primer, and 0.125 U GoTaq polymerase. Reactions were run for 40 PCR cycles (95 °C for 30 s, 55 °C for 60 s, 72 °C for 60 s).

Results

DNA constructs

Based on previous characterization of a proximal enhancer 63 base pair sequence (7656–7719) in the HPV16 LCR, the RG element, and its established preferential function in human keratinocytes of genital skin and mucosa [6,11], we chose to test this sequence for its potential to target DT-A expression specifically to HPV16-infected cells (Fig. 1). In addition to binding motifs recognizing the ubiquitously expressed transcription factors NF-1 and AP-1, the RG element contains TEF1 and AP-2 transcription factor binding sites as well as the cytoke- ratin element that contributes to epithelial cell preference. In this study, we used four DNA constructs that each contains 4 head-to-tail tandem repeats of the RG element and an enhancer-negative SV40 promoter upstream of either green fluorescent protein (GFP), luciferase (Luc), or the diphtheria toxin A chain (DT-A), as well as a control construct that has no coding sequence (Fig. 1). In prior work, 4 tandem repeats of RG yielded significantly higher expression of CAT than a single copy in spontaneously immortalized human keratinocytes [6].

RG4X-regulated DT-A suppresses protein synthesis and cervical tumor growth

DT-A toxin inhibits protein synthesis by catalyzing the ADP-ribosylation of EF2 elongation factor [12]. To assess how effectively HPV16 RG element-regulated DT-A expression suppresses protein

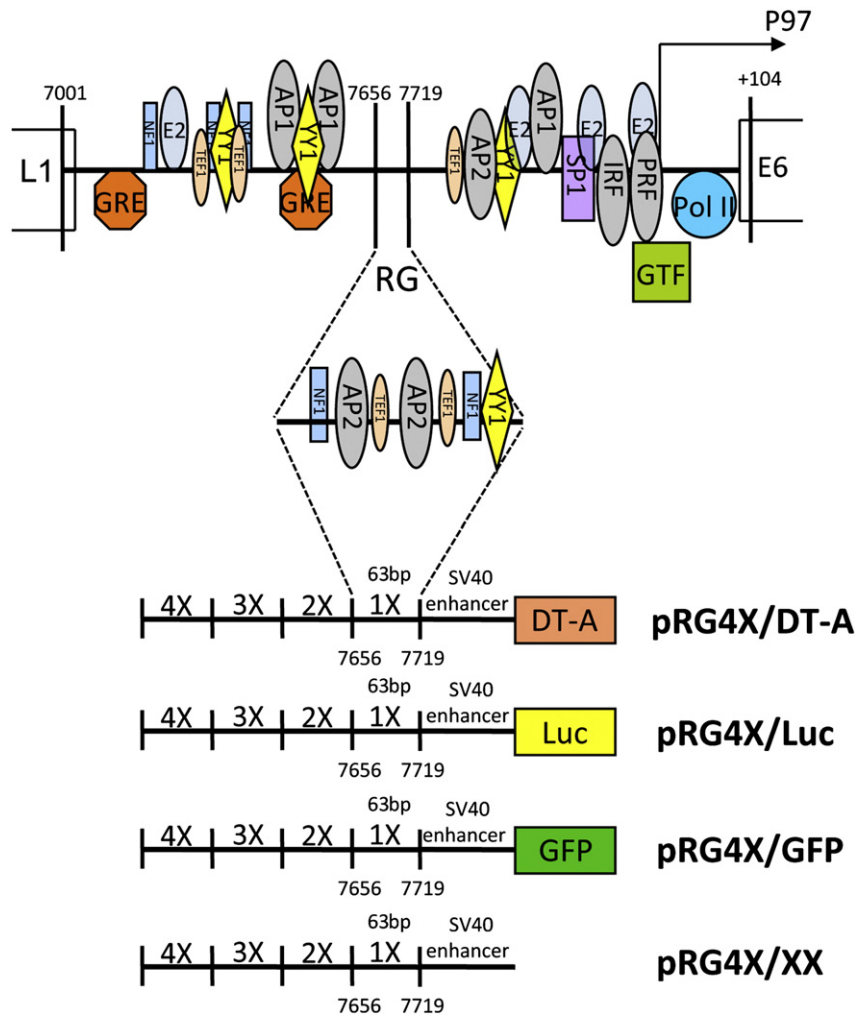


Fig. 1. Top: Map of transcription factor binding sites in the HPV16 LCR. Bottom: DNA constructs used in this study. Each contains 4 tandem repeats of the RG region of the LCR.

synthesis, we co-transfected HPV16⁺ CaSki and SiHa human cervical cancer cells with pRG4X/DT-A and pCAG/Luc, and measured luciferase activity 24 h later. Following co-transfection with RG4X/DT-A + CAG/Luc plasmids, we observed a >90% inhibition of luciferase activity in these cells compared to activity following transfection with CAG/Luc alone (Fig. 2A). Less inhibition of luciferase activity was observed following co-transfection of HPV16⁻ cell lines, HeLa and HepG2 (84% and 15%, respectively).

To evaluate whether nanoparticle delivery of RG4X/DT-A DNA suppresses the growth of HPV16⁺ cells, subcutaneous xenografts of CaSki human cervical cancer cells in nude mice were injected intratumorally with RG4X/DT-A nanoparticles biweekly for four weeks (n = 8). Control mice were injected with RG4X/XX nanoparticles (*i.e.*, no protein coding DNA; n = 5) or buffer only (n = 7). Tumor volumes were measured prior to each injection and 4 days after the last injection prior to sacrifice. The growth rate of tumors injected with RG4X/DT-A nanoparticles was slower than that of tumors in both control groups (Fig. 2B), although the difference was not statistically significant (DT vs buffer, p = 0.12; DT vs XX, p = 0.27).

RG4X activity is not restricted to HPV16⁺ cells

To determine whether the RG4X element was specifically active in HPV16-infected cells, we injected mice intraperitoneally with RG4X/Luc-nanoparticles and optically imaged the mice 24 h later. Bioluminescence was observed in multiple organs, indicating that the RG4X element was active in non-HPV16 infected cells (Fig. 3).

To further examine RG4X specificity, we obtained normal-appearing cervical tissue from eight women that had undergone hysterectomies for reasons unrelated to cancer. Using HPV16-specific oligonucleotides for PCR amplification of DNA isolated from a piece of each specimen, we determined that all specimens were HPV16 negative. The balance of each specimen was incubated with RG4X/Luc nanoparticles and then optically imaged. Bioluminescence was detected in 5 of the 8 HPV⁻ specimens. Luciferase expression was also observed in HPV⁻ human lung cancer A549 cells following transfected with RG4X/Luc DNA, providing further evidence for the non-specific activity of the RG4X element (data not shown). In sum, these data clearly indicate that the RG4X sequence lacks specificity and is active in both HPV16-infected and non-infected cells.

Gene expression following exposure of cervical cells to DNA nanoparticle/Lutrol F127

As an alternative to targeting gene expression to HPV16 infected cervical cells through RG4X transcriptional regulation, we considered

targeting nanoparticle delivery of DNA to infected cells. Lutrol F127 (also known as Pluronic® F127 and Poloxamer 407) is a hydrophilic thermal sensitive triblock copolymer surfactant that has previously been used in the direct production of solid lipid and poly(lactic-co-glycolic acid) (PLGA) nanoparticles for drug delivery [13,14], and combined with Poloxamer 181 for gene transfer and expression of plasmid DNA to skeletal muscle [15]. The phase transition of Lutrol F127 from an aqueous solution to a gel that occurs as temperature increases to 15 °C to 25 °C, dependent on polymer concentration and co-formulation components, makes it an attractive vehicle with which to mix with nanoparticles at cold temperature and then deliver to a defined area at physiological temperature (37 °C) where the mixture gels [16].

We tested a mix of DNA nanoparticles and Lutrol F127 for ability to deliver and express DNA when the mix was applied to cervical cells in *ex vivo* and *in vivo* assays. Luciferase was expressed in cervical tissue following incubation of resected mouse cervix with RG4X/Luc nanoparticles in Lutrol F127 and following intravaginal administration of the mix in mice (Figs. 4A and B). RG4X-regulated luciferase expression was comparable to luciferase expression driven by a CAG regulatory sequence, a robust, constitutively active sequence with no apparent tissue restriction, consisting of the CMV IE enhancer and the chicken β -actin gene promoter [17]. Following intravaginal administration in mice of a mix of RG4X/GFP nanoparticles with Lutrol F127, immunostained sections of fixed, resected cervical tissue showed GFP protein present in cervical epithelial cells, but not in underlying stromal mucosa (Fig. 4C, top row). Consistent with this observation, following intravaginal delivery of RG4X/DT-A nanoparticles suspended in Lutrol F127, mouse cervical epithelial cells underwent apoptosis as a consequence of DT-A expression as demonstrated by TUNEL assay of sectioned cervical tissue harvested 24 h after administration of the nanoparticle/gel mix (Fig. 4C, bottom row).

Gene expression following exposure of LEEP surgical specimens to DNA nanoparticle/Lutrol F127

To determine whether nanoparticles in Lutrol F127 gel can deliver DNA to HPV-infected cervical cells, and whether the DNA can be expressed, pieces of surgical LEEP specimens were overlaid with nanoparticles suspended in gel and incubated for 24 h. Following incubation, specimens were optically imaged. Specimens treated with either RG4X/Luc and CAG/Luc nanoparticles emitted bioluminescence, while control specimens treated with pGL4 nanoparticles did not (Fig. 4D). The observed bioluminescence is the result of expression of nanoparticle-delivered DNA encoding luciferase.

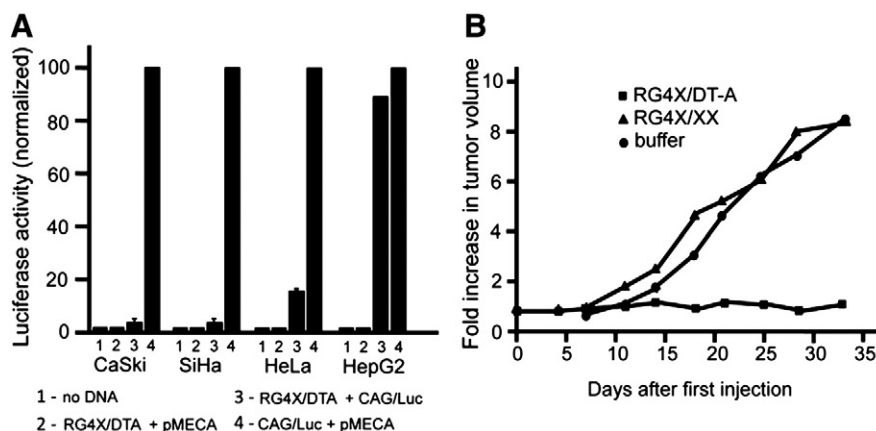


Fig. 2. RG4X-regulated DT-A suppresses protein synthesis and cervical tumor growth. A. Luciferase activity in CaSki cells 24 h after co-transfection with (RG4X/DT-A + CAG/Luc) and control co-transfections, or no DNA. Activity is normalized to the activity of cells transfected with (CAG/Luc + pMECA). B. Fold increase in CaSki xenograft volumes. Tumors were injected twice a week for 3 weeks with RG4X/DT- or with RG4X/XX-nanoparticles, or with PBS buffer.

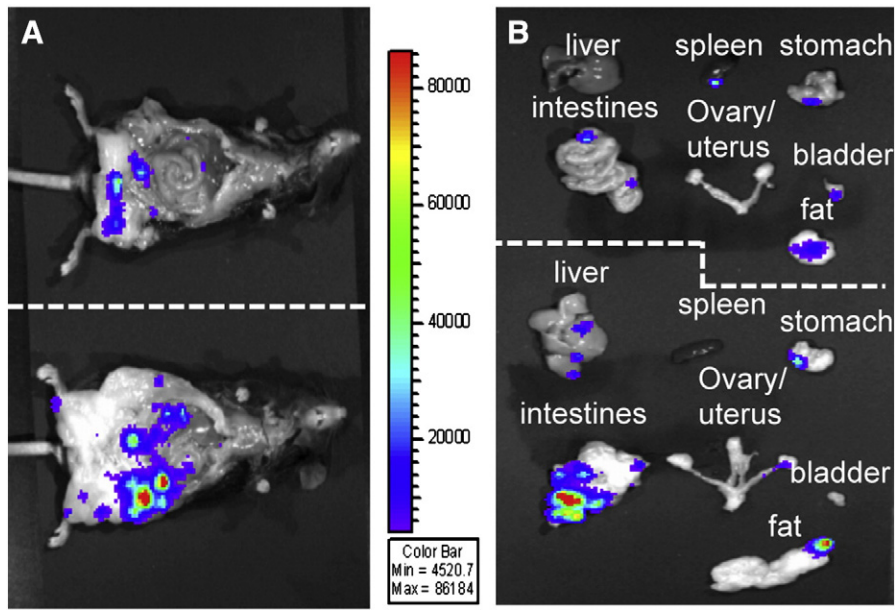


Fig. 3. Optical images of (A) whole carcasses and (B) individual resected organs 24 h after intraperitoneal injection of C57BL/6J mice with RG4X/Luc nanoparticles.

Discussion

It is now recognized that infection with human papilloma virus (HPV) is the inciting event for all cervical cancer and a significant portion of head and neck cancers [18,19]. Together, infection with HPV16 and HPV18 accounts for approximately 70% of cervical cancers. The association of HPV16 infection with ~50% of cervical cancer cases led us to test a DNA sequence of the HPV16 LCR for its ability to

activate diphtheria toxin (DT-A) suicide gene expression in HPV16-infected cells, but not in non-infected cells. Protein synthesis is inhibited in cells that produce DT-A toxin due to catalysis of ADP-ribosylation of the EF2 translational elongation factor [12]. In the absence of the B chain that is present in the native DT protein, if DT-A produced by a cell were to be secreted, it could not be taken up by neighboring cells, thus avoiding non-specific expression and diphtheria-associated pathology.

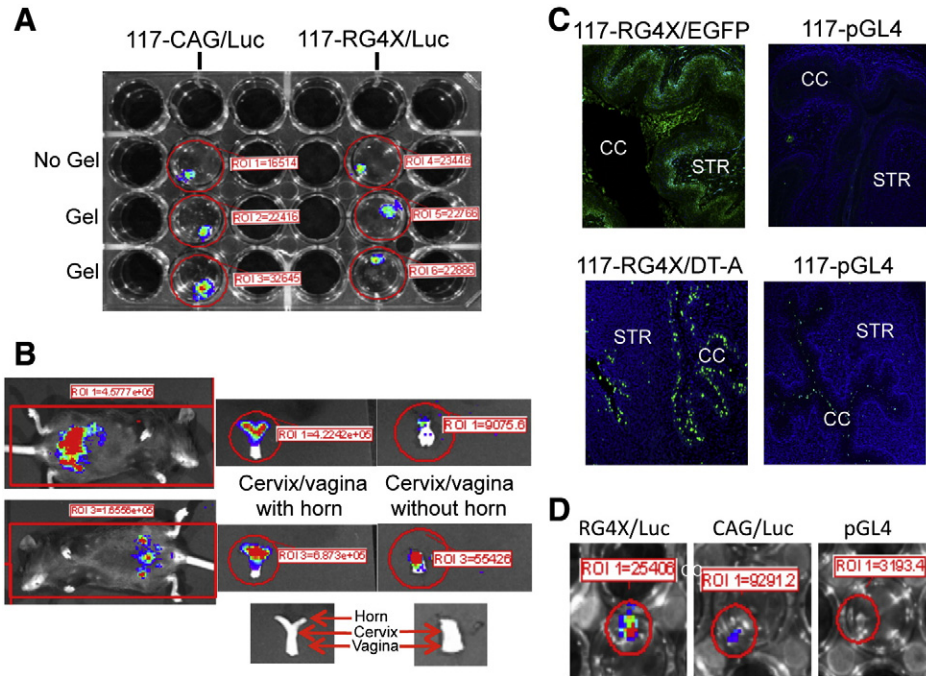


Fig. 4. DNA expression following *ex vivo* and *in vivo* exposure of cervical cells and tissue to DNA nanoparticles suspended in Lutrol F127 gel. A. Bioluminescence 24 h after *ex vivo* overlay of 117-nanoparticles +/- gel onto mouse cervical tissue. B. Bioluminescence 24 h after intravaginal injection of 117-RG4X/Luc + gel (100 µg DNA). Whole mouse images (left) and images of resected horn/cervix/vagina and cervix/vagina without horn from the same mice (right). C. Top row: Sections of mouse cervix 24 h after intravaginal injection of gel mixed with 117-RG4X/EGFP (left) or 117-pGL4 control nanoparticles (right) and immunostained for GFP. Bottom row: Terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL) of sections of mouse cervix 24 h after intravaginal injection of gel mixed with of 117-RG4X/DT-A (left) or 117-pGL4 control nanoparticles (right). CC, cervical canal; STR, stroma. D. Bioluminescence of HPV16⁺ LEEP sample 24 h after *ex vivo* incubation with overlaid Lutrol gel/DNA nanoparticles.

One way to target DNA-based nanotherapies to specific cells is to use gene promoters that activate gene expression in a subset of cells, but not in others. Previous work suggested that a 63 bp LCR DNA sequence, RG, might have the desired specificity for activating DT-A gene expression specifically in HPV16-infected cells and that four tandem repeats of this sequence would enhance expression above that obtained using one copy [6]. However, when we injected RG4X/Luc nanoparticles into the peritoneal space of mice and then assessed luciferase expression 24 h later by optical imaging, we observed bioluminescence in multiple organs. The fact that RG was active in non-HPV infected mouse cells and failed to retain species specificity, a hallmark of HPV16 infection, indicates that RG lacks the ability to target gene activity specifically to HPV-infected cells. Interestingly, the cytokeratin element in the RG sequence retained its ability to restrict gene activation to epithelial cells in the cervix. Future studies will test the targeting ability of other HPV16 regulatory sequences.

A second way to achieve specificity of nanotherapeutics is to limit the exposure of cells to nanoparticles, thus restricting DNA delivery. In this report, we treated mouse cervical tissue and HPV16⁺ precancerous LEEP specimens with DNA-nanoparticles suspended in Lutrol F127 thermo-sensitive gel. The results of these proof-of-principal experiments demonstrate that nanoparticle delivery and expression of DNA occur in cells that are in contact with the gel. The ability of Lutrol gel to adhere specifically to precancerous foci on the cervix for any length of time, however, is questionable. We are currently testing other delivery vehicles that may have better mucosal adherence properties.

We note that in prior preclinical studies of DT-A nanotherapy in mouse models of prostate and ovarian cancer, there has been no evidence of adverse physiological effects, including an immune response, to long-term treatment with poly(β -amino ester)-formulated nanoparticles [7,9]. Thus, multiple administrations of DT-A nanotherapy to cervical lesions may be possible if needed. In the 2–10% of cases where recurrent CIN3 develops following surgical procedures despite attainment of negative margins, DT-A nanotherapy could also be considered as a preferred follow-up treatment over further surgery [20].

We observed a reduction in the rate of tumor growth of xenografts in mice treated with RG4X/DT-A nanoparticles as compared to control-treated tumors. This therapeutic response suggests the utility of DT-A nanotherapy for the treatment of pre-invasive and early invasive cervical cancer, perhaps by direct injection of nanoparticles to the tumors.

Despite the development of two prophylactic HPV vaccines, Gardasil and Cervarix, HPV infection leading to cervical cancer remains a huge global problem and is expected to remain so for many years. Approximately 500,000 cases of cervical cancer worldwide are diagnosed each year, with around 270,000 deaths, of which 85% occur in developing countries [21]. Financing a vaccine, distributing it, and treating the primary target population of preadolescent girls, particularly in these countries, represent some of the major challenges facing the healthcare community. Together with improved screening for HPV, a topically applied nanotherapy for precancerous lesions would provide a practical and cost-effective alternative to surgery in those countries that can least afford it, and stem the cancer death rate of women around the world.

Finally, in addition to the causative role that HPV infection plays in cervical cancer, HPV infections are commonly associated with other pathogenic conditions affecting skin and mucosa. Some conditions, e.g., warts on the hands and feet, are benign and easily treatable, while other HPV infections, primarily those that are sexually transmitted, are associated with more serious conditions including anal, nasopharyngeal, oral, and penile cancer. The incidence of oropharyngeal cancers caused by HPV16 infection, in particular, has increased significantly in the United States in recent years and is expected to surpass the incidence of cervical cancer by 2020 [22]. Current treatments for oropharyngeal cancers include surgery, radiation, and chemotherapy

as monotherapies or in combination, and are often associated with severe side effects. Further refinement of the DNA nanotherapy we describe for cervical HPV lesions may offer an alternative, more palliative, and effective treatment for oral/throat cancers as well.

Conflict of interest statement

This paper has not been published elsewhere and all authors have contributed substantially to its contents. Furthermore, there are no financial relationships, and/or conflict of interest between the authors and the subject of this manuscript.

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