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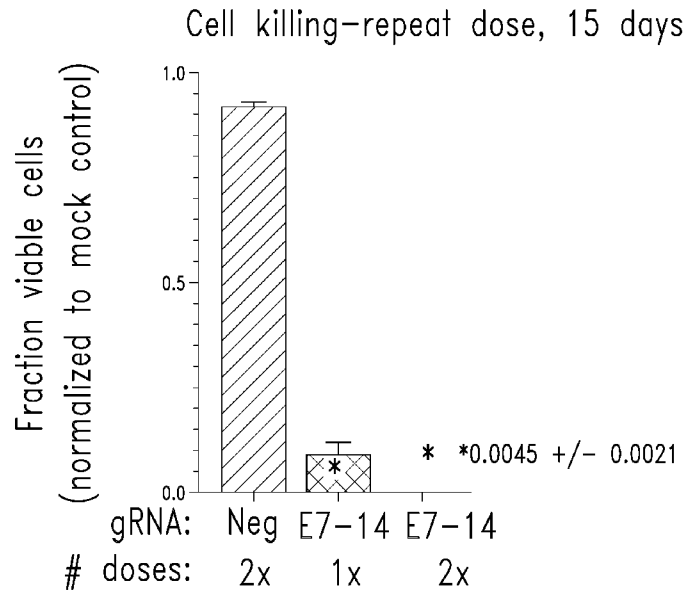

FIG. 3B

(57) Abstract: The present disclosure provides guide RNAs for use in cleaving target human papillomavirus (HPV) nucleic acids. The present disclosure also provides compositions that include an mRNA encoding a Cas endonuclease and a guide RNA having particular sequences and modifications, with both RNA molecules encapsulated by nanoparticles, for the treatment of HPV infection. In some embodiments, the guide RNAs and mRNA encoding a Cas endonuclease are packaged in a lipid nanoparticle, which may be suspended in a carrier formulation for topical or local delivery to infected tissue. In some embodiments, the guide RNA includes features such as modified nucleotides that promote the delivery of the RNAs to, and retention within, infected cells.

[Continued on next page]

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COMPOSITIONS AND METHODS FOR TREATMENT OF HUMAN PAPILLOMAVIRUS

STATEMENT REGARDING SEQUENCE LISTING

The Sequence Listing associated with this application is provided in text format in lieu of a paper copy, and is hereby incorporated by reference into the specification. The name of the text file containing the Sequence Listing is 930285_428WO_SEQUENCE_LISTING.txt. The text file is 36.5 KB, was created on January 19, 2020, and is being submitted electronically via EFS-Web.

BACKGROUND

Viral infections are a significant cause of disease and death worldwide. Cervical cancer is caused by infection with certain types of human papillomavirus (HPV). Two HPV types (16 and 18) cause 70% of cervical cancers and precancerous cervical lesions. HPV is also linked to cancers of the head and neck (oropharynx), anus, vulva, vagina, and penis. According to the World Health Organization, approximately 311,000 women died from cervical cancer in 2018. Worldwide, cervical cancer is the fourth most frequent cancer in women, with an estimated 570,000 new cases in 2018 representing 7.5% of all female cancer deaths.

HPV is a member of the Papillomaviridae, a family of DNA viruses collectively known as papillomaviruses. Papillomaviruses replicate in the basal layer of the body surface tissues. Papillomaviruses are non-enveloped, meaning that the outer shell or capsid of the virus is not covered by a lipid membrane. A single viral protein, known as Ll, forms a 55-60 nanometer capsid. Like most non-enveloped viruses, the capsid is geometrically regular and presents icosahedral symmetry.

HPV infects anogenital and oral mucosa and persists in local basal epithelium. The HPV genome is a double-stranded circular DNA molecule about 8,000 base pairs in length. It is packaged within the Ll shell along with cellular histone proteins, which package the genomic viral DNA. HPV viral DNA exists in both episomal and integrated forms. The HPV genes E6 and E7 are involved in malignant conversion and inhibit the tumor suppressors p53 and RB.

HPV vaccines based on the Ll coat protein must be administered prior to exposure to the virus. Such vaccines cannot treat HPV infection or HPV-associated disease such as cancer. Additionally, there are no approved inhibitors of E6 or E7.

There is no known cure for an HPV infection, and current vaccines do not treat existing infections. There remains a need for therapeutic approaches to preventing and treating HPV infection.

SUMMARY

In certain aspects, the present disclosure provides guide RNAs that target viral nucleic acids, such as HPV16 nucleic acids. In additional aspects, the present disclosure provides a composition comprising an mRNA encoding a Cas endonuclease and a guide RNA. The mRNAs and guide RNAs may include particular sequences and modifications, and may be encapsulated by nanoparticles.

In some embodiments, the composition includes mRNA encoding a modified Cas endonuclease as well as one or more guide RNAs that target the Cas endonuclease to the HPV genome, including full-length and partial fragments of the HPV genome that exist in episomal form and/or as integrated into the host genomic DNA. In certain embodiments, guide RNAs target the E7 region of the HPV genome.

In some embodiments, the guide RNAs may include features such as modified nucleotides that promote the delivery of the guide RNAs to, and retention within, infected cells. In some embodiments, modified nucleotides in RNAs may function to improve RNA stability, reduce immunogenicity, or improve specificity of the endonuclease activity. For example, the guide RNAs can include features such as one or more 2′-O-methyl groups on a ribose ring, one or more phosphorothioate bonds between nucleotides, or both, and particularly located proximal to the 5′ and 3′ termini of the guide RNAs, which may protect against exonuclease digestion.

In some embodiments, the mRNA preferably encodes a programmable nuclease such as a Cas endonuclease or a modified Cas endonuclease. In some embodiments, the Cas endonuclease can include mutations, relative to wild-type Cas9, that may enhance specificity and decrease off-target activity (*e.g.*, by destabilizing interactions with target

DNA at locations outside of the guide RNA targets). In still further embodiments, the RNAs may include modifications such as 5-methoxyuridine that may minimize an immune response to the RNAs by the patient.

In some embodiments, the Cas9-encoding mRNA and guide RNAs are packaged in a lipid nanoparticle (LNP), solid nanoparticle, or liposome. In such embodiments, the RNA-encapsulating nanoparticles can be formulated for topical, mucosal, or local delivery to infected tissue, which avoids systemic delivery and circulation, thus minimizing drug exposure, off-target activity, and immunogenicity of the Cas endonuclease. In some embodiments, the RNAs are packaged in lipid nanoparticles that include, for example, cationic lipids, which balance the charge of the phosphate backbone and promote penetration through tissue and into cells and release of RNA within the cell. Lipid nanoparticles encapsulating a Cas9 encoding mRNA and guide RNA may further be provided in a topical formulation that contains a carrier formulation, which may be a suitable gel or suspension, such as an aqueous suspension, and which may include a tissue retention-enhancing or thickening agent such as, for example, hydroxyethyl cellulose or carboxymethyl cellulose. An LNP formulation as contemplated herein may include one or more excipients to enhance LNP stability such as, for example, sucrose or mannitol. In some embodiments, an LNP formulation as described herein is prepared for topical delivery, and the formulation includes excipients to enhance tissue penetration such as, for example, sodium lauryl sulfate, ethanol, diethylene glycol monoethyl ether (Transcutol), propylene glycol, polyethylene glycol (PEG) esters, sucrose esters, or N-methyl pyrrolidone. In certain embodiments, an LNP formulation according to the present description may be administered with a device to enhance RNA delivery to the basal epithelium, such as, for example, a microneedle array.

In some embodiments, a LNP formulation as described herein is applied topically or locally to a site of infection such as a high-grade pre-cancerous lesion associated with an HPV infection. In such an embodiment, the guide RNA(s) encapsulated by the LNPs are released within the cells, and the mRNAs encapsulated by the LNPs are released and translated by the cell's ribosomes to produce a Cas endonuclease. Cas9 endonucleases for use in the context of the present compositions

and formulations may include linker sequences and one or more nuclear localization sequences (NLS) at the N-terminus and/or C-terminus designed for optimal nuclear localization. Once translated, the Cas endonuclease complexes with the provided guide RNA or guide RNAs to form active RNP. The RNP traffics to the nucleus and binds to the viral genome by virtue of sequence-specific interaction between the complementary portions of the guide RNA and the target within the viral genome. Upon binding to the viral target, the Cas endonuclease cleaves the viral genome. Resultant viral DNA fragments may be degraded or repaired by cellular pathways, thereby clearing or disrupting the infection.

In some aspects, the present disclosure provides a synthetic guide RNA comprising a nucleotide sequence having at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO: 6 or SEQ ID NO: 7. In certain embodiments, the guide RNA comprises the nucleotide sequence as set forth in SEQ ID NO: 6 or SEQ ID NO: 7. In certain embodiments, the guide RNA comprises the nucleotide sequence as set forth in SEQ ID NO: 7.

In some embodiments, the guide RNA comprises one or more modified bases, which may be 2′-O-methylcytidine; 2′-O-methylguanadine; 2′-O-methyluridine; or 2′-O-methyladenine. In some embodiments, the guide RNA comprises a plurality of modified bases, wherein each of the modified bases is independently selected from the group consisting of 2′-O-methylcytidine; 2′-O-methylguanadine; 2′-O-methyluridine; or 2′-O-methyladenine. In some embodiments, the guide RNA is SEQ ID NO: 6, optionally with one or more modified bases each selected from the group consisting of 2′-O-methylcytidine; 2′-O-methylguanadine; 2′-O-methyluridine; and 2′-O-methyladenine. In some embodiments, the guide RNA is SEQ ID NO: 7.

In some embodiments, the one or more of the internucleoside bonds in the guide RNA is a phosphorothioate bond. In some embodiments, one or more of the internucleoside bonds within the ten terminal nucleotides at each of a 5′ end and at a 3′ end of the guide RNA is a phosphorothioate bond. In some embodiments, the guide RNA is SEQ ID NO: 6, optionally with one or more of the internucleoside bonds being phosphorothioate bonds. In some embodiments, the guide RNA is SEQ ID NO: 7.

In some aspects, the present disclosure provides a composition that includes an mRNA encoding a Cas endonuclease; a guide RNA comprising a nucleic acid sequence as set forth in SEQ ID NO: 6 or SEQ ID NO: 7, or versions thereof with modified bases; a plurality of nanoparticles comprising a cationic lipid (*i.e.*, "lipid nanoparticles" or "LNPs") and encapsulating the mRNA and the guide RNA; and a carrier formulation. In specific embodiments, the carrier formulation may include one or more constituents that stabilize the LNPs encapsulating the mRNA and the guide RNA. In further embodiments, the carrier formulation may include one or more constituents that stabilize the LNPs encapsulating the mRNA and the guide RNA as well as one or more constituents that enhance topical or local delivery by promoting tissue retention and/or tissue penetration. The cationic lipid used in the lipid nanoparticles may include palmitoyl-oleoyl-nor-arginine (PONA). In some embodiments, the LNPs further comprise one or more of cholesteryl hemisuccinate (CHEMS), cholesterol, and 1,2-dimyristyl-sn-glycero-3-phosphoethanolamine-N-[poly(ethylene glycol) 2000 (DMPE-PEG2K). In some embodiments, the LNPs comprise PONA, CHEMS, cholesterol and DMPE-PEG2K. In some embodiments, the plurality of LNPs are solid lipid nanoparticles dispersed within the carrier formulation, and the carrier formulation comprises a carrier liquid, oil, or gel. In certain embodiments, the mRNA encapsulated in the LNPs comprises a sequence selected from SEQ ID NO: 2 to SEQ ID NO: 5; the guide RNA encapsulated in the LNPs comprises SEQ ID NO: 6 or SEQ ID NO: 7; and the plurality of LNPs are dispersed within a carrier liquid, oil, or gel provided by the carrier formulation.

In some embodiments, the Cas endonuclease may be Cas9, optionally including at least one variation, relative to wild type Cas9. In some embodiments, an mRNA encoding a Cas9 comprises a sequence selected from SEQ ID NO: 2 to SEQ ID NO: 5. In some embodiments, the mRNA comprises 5-methoxyuridine. In some embodiments, the mRNA comprises 5-methoxyuridine in place of each uridine present in the wild-type Cas9 endonuclease mRNA sequence.

In some embodiments, the compositions include a Cas endonuclease, or a polynucleotide encoding the Cas endonuclease, with one or more substitutions (*e.g.*, between 1 and 25) within, relative to SEQ ID NO: 2 or SEQ ID NO: 3. In some

embodiments, the compositions include a Cas9-HF2 variant endonuclease, or a polynucleotide encoding a Cas9-HF2 variant endonuclease. In some embodiments, the mRNA encoding the Cas endonuclease comprises SEQ ID NO: 4 or SEQ ID NO: 5.

Methods of treating a viral infection are also provided herein. Embodiments of such methods include administering to a subject a therapeutically effective amount of a composition described herein. In some embodiments, such methods include multiple, sequential administration to a subject of a therapeutically effective amount of a composition described herein. In some embodiments, administration comprises topical application to epithelium of the subject. The epithelium may be mucosal epithelium, such as vaginal or anal epithelium.

In some embodiments, the subject is human. In some embodiments, the viral infection is a human papillomavirus infection (HPV). In some embodiments, the subject has an HPV infection and has been diagnosed with HPV-positive pre-cancerous low-grade or high-grade lesions, HPV-positive squamous cell carcinoma *in situ*, or HPV-positive invasive cancer.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a schematic summarizing the selection process used to identify guide RNA molecules.

Fig. 2A-D shows potent and specific activity of Cas9 mRNA + E7-14 gRNA compositions in HPV16-positive SiHa cells. Fig. 2A shows that, in a single dose exposure model, treatment with *Spy* Cas9 mRNA + E7-14 gRNA induced high indel activity of HPV16 E7 target DNA relative to buffer-only treatment two days post-treatment. The fraction of wild-type E7 gene sequence is shown for cells receiving either E7-14 gRNA (*upper bar*) or buffer only ("neg ctrl", *lower bar*). Fig. 2B shows NGS on-target amplicon analysis of Cas9 mRNA + E7-14 gRNA-induced on-target activity, which indicates that repaired DNA was composed predominantly of small deletions and insertions out-of-frame with E7 coding sequence two days post-treatment. The percent of indels, including single nucleotide variants, at various locations within

the 172 bp amplicon window for cells receiving the *Spy* Cas9 mRNA + E7-14 gRNA treatment is shown. The peak of indels corresponds to the target site for the E7-14 gRNA. Fig. 2C illustrates the method by which a larger amplicon of 5916 bp was analyzed, which also detected large deletions. Fig. 2D shows that most deletions were less than 10 bp, but deletions up to several thousand base pairs were observed. Due to the size of the HPV genome and the location of the E7-14 target, even the largest deletions observed were still within the HPV genome and no deletions within the human portion of the amplicon were observed.

Fig. 3A-B shows cytotoxicity of Cas9 mRNA + E7-14 gRNA compositions in HPV16-positive SiHa cells or Cerv186 derived from human cervical cancer tissue. Fig. 3A shows that treatment with *Spy* Cas9 mRNA + E7-14 gRNA (*circles*) induced maximal cytotoxicity by 7 days post-single dose exposure with minimal background activity induced by non-targeting gRNA (*square*). Fig 3B shows that using a repeat dose exposure model, treatment with Cas9-HF2 + E7-14 sgRNA induced cumulative HPV16-positive Cerv186 cell cytotoxicity by day 15 when treated at day 1 and again at day 7. Right two bars indicate Cas9-HF2 + E7-14 sgRNA and * indicates measured cell count was < 0.005 of control; left bar indicates Cas9-HF2 + a non-targeting guide with a sequence specific to HPV18.

Fig. 4A-B shows that cytotoxicity of Cas9 mRNA + E7-14 gRNA compositions in HPV16-positive SiHa cells is limited by transfection rates. Fig. 4A shows a flow chart of the procedure. SiHa cells were dosed with a combination of 1:1 mass ratio of Cas9 mRNA and E7-14 gRNA at 5 ng total RNA per 1,000 cells and a trace amount of either enhanced green fluorescent protein (EGFP) mRNA or a reporter plasmid. Negative control samples were performed using a non-targeting sgRNA in place of the E7-14 gRNA. The EGFP mRNA is converted to detectable green protein; cells expressing the green protein were sorted into single cell aliquots of 96 well plates and were allowed to grow for 4 weeks. The reporter plasmid contained the RFP gene followed by an out-of-frame GFP gene with the E7-14 target site between the two genes. Cas9 editing events could shift the GFP gene into frame, resulting in expression of the green reporter protein. SiHa cells expressing both the red and green proteins were sorted into single cell aliquots and allowed to grow for 4 weeks. After this, all of

the cells in each well of the plates were counted and binned according to the cell growth. As shown in Fig. 4B, most cells that received drug died while most of the cells that had received the negative control gRNA began to grow and divide.

Fig. 5A-E shows components, preparation, characterization, and cellular transfection application of PONA-based LNPs. Fig. 5A shows an outline of the process for PONA LNP preparation. The PONA-based LNPs have neutral PEG-lipids on the surface and PONA/CHEMS/Cholesterol/RNA components inside. Fig. 5B-C show the results of DLS and RiboGreen assays. Fig. 5D shows the results of TapeStation assay, indicating the presence of integrated RNA (EGFP, mCherry, Cas9, gRNA) structures post-LNP encapsulation. Fig. 5E shows stability and 293T transfection ability of LNP frozen for longer than 3 months. Flow cytometry was used to measure the relative proportion of viable EGFP+ cells, which are represented normalized to untreated cells. No significant EGFP expression differentiation was observed among samples following up to 3 months of storage under -80 °C.

Fig. 6A-B shows CRISPR activity of Cas9 mRNA and guide RNAs encapsulated in palmitoyl-oleoyl-nor-arginine (PONA)-based lipid nanoparticles (LNPs). Expression of LNP-encapsulated mRNA after transfection into HPV16-negative C33a cells (Fig. 6A). Relative DNA cleavage for LNP formulations containing either a 1:1 mass ratio of Cas9 mRNA and E7-14 sgRNA or Cas9 mRNA alone, which served as a normalization control) after transfection into HPV16 positive SiHa cells (Fig. 6B). The relative fraction of disrupted E7 target normalized to sample from Cas9 mRNA alone LNPs is shown as the average of 4 independent replicates with error bars representing the standard error of the mean.

Fig. 7A-B shows expression of reporter mRNA *in vivo* after administration of PONA LNP. Fig. 7A shows preclinical models and *in vivo* protocols developed for the topical application of LNP to mucosal epithelial cells in the vagina and anus of mice and rats. Fig. 7B shows the effect that particle size of PONA LNP-formulated RNA has on *in vivo* delivery efficiency.

Fig. 8A-B shows expression of EGFP reporter protein expression in the upper epithelium following topical intravaginal delivery of PONA LNP-formulated EGFP mRNA. Fig. 8A shows exemplary histological data following single dose or multiple

dose delivery of PONA-EGFP. Multiple doses of PONA-EGFP enhance EGFP protein expression when delivered topically. Fig. 8B shows expression of EGFP following single or multiple doses of PONA-EGFP as compared to a PONA-luciferase control.

Fig. 9A-B demonstrates topical delivery of Cas9 mRNA combined with gRNA formulated in PONA LNP to target primary Ai9 mouse fibroblasts, and the induction of local *in situ* CRISPR activity. Fig. 9A is a schematic showing editing of the Tdtomato reporter gene after treating with Cre mRNA or Cas9mRNA + surrogate gRNA. Fig. 9B shows the results of treating Ai9 mice with Cre mRNA or Cas9mRNA + surrogate gRNA.

Fig. 10 demonstrates topical intravaginal delivery of Cas9 mRNA combined with gRNA formulated in PONA LNP to female Ai9 mice, and the induction of *in vivo* CRISPR activity.

DETAILED DESCRIPTION

I. Definitions

Prior to setting forth this disclosure in more detail, it may be helpful to an understanding thereof to provide definitions of certain terms to be used herein. As used herein, certain items have the following defined meanings. Unless defined otherwise, all technical and scientific terms used herein have the same meaning as is commonly understood by one of skill in the art to which this invention belongs.

Unless the context requires otherwise, throughout the present specification and claims, the word "comprise" and variations thereof, such as "comprises" and "comprising," are to be construed in an open, inclusive sense, that is, as "including, but not limited to". "Consisting of" shall mean excluding more than trace elements of other ingredients and substantial method steps disclosed herein. The term "consisting essentially of" limits the scope of a claim to the specified materials or steps, or to those that do not materially affect the basic characteristics of a claimed invention. For example, a composition consisting essentially of the elements as defined herein would not exclude trace contaminants from the isolation and purification method and pharmaceutically acceptable carriers, such as phosphate buffered saline, preservatives,

and the like. Similarly, a protein consists essentially of a particular amino acid sequence when the protein includes additional amino acids that contribute to at most 20% of the length of the protein and do not substantially affect the activity of the protein (*e.g.*, alters the activity of the protein by no more than 50%). Embodiments defined by each of the transitional terms are within the scope of this invention.

Reference throughout this specification to "some embodiments" or "certain embodiment" means that a particular feature, structure, or characteristic described in connection with the embodiment is included in at least one embodiment of the present invention. Thus, the appearances of the phrases "in some embodiments" or "in certain embodiments" in various places throughout this specification are not necessarily all referring to the same embodiments. Furthermore, the particular features, structures, or characteristics may be combined in any suitable manner in one or more embodiments.

As used in the specification and claims, the singular for "a", "an", and "the" include plural references unless the context clearly dictates otherwise. For example, the term "a cell" includes a plurality of cells, including mixtures thereof. Similarly, use of "a composition" for treatment of preparation of medicaments as described herein contemplates using one or more compositions of the disclosure for such treatment or preparation unless the context clearly dictates otherwise.

The use of the alternative (*e.g.*, "or") should be understood to mean one of the alternatives or any combination of the alternatives. For example, "a nucleic acid molecule or peptide" refers to either the nucleic acid molecule or the peptide, or both of them.

"Optional" or "optionally" means that the subsequently described event or circumstances may or may not occur, and that the description includes instances where said event or circumstance occurs and instances in which it does not.

As used herein, when describing a sequence, "a plurality of" means between at least two and all possible. To illustrate, SEQ ID NO: 6 is 102 nucleotides long and includes 31 uridines. To state that a plurality of bases in SEQ ID NO: 6 are modified means that between 2 and 102 bases are modified. To state that SEQ ID NO: 6 contains a plurality of 5-methyoxyuridine modifications means that between 2 and 31 of the uridines have 5′ methoxy groups.

As used herein, "about" and "approximately" generally refer to an acceptable degree of error for the quantity measured, given the nature or precision of the measurements. Typical, exemplary degrees of error may be within 20%, 10%, or 5% of a given value or range of values. Alternatively, and particularly in biological systems, the terms "about" and "approximately" may mean values that are within an order of magnitude, potentially within 5-fold or 2-fold of a given value. When not explicitly stated, the terms "about" and "approximately" mean equal to a value, or within 20% of that value.

As used herein, numerical quantities are precise to the degree reflected in the number of significant figures reported. For example, a value of 0.1 is understood to mean from 0.05 to 0.14. As another example, the interval of values 0.1 to 0.2 includes the range from 0.05 to 0.24. Thus, a concentration of from 0.1 mg/mL to 2 mg/mL means a concentration range of from 0.05 mg/mL to 2.4 mg/mL.

In the present description, any concentration range, percentage range, ratio range, or integer range is to be understood to include the value of any integer within the recited range and, when appropriate, fractions thereof (such as one tenth and one hundredth of an integer), unless otherwise indicated.

As used herein, the terms "peptide," "polypeptide," and "protein" are used interchangeably, and refer generally to a polymer of amino acids linked by peptide (amide) bonds. It may be of any length and may be linear, branched, or cyclic. The amino acid may be naturally-occurring, non-naturally-occurring, or may be an altered amino acid. This term can also include an assembly of a plurality of polypeptide chains into a complex. This term also includes natural or artificially altered amino acid polymers. Such alteration includes disulfide bond formation, glycosylation, lipidation, acetylation, phosphorylation, or any other manipulation or alteration. This definition also includes, for example, polypeptides including one or two or more analogs of amino acids (*e.g.*, including non-naturally-occurring amino acids), peptide-like compounds (*e.g.*, peptoids) and other alterations known in the art.

As used herein the term "a functionally equivalent peptide" refers to a peptide that may vary in terms of structure (sequence) but is the same or similar to the original peptide. Functionally equivalent proteins or peptides may be created via the application

of recombinant DNA technology, in which changes in the protein structure may be engineered, based on considerations of the properties of the amino acids being exchanged. Those skilled in the art may introduce designed changes through the application mutagenesis techniques.

As used herein, "nucleic acid" or "nucleic acid molecule" refers to any of deoxyribonucleic acid (DNA), ribonucleic acid (RNA), oligonucleotides, fragments generated, for example, by the polymerase chain reaction (PCR) or by *in vitro* translation, and fragments generated by any of ligation, scission, endonuclease action, or exonuclease action. In certain embodiments, the nucleic acids of the present disclosure are produced by PCR. Nucleic acids may be composed of monomers that are naturally occurring nucleotides (such as deoxyribonucleotides and ribonucleotides), analogs of naturally occurring nucleotides (*e.g.*, α-enantiomeric forms of naturally-occurring nucleotides), or a combination of both. Modified nucleotides can have "modifications" or be "modified," wherein the nucleotide differs from the wild-type or original or comparator nucleotide molecule in, for example, replacement of or modification of sugar moieties (*e.g.*, 2'-O-methylation), pyrimidine or purine base moieties (*e.g.*, methylation), or linkages between nucleotides (*e.g.*, a phosphorothioate linkage). Nucleic acid monomers can be linked by phosphodiester bonds or analogs of such linkages. Analogs of phosphodiester linkages include phosphorothioate, phosphorodithioate, phosphoroselenoate, phosphorodiselenoate, phosphoroanilothioate, phosphoranilidate, phosphoramidate, and the like. Nucleic acid molecules can be either single stranded or double stranded.

The term "gene" means the segment of DNA involved in producing a polypeptide chain; it includes regions preceding and following the coding region "leader and trailer" as well as intervening sequences (introns) between individual coding segments (exons).

The term "isolated" means that the material is removed from its original environment (*e.g.*, the natural environment if it is naturally occurring). For example, a naturally occurring nucleic acid or polypeptide present in a living animal is not isolated, but the same nucleic acid or polypeptide, separated from some or all of the co-existing materials in the natural system, is isolated. Such nucleic acid could be part of a vector

and/or such nucleic acid or polypeptide could be part of a composition (*e.g.*, a cell lysate), and still be isolated in that such vector or composition is not part of the natural environment for the nucleic acid or polypeptide.

As used herein, the term "recombinant" refers to a cell, microorganism, nucleic acid molecule, or vector that has been modified by introduction of an exogenous nucleic acid molecule, or refers to a cell or microorganism that has been altered such that expression of an endogenous nucleic acid molecule or gene is controlled, deregulated or constitutive, where such alterations or modifications may be introduced by genetic engineering. Genetic alterations may include, for example, modifications introducing nucleic acid molecules (which may include an expression control element, such as a promoter) encoding one or more proteins or enzymes, or other nucleic acid molecule additions, deletions, substitutions, or other functional disruption of or addition to a cell's genetic material.

As used herein, "mutation" refers to a change in the sequence of a nucleic acid molecule or polypeptide molecule as compared to a reference or wild-type nucleic acid molecule or polypeptide molecule, respectively. A mutation can result in several different types of change in sequence, including substitution, insertion or deletion of nucleotide(s) or amino acid(s).

As used herein, a "conservative mutation" or "conservative substitution" with respect to a polypeptide sequence refers to a substitution of one amino acid for another amino acid that has similar properties. Exemplary conservative substitutions are well known in the art (*see*, *e.g.*, Lehninger, Biochemistry, 2nd Edition; Worth Publishers, Inc. NY, NY, pp.71-77, 1975; Lewin, Genes IV, Oxford University Press, NY and Cell Press, Cambridge, MA, p. 8, 1990).

"Sequence identity" or "percent identity" as used herein, refers to the percentage of nucleic acid or amino acid residues in one sequence that are identical to the nucleic acid or amino acid residues in another reference polynucleotide or polypeptide sequence (*i.e.*, % identity = number of identical positions/total number of positions x 100), taking into account the number of gaps, and the length of each gap that needs to be introduced to optimize alignment of two or more sequences. The percentage sequence identity values can be generated using the NCBI BLAST2.0 software as

defined by Altschul *et al.* (1997) "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs", Nucleic Acids Res. 25:3389-3402, with the parameters set to default values.

Additional definitions are set forth throughout this disclosure.

II. Guide RNA Molecules

As used herein, a "guide RNA" (gRNA) refers to the inclusion of both trans-activating RNA (tracrRNA) and crispr RNA (crRNA), which function together to form the active ribonucleoprotein (RNP) with an endonuclease, or to the single-molecule version known as single guide RNA, or sgRNA. As used herein, a "ribonucleoprotein" or RNP" refers to a CRISPR/Cas protein complex formed by the association of a guide RNA with a Cas endonuclease. RNPs can form *in vivo* as a result of the natural association of the guide RNA and endonuclease, or they can be assembled *in vitro*, and delivered directly to cells using electroporation or transfection techniques.

In some embodiments, the guide RNA is present in the single guide RNA form.

In some embodiments, the guide RNA includes one or more modifications in the form of modified nucleotides or modifications to the canonical phosphate backbone bonds.

In some embodiments, guide RNAs comprise targeting regions or targeting portions that are complementary to sites within the genome of a virus, which sites do not also appear in a human genome. In some embodiments, the guide RNA includes a targeting region that hybridizes specifically to a target within an HPV genome.

In some embodiments, the guide RNA is provided in the form of an RNA molecule. In some embodiments, the guide RNA is provided in the form of a DNA molecule encoding the guide RNA, such as a viral or plasmid vector, which is transcribed within the target cell to form the active guide RNA.

In some embodiments, the guide RNAs may include features such as modified nucleotides that promote the delivery of the RNAs to, and retention within, infected cells. In some embodiments, modified nucleotides in RNAs may function to improve RNA stability, reduce immunogenicity, or improve specificity of the endonuclease activity. For example, the guide RNAs can include features such as one or more 2′-O-

methyl groups on a ribose ring, one or more phosphorothioate bonds between nucleotides, or both. Such modifications may, in some embodiments, be located proximal to the 5′ and 3′ termini of the guide RNAs, which may protect against exonuclease digestion.

In some embodiments, the guide RNA comprises a plurality of (*e.g.*, between about 20 and 100%) modified bases each selected from the group consisting of 2′-O-methylcytidine ("Cm"); 2′-O-methylguanadine ("Gm"); 2′-O-methyluridine ("Um"); and 2′-O-methyladenine ("Am"). In some embodiments, the guide RNA comprises between one and nine phosphorothioate bonds between the ten terminal nucleotides at each of a 5′ end and at a 3′ end of the guide RNA.

For compositions wherein an mRNA encoding a Cas9 is delivered, once the composition is delivered to cells, the mRNA is translated to form the Cas9 protein, which complexes with the guide RNA to form the enzymatically active RNP. In certain embodiments, the guide RNA is SEQ ID NO: 6, optionally with one or a plurality of modifications. Modifications may include, for example, a 2′-O-methyl group on a ribose ring of a nucleotide or a phosphorothioate bond between nucleotides.

The guide RNA may include 2′-O-methylated nucleotides. The 2′-O-methyl oligo modification may be characterized as a RNA analog protecting against general base hydrolysis and nucleases, as well as increased Tm of duplexes by 1-4 °C per addition. It may be found that the configuration of a 2′-O-methyl oligonucleotide is in the A-form like RNA, and not the B-form like DNA. It may only require a two or three of those modifications in a row to effect the transition from one form to the other. Another benefit of 2′ O-methyl groups includes the stabilization of RNA molecules from nuclease activity. 2′-O-methylated nucleotides are available from TriLink BioTechnologies, LLC (San Diego, CA). In some embodiments, between 1 and about 10, preferably about 3, 2′-O-methyl groups are included within the first 3 or 4 or 5 bases at both the 5′ and the 3′ end of the guide RNA to protect from exonuclease activity when the composition is delivered to cells.

In some embodiments, the guide RNA targets an HPV genome, such as, for example, and HPV16 genome. In some embodiments, the guide RNA comprises a nucleic acid sequence as set forth in SEQ ID NO: 6, and optionally wherein one or

more of the nucleotides of the guide RNA are substituted with a modified base, each selected from the group consisting of 2′-O-methylcytidine (Cm); 2′-O-methylguanadine (Gm); 2′-O-methyluridine (Um); and 2′-O-methyladenine (Am). In some embodiments, the guide RNA comprises a nucleic acid sequence as set forth in SEQ ID NO: 7. In some embodiments, the guide RNA In some embodiments, the one or more of the internucleoside bonds in the guide RNA is a phosphorothioate bond. In some embodiments, one or more of the internucleoside bonds within the ten terminal nucleotides at each of a 5′ end and at a 3′ end of the guide RNA is a phosphorothioate bond. In some embodiments, the guide RNA is SEQ ID NO: 6, optionally with one or more of the internucleoside bonds being phosphorothioate bonds. In some embodiments, the guide RNA is SEQ ID NO: 7.

III. Cas9 Molecules and Nucleic Acids

In some embodiments, the compositions as disclosed herein comprise a Cas endonuclease, *e.g.*, any member of the family of CRISPR associated bacterial endonucleases.

In some embodiments, the Cas endonuclease is a wild-type Cas9 endonuclease, *e.g.*, one encoded by a nucleic acid molecule as set forth in SEQ ID NO: 3 and/or comprising an amino acid sequence as set forth in SEQ ID NO: 4. In other embodiments, the Cas endonuclease is a modified Cas9 endonuclease, wherein the modified Cas9 endonuclease is modified relative to the wild-type molecule. For example, the Cas9 may be modified to enhance specificity and decrease off-target activity (*e.g.*, by destabilizing interactions with target DNA at locations outside of the guide RNA targets).

The mRNA can be manufactured by *in vitro* transcription by a company such as TriLink BioTechnologies, LLC (San Diego, CA) or AmpTec GmbH (Hamburg, Germany). *In vitro* transcription manufacturing of mRNA typically uses double stranded DNA template in buffer with an RNA polymerase and a mix of NTPs. The polymerase synthesizes the mRNA. The DNA is then enzymatically degraded. The mRNA is purified away from polymerase, free NTPs, and degraded DNA.

In some embodiments, the Cas9 endonuclease is delivered to cells encoded in a DNA vector, in protein form (*e.g.*, as an active RNP with the modified Cas9 endonuclease complexed with an antiviral guide RNA), or as an mRNA to be translated within the target cells. In some embodiments, the Cas9 endonuclease is delivered in mRNA form along with one or more guide RNAs.

In some embodiments, the mRNA includes a Cap1 structure on the 5' end. In some embodiments, the Cap1 includes a 7-methyl-guanosine linked to the first 5′ nucleotide via 5′-5′ triphosphate bridge.

One of skill in the art will understand that, because of the degeneracy of the genetic code, certain predictable variations of SEQ ID NOs: 2-5 will provide functional equivalents of each. For example, making reference to the mRNA sequence of a Cas endonuclease gene, a portion of the ORF may include CAA CCU CAA, which encodes MPQ. However, proline (P) is incorporated by the ribosome when any base is in the wobble position, so the codon for proline could be represented as CCN. Glutamine (Q) is incorporated when the wobble base is a purine, and thus the Q codon could be represented (again, in mRNA) as CAR (where one of skill in the art will recognize that N and R are the IUPAC ambiguity codes for any base and a purine, respectively). Using a standard codon chart and an understanding of the genetic code, it is possible to generate a sequence with as low as about 85% identity to one of SEQ ID NOs: 2-5 yet that nevertheless still encodes the same protein as is encoded by SEQ ID NOs: 2-5, respectively. As such, it will be understood that the ORF of the mRNA can include one of SEQ ID NOs: 2-5 or a sequence that has between 85% and 100% identity to one of SEQ ID NOs: 2-5 (as determined by aligning the two sequences and calculating [(matched bases/total bases)* 100]). However, a sequence having or approaching 100% identity to SEQ ID NOs: 2-5 (*e.g.*, sequences having between 95% and 100% identity) may be preferable due to codon optimization and, for example, GC content or avoidance of RNA secondary structures.

In some embodiments, the mRNA encodes a Cas endonuclease such as a version of Cas9. In some embodiments, the Cas9 endonuclease is a wild-type *S. pyogenes* Cas9 (*Spy* Cas9), and is encoded by an mRNA comprising SEQ ID NO: 2 or SEQ ID NO: 3.

In some embodiments, the mRNA encodes a modified Cas9 endonuclease, such as a Cas9-HF2 endonuclease, and is encoded by an mRNA comprising SEQ ID NO: 4 or SEQ ID NO: 5.

Spy Cas9-sgRNA complexes cleave target sites composed of an NGG PAM sequence (recognized by *Spy* Cas9) and an adjacent 20 bp protospacer sequence (which is complementary to the 5′ end of the sgRNA). Structural studies have suggested that the *Spy* Cas9-sgRNA-target DNA complex includes several *Spy* Cas9-mediated DNA contacts, including direct hydrogen bonds made by four *Spy* Cas9 residues (N497, R661, Q695, Q926) to the phosphate backbone of the target DNA strand. Alanine substitution of one, more than one, or all of those residues does not reduce on-target cleavage efficiency of *Spy* Cas9. It has been indicated that the triply substituted variants (R661A/Q695A/Q926A) and the quadruple substitution variant (N497A/R661A/Q695A/Q926A) show good activity. The quadruple substitution variant has been dubbed *Spy* Cas9-HF1 for High-Fidelity variant #1, aka HF S.p. Cas9. Off-target activity may be assessed using the genome-wide unbiased identification of double-stranded breaks enabled by sequencing (GUIDE-seq) method. *See* Tsai, 2015, GUIDE-seq enables genome-wide profiling of off-target cleavage by CRISPR-Cas nucleases. Nat Biotechnol 33:187-197, incorporated by reference. *Spy* Cas9-HF1 with an additional D1135E substitution (dubbed *Spy* Cas9-HF2) retains 70% or more activity of wild-type *Spy* Cas9 in studies and may be included in the compositions. Additionally, variants harboring additional L169A or Y450A substitutions (positions whose side chains are believed to mediate non-specific hydrophobic interactions with the target DNA on its PAM proximal end) may be included. *See* Kleinstiver, 2016, High-fidelity CRISPR-Cas9 variants with undetectable genome-wide off targets, Nature 529(7587):490-495, incorporated by reference.

Slaymaker *et al.* report that Cas9-mediated DNA cleavage may be dependent on DNA strand separation. *See* Slaymaker, 2016, Rationally designed Cas9 nucleases with improved specificity, Science 351(6268):84-88, incorporated by reference. Nuclease activity is activated by strand separation and it is thought that by attenuating the helicase activity of Cas9, mismatches between the sgRNA and target DNA would reduce cleavage activity at off-target sites. *Streptococcus pyogenes* Cas9 crystal

structures exhibit a positively-charged groove, positioned between the HNH, RuvC, and PAM-interacting domains in *Spy* Cas9, that is likely to be involved in stabilizing the non-target strand of the target DNA. Neutralization of positively-charged residues in that non-target strand groove may weaken non-target strand binding and encourage re-hybridization between the target and non-target DNA strands, thereby requiring more stringent Watson-Crick base pairing between the RNA guide and the target DNA strand. Five substitutions within that groove may reduce activity at off-target sites compared to wild type *Spy* Cas9 while maintaining on-target cleavage efficiency. Variants with both high efficiency (wild type levels of on-target indel formation) and specificity (no detectable indel formation at EMX(1) and VEGFA(1) off-targets) include: *Spy* Cas9 (K855A), *Spy* Cas9 (K810A/K1003A/R1060A) [also referred to as eSpCas9(1.0)], and *Spy* Cas9 (K848A/K1003A/R1060A) [also referred to as eSpCas9(1.1)]. Some embodiments may include mRNA encoding any of these variant Cas9 endonucleases.

In any of the embodiments, the mRNA may include one or a plurality of 5-methoxyuridine (m5u). Modified nucleosides may reduce innate immune activation and increase translation of mRNA. Unmodified mRNA may induce undesirable cytokine secretion. Large quantities of RNA may be prepared by *in vitro* transcription from DNA templates using phage RNA polymerase or solid-phase chemical synthesis. Triphosphate-derivatives of m5u (TriLink) may be used to generate RNA containing a modified nucleoside. Incorporation of modified nucleotides into RNA may reduce its ability to activate RNA sensors such as Toll-like receptor (TLR)3, TLR7 and TLR8, retinoic acid inducible gene I (RIG-I), and RNA-dependent protein kinase (PKR). HPLC purification removes dsRNA and other contaminants from *in vitro*-transcribed RNAs containing m5C nucleosides, yielding RNA with high levels of translation. *See* Kariko, 2011, Generating the optimal mRNA for therapy: HPLC purification eliminates immune activation and improves translation of nucleoside-modified, protein-encoding mRNA, Nucleic Acids Res 39(21):e142, incorporated by reference.

The mRNAs may be transcribed to contain, *e.g.*, 30, 51, or 120-nt long poly(A) tails. Poly(A) tail may be added in a template-dependent fashion during transcription and/or may be added enzymatically post-transcription. For example, after transcription,

additional poly(A) tail may be added with yeast poly(A) polymerase. RNAs may be capped with, *e.g.*, an m7G capping kit with or without 2′-O-methyltransferase (ScriptCap, CellScript) to obtain Cap1 or Cap0. Capping may be done using TriLink's CleanCap or AmpTec's enzymatic capping, for example, to result in Cap1.

In some embodiments, the Cas endonuclease comprises a wild-type *Streptococcus pyogenes* Cas9 amino acid sequence. Amino acid sequences of wild-type *Streptococcus pyogenes* Cas9 can be found at, for example, UniProt accession number Q99ZW2, which is set forth in SEQ ID NO: 1. In some embodiments, the Cas endonuclease comprises a wild-type *Streptococcus pyogenes* Cas9 encoded by an mRNA having the nucleic acid sequence as set forth in SEQ ID NO:2. In some embodiments, the Cas endonuclease comprises a variant *Streptococcus pyogenes* Cas9 encoded by an mRNA having the nucleic acid sequence as set forth in SEQ ID NO:4.

IV. Compositions

In certain aspects, the present disclosure provides compositions comprising a Cas endonuclease and one or more guide RNAs that target a viral nucleic acid, or a nucleic acid encoding such a Cas9 endonuclease and/or guide RNAs. In some embodiments, the present disclosure provides a composition that comprises an mRNA encoding a Cas endonuclease and one or more guide RNAs, for the treatment of a viral infection, such as an HPV infection. In some embodiments, the composition comprises a Cas9 endonuclease and one or more guide RNAs. In some other embodiments, the composition comprises a ribonucleoprotein (RNP) complex, wherein the RNP comprises a Cas9 endonuclease complexed or associated with a guide RNP. In any of the aforementioned embodiments, the mRNA and guide RNA may include particular sequences and modifications, and may be encapsulated by nanoparticles. In particular embodiments, the guide RNA targets an HPV nucleic acid.

In some embodiments, the composition includes mRNA encoding a modified Cas endonuclease as well as one or more guide RNAs that target the Cas endonuclease to the HPV genome. In some embodiments, he RNAs are packaged in a lipid nanoparticle, solid nanoparticle, or liposome. In some embodiments, the RNA-encapsulated nanoparticles are optimally formulated for topical, mucosal, or local

delivery to infected tissue, which avoids systemic delivery and circulation, thus minimizing drug exposure, off-target activity, and immunogenicity of the Cas endonuclease.

In some embodiments, the mRNA encodes a programmable nuclease such as a Cas endonuclease or a modified Cas endonuclease. In some embodiments, the Cas endonuclease or modified endonuclease is a Cas9 endonuclease or modified Cas9 endonuclease. In certain embodiments, the mRNA encodes a modified Cas endonuclease that is modified, relative to a wild-type version, to enhance specificity and decrease off-target activity. In still further embodiments, the mRNA may include modifications such as 5-methyluridine that may minimize an immune response by the patient.

In some embodiments, guide RNAs comprise targeting regions that are complementary to sites within a viral genome, such as the genome of HPV16, which sites do not also appear in a human genome.

In some embodiments, the guide RNAs may include features such as modified nucleotides that promote the delivery of the RNAs to, and retention within, infected cells. In some embodiments, modified nucleotides in RNAs may function to improve RNA stability, reduce immunogenicity, or improve specificity of the endonuclease activity. For example, the guide RNAs can include features such as one or more 2′-O-methyl groups on a ribose ring, one or more phosphorothioate bonds between nucleotides, or both, and particularly located proximal to the 5′ and 3′ termini of the guide RNAs, which may protect against exonuclease digestion.

In some embodiments, the RNAs are packaged in lipid nanoparticles that include, for example, cationic lipids, which balance the charge of the phosphate backbone and promote penetration through tissue and into cells and release of RNA within the cell. The lipid nanoparticles may further be provided in a topical formulation that contains a suitable gel or suspension, such as an aqueous suspension, which may include a tissue retention-enhancing or thickening agent such as, for example, hydroxyethyl cellulose or carboxymethyl cellulose. The formulation may include excipients to enhance LNP stability such as, for example, sucrose or mannitol. The formulation may include excipients to enhance tissue penetration such as, for example,

sodium lauryl sulfate, ethanol, diethylene glycol monoethyl ether (Transcutol), propylene glycol, polyethylene glycol (PEG) esters, sucrose esters, or N-methyl pyrrolidone (NMP).

The cationic lipid used in the lipid nanoparticles may include palmitoyl-oleoyl-nor-arginine (PONA). In some embodiments, the LNPs further comprise one or more of cholesteryl hemisuccinate (CHEMS), cholesterol, and PEGylated 1,2-bis(dimethylphosphino)ethane (DMPE-PEG2K). In some embodiments, the LNPs comprise PONA, CHEMS, cholesterol and DMPE-PEG2K. The LNPs are optionally carried by a carrier formulation, such as water, an aqueous solution, an oil, or a gel. In certain embodiments, the mRNA encapsulated in the LNPs comprises a sequence selected from SEQ ID NO: 2 to SEQ ID NO: 5; the guide RNA encapsulated in the LNPs comprises SEQ ID NO: 6 or SEQ ID NO: 7; and the plurality of LNPs are dispersed within a carrier liquid, oil, or gel provided by the carrier formulation. In some embodiments, the carrier is an aqueous buffer. Described herein are the mRNA, the guide RNA, the lipid nanoparticles, and the carrier formulation, as well as methods of treating an infection and methods of making a medicament.

In certain embodiments, the Cas9 mRNA comprises a nucleic acid as set forth in SEQ ID NOs: 2, 3, 4, or 5; the guide RNA comprises a nucleic acid as set forth in SEQ ID NOs: 6 or 7; and the Cas9 mRNA and guide RNA are encapsulated within a plurality of nanoparticles, wherein the plurality of nanoparticles are dispersed within a carrier formulation.

In some embodiments, the present disclosure provides a composition comprising an mRNA encoding a Cas endonuclease, a guide RNA as described herein, a plurality of nanoparticles comprising a cationic lipid and encapsulating the mRNA and the guide RNA, and a carrier formulation. In a further embodiment, the carrier formulation stabilizes the lipid nanoparticle and enhances topical or local delivery by promoting tissue retention and tissue penetration. In some embodiments, the guide RNA comprises SEQ ID NO: 6 or SEQ ID NO: 7.

In any of the aforementioned embodiments, the cationic lipid may comprise PONA. In some embodiments, the nanoparticles comprise PONA, CHEMS, cholesterol, and DMPE-PEG2K. In some embodiments, the plurality of nanoparticles

are dispersed within a carrier formulation, and the carrier formulation comprises a liquid, oil, or gel.

In certain embodiments, the Cas endonuclease may be a Cas9 endonuclease, which may be a wild-type or modified Cas9 endonuclease. In some embodiments, the mRNA comprises a plurality of 5-methoxyuridine.

In some embodiments, the mRNA comprises SEQ ID NOs: 2, 3, 4, or 5, or a sequence having at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to any one of SEQ ID NOs: 2, 3, 4, or 5; the guide RNA comprises SEQ ID NO: 6 or SEQ ID NO: 7, or a sequence having at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to any one of SEQ ID NO: 6 or SEQ ID NO: 7; and the mRNA and the guide RNA are encapsulated within the lipid nanoparticle.

In any of the aforementioned embodiments, the cationic lipid may comprise PONA. In some embodiments, the nanoparticles comprise PONA, CHEMS, cholesterol, and DMPE-PEG2K. In some embodiments, the plurality of nanoparticles are dispersed within a carrier formulation, and the carrier formulation comprises a liquid, oil, or gel.

In some embodiments, the present disclosure provides methods of using any of the aforementioned compositions for treating a viral infection. In some embodiments, the present disclosure provides pharmaceutical compositions according to any of the aforementioned embodiments for use in the manufacture of a medicament for the treatment of a viral infection.

V. Nanoparticles

In some embodiments, the guide RNAs and Cas mRNAs as disclosed herein are packaged in lipid nanoparticles that include, for example, cationic lipids, which balance the charge of the phosphate backbone and promote penetration through tissue and into cells and release of RNA within the cell. The lipid nanoparticles may further be provided in a topical formulation that contains a suitable gel or suspension, such as an aqueous suspension, which may include a tissue retention-enhancing or thickening agent such as, for example, hydroxyethyl cellulose or carboxymethyl cellulose. The formulation may include excipients to enhance LNP stability such as, for example,

sucrose or mannitol. The formulation may include excipients to enhance tissue penetration such as, for example, sodium lauryl sulfate, ethanol, diethylene glycol monoethyl ether (Transcutol), propylene glycol, polyethylene glycol (PEG) esters, sucrose esters, or N-methyl pyrrolidone.

In some embodiments, the cationic lipid may include PONA. The nanoparticles may further include one or more of CHEMS, cholesterol, and DMPE-PEG2K. In some embodiments, the nanoparticles include PONA, CHEMS, cholesterol, and DMPE-PEG2K.

In some embodiments, the composition includes a plurality of nanoparticles comprising a cationic lipid. The nanoparticles encapsulate the mRNA and the guide RNA. Any suitable nanoparticles may be included. The nanoparticle may be a solid lipid nanoparticle as shown in Fig. 5A. Additionally or alternatively, liposomes may be used to deliver the mRNA and the guide RNA due to multiple cationic surface groups, which interact with anionic nucleic acids and form lipoplexes.

LNPs may typically range in size from 50-200 nm in diameter, and preferably range in size from 50-110 nm, and may optionally include a surface coating of a neutral polymer such as PEG to minimize protein binding and unwanted uptake. The nanoparticles are optionally carried by a carrier, such as water, an aqueous solution, suspension, or a gel. LNPs may be included in a formulation or preparation for topical delivery such as a suspension or gel. Such as a formulation may include chemical enhancers, such as fatty acids, surfactants, esters, alcohols, polyalcohols, pyrrolidones, amines, amides, sulfoxides, terpenes, alkanes and phospholipids (to enhance topical drug penetration by perturbing the highly ordered structure of the epithelium or stratum corneum).

Use of an LNP may enhance the solubility of the payload RNA, provide sustained and controlled release, and deliver higher concentrations of RNA to target areas due to an Enhanced Permeation and Retention (EPR) effect. Lipid-based nanoparticles (liposomes and solid-lipid nanoparticles) may be used.

Topical drug delivery of nanoparticles may provide therapeutic action directly to the targeted site, potentially reducing unwanted systemic side effects. There is a variety of ways that nanoparticles can be formulated for topical use in the clinic, for example,

as solution/liquid formulations, dry formulations, or viscous formulations (*i.e.*, creams, lotions, gels, ointments). The medium used to suspend the nanoparticles should be biocompatible and used to facilitate percutaneous absorption. The suspending medium may also alter the release kinetics of the drug from the nanoparticles. *See* Goyal, 2016, Nanoparticles and nanofibers for topical drug delivery, J Control Release 240:77-92, incorporated by reference.

The medium may aid penetration of barriers such as stratum corneum. *E.g.*, where the stratum corneum includes corneocytes embedded in a double-layered matrix of free sterols, free fatty acids, triglycerides, and ceramides, skin penetration enhancers may increase the penetration of the RNA.

In certain embodiments, LNPs are suspended in a buffer, such as an aqueous buffer. The buffer may include a penetration enhancing agent such as sodium lauryl sulfate (SLS). SLS is an anionic surfactant that enhances penetration into the skin by increasing the fluidity of epidermal lipids. The increase in lipid fluidity below the applied site may allow SLS to diffuse optimally. SLS could thus increase intra-epidermal drug delivery without increasing transdermal delivery. Methods may include use of a buffer such as a pH=6 200 mM phosphate buffer, optionally with SLS at about 1 to 10% wt/wt, *i.e.*, about 35 to 250 mM SLS. *See* Piret, 2000, Sodium Lauryl Sulfate Increases the Efficacy of a Topical Formulation of Foscarnet against Herpes Simplex Virus Type 1 Cutaneous Lesions in Mice, Antimic Ag Chemother 44(9):2263-2270, incorporated by reference.

Lipid nanoparticles optionally may be delivered via a gel, such as a polyoxyethylene-polyoxypropylene block copolymer gel (optionally with SLS). Poloxamers are nonionic triblock copolymers composed of a central hydrophobic chain of polyoxypropylene (poly(propylene oxide)) flanked by two hydrophilic chains of polyoxyethylene (poly(ethylene oxide)). Because the lengths of the polymer blocks can be customized, many different poloxamers exist that have slightly different properties. For the generic term “poloxamer”, these copolymers are commonly named with the letter “P” (for poloxamer) followed by three digits: the first two digits x 100 give the approximate molecular mass of the polyoxypropylene core, and the last digit x 10 gives the percentage polyoxyethylene content (*e.g.*, Kolliphor P 407 = poloxamer with a

polyoxypropylene molecular mass of 4,000 g/mol and a 70% polyoxyethylene content). For the Pluronic and Synperonic tradenames, coding of these copolymers starts with a letter to define its physical form at room temperature (L = liquid, P = paste, F = flake (solid)) followed by two or three digits, The first digit (two digits in a three-digit number) in the numerical designation, multiplied by 300, indicates the approximate molecular weight of the hydrophobe; and the last digit x 10 gives the percentage polyoxyethylene content (*e.g.*, L61 indicates a room temperature liquid comprising polyoxypropylene with a molecular mass of 1,800 g/mol and a 10% polyoxyethylene content).

Lipid nanoparticles may be frozen (*e.g.* at -80°C) for storage for up to three months or more. LNPs may be held in an aqueous suspension or in an emulsion, *e.g.*, with lecithin.

VI. Methods of Treatment

Another aspect of the disclosure is the use of the guide RNAs and related compositions described herein to treat a viral infection. For example, in some embodiments, a method of treating a viral infection in a subject is provided, comprising administering to the subject a therapeutically effective amount of a composition comprising a guide RNA and a mRNA encoding a Cas endonuclease.

In some embodiments, the present disclosure provides a method of treating a Human Papillomavirus (HPV) infection. Some HPV types can cause precancerous lesions, which are abnormal growths that can turn into cancer, or cancer. Certain HPV types infect genital and other areas, including inside and outside the vagina, the penis, the anus or some areas of the head and neck. "High-risk" HPVs are types of HPV that are more likely to cause cancer. Such an infection transforms normal cells into precancerous lesions or cancer. Cancers associated with HPV include cervical cancer, oral cancer, anal cancer, vulvar cancer, vaginal cancer, and penile cancer.

Of the cervical cancers related to HPV, about 70% are caused by HPV16 or HPV18. Cervical cancer from HPV may manifest as a precancerous cervical lesion. A precancerous cervical lesion, which is also called an intraepithelial lesion, is an abnormality in the cells of the cervix that could eventually develop into cervical cancer.

There are two main types of cervical cells, squamous and glandular, and abnormalities can occur in either type. The most common types of precancerous cervical lesions include: atypical squamous cells (abnormalities in the squamous cells of the cervix); squamous intraepithelial lesion (classified as either low- or high-grade, with high-grade lesions being more likely to progress to cervical cancer); and atypical glandular cells (possible precancerous lesion in the upper area of the cervix or inside the uterus). Recommended actions may include HPV testing. Upon positive test results, the lesion may be treated to clear the HPV virus. *See* Senapati, 2016, Molecular mechanisms of HPV mediated neoplastic progression, Infect Agent Cancer 11:59 eCollection; Ghittoni, 2015, Role of human papillomavirus in carcinogenesis, Ecancermedicalscience 9:526; and World Health Organization, 2013, WHO Guidelines for screening and treatment of precancerous lesions for cervical cancer prevention, WHO, Geneva, all incorporated by reference.

In some embodiments, the composition is applied topically or locally to the epithelium of the subject. The composition may be applied to the mucosal epithelium, such as vaginal or anal epithelium. In some embodiments, the composition is applied topically or locally to a site of infection such as a high-grade pre-cancerous lesion associated with an HPV infection. The RNAs are released within the cells, and the mRNAs are translated by the cell's ribosomes to produce a Cas endonuclease. The Cas9 endonuclease includes linker sequences and one or more nuclear localization sequences (NLS) at the N-terminus and/or C-terminus designed for optimal nuclear localization. The Cas endonuclease complexes with the provided guide RNA or guide RNAs to form active ribonucleoprotein (RNP). The RNP traffics to the nucleus and binds to the viral genome by virtue of sequence-specific interaction between the complementary portions of the guide RNA and the target within the viral genome. Upon binding to the viral target, the Cas endonuclease cleaves the viral genome. Resultant viral DNA fragments may be degraded or repaired by cellular pathways, thereby clearing or disrupting the infection.

In some embodiments, methods of treatment include providing a composition according to the embodiments described herein. An effective amount of the composition is administered at a site of infection in a patient in need thereof. In some

embodiments, the composition includes an mRNA encoding a Cas endonuclease and a guide RNA comprising one of SEQ ID NO: 6 or SEQ ID NO: 7. In some embodiments, a plurality of nanoparticles encapsulate the mRNA and the guide RNA. In some embodiments, the composition is administered topically, and systemic circulation is avoided. For example, the composition may be applied directly to a surface of the site of infection. By such means, the method may be used to treat a site of infection such as a squamous cell carcinoma lesion, *e.g.*, a high-grade, pre-cancerous HPV lesion. The method may be used to prevent the onset of a cancer, such as cervical, anal, oral, penile, or vaginal cancer. In some embodiments, the method is used to prevent cervical cancer. In some embodiments, the method is used to treat HPV infection in a subject at risk of HPV-positive pre-cancerous low-grade or high-grade lesions, HPV-positive squamous cell carcinoma in situ, or HPV-positive invasive cancer. In certain embodiments, the E7 gene of HPV is targeted. In some embodiments, a method of treating an HPV infection in a subject is provided, comprising administering a therapeutically effective amount of a guide RNA comprising SEQ ID NO: 7 and an mRNA encoding a Cas9 endonuclease.

VII. Methods of Making a Medicament

Aspects of this disclosure provide a method of making a medicament for the treatment of a viral infection.

In some embodiments, the method includes preparing an mRNA encoding a Cas endonuclease; preparing a guide RNA comprising one of SEQ ID NO: 7 or SEQ ID NO: 8; and encapsulating the mRNA and the guide RNA in a plurality of nanoparticles comprising a cationic lipid. The nanoparticles may be introduced into a pharmaceutically acceptable carrier, *e.g.*, a gel or suspension such as an aqueous suspension.

In some embodiments, the guide RNA comprises SEQ ID NO: 7, optionally with one or a plurality of modifications. The guide RNA is preferably synthesized by solid-phase synthesis. Solid-phase synthesis is carried out on a solid support that may be held between filters, in columns that enable all reagents and solvents to pass through freely. With solid-phase synthesis, a large excess of solution-phase reagents can be

used to drive reactions quickly to completion. Impurities and excess reagents are washed away and no purification is required. The process may be automated and is amenable to automation on computer-controlled solid-phase synthesizers. Solid supports (aka resins) are the insoluble particles, typically 50-200 μm in diameter, to which the oligonucleotide is bound. Suitable supports include controlled pore glass and polystyrene.

Solid supports are typically manufactured with a loading of 20-30 μmol of nucleoside per gram of resin. Any suitable method may be used including, for example, the H-phosphonate and phosphotriester methods, and Khorana's phosphodiester approach. In some embodiments, the phosphoramidite method using solid-phase technology and automation is used.

Phosphoramidite oligo synthesis proceeds in the 3′- to 5′-direction with one nucleotide added per synthesis cycle. Building blocks used for synthesis are commonly referred to as "monomers", which are activated RNA nucleosides (phosphoramidites). The dimethoxytrityl (DMT) group is used to protect the 5′-end of the nucleoside. A β-cyanoethyl group protects the 3′-phosphite moiety, and additional groups may also be included that serve to protect reactive primary amines in the heterocyclic nucleobases. The protecting groups are selected to prevent branching or other undesirable side reactions from occurring during synthesis. Oligonucleotides are synthesized on solid supports. Typically, the support is a small column filled with control pore glass (CPG), polystyrene or a membrane. The oligonucleotide is usually synthesized from the 3′to the 5′. The synthesis begins with the addition of a reaction column loaded with the initial support-bound protected nucleotide into the column holder of the synthesizer. The first nucleotide building block or monomer is usually anchored to a long chain alkylamine-controlled pore glass (LCAA-CPG). The phosphoramidite approach to oligonucleotide synthesis proceeds in four steps on solid support, usually controlled pore glass (CPG) or polystyrene. Synthesis is initiated with cleavage of the 5′-trityl group by brief treatment with dichloroacetic acid (DCA) dissolved in dichloromethane (DCM). Next, the monomer activated with tetrazole is coupled to the available 5′-hydroxyl resulting in a phosphite linkage. Subsequent phosphite oxidation by treatment with iodine using a THF/pyridine /H2O solution yields a phosphate backbone. The

capping step with acetic anhydride, which terminates undesired failure sequences, completes the cycle of oligonucleotide synthesis. *See* McBride, 1983, An investigation of several deoxynucleoside phosphoramidites useful for synthesizing deoxyoligonucleotides. Tetrahedron Lett 24:245-248, 1983; and Kosuri, 2014, Large-scale de novo DNA synthesis: technologies and applications Nat Meth 11:499-507, both incorporated by reference.

In some embodiments, the mRNA encoding a Cas endonuclease is prepared by synthesizing the mRNA. Any suitable synthesis method may be used. In some embodiments, the mRNA is made by *in vitro* transcription. *In vitro* transcription uses a purified linear DNA template containing a promoter, ribonucleotide triphosphates, a buffer system that includes DTT and magnesium ions, and an appropriate phage RNA polymerase. The DNA template preferably includes a double-stranded promoter for binding of the phage polymerase. The template may include plasmid constructs engineered by cloning, cDNA templates generated by first- and second-strand synthesis from an RNA precursor, or linear templates generated by PCR or by annealing chemically synthesized oligonucleotides. The template may be an (*e.g.*, linearized) plasmid. Many plasmids include phage polymerase promoters. Any suitable promoter may be used, *e.g.*, the promoter for any of three common polymerases, SP6, T7 or T3, may be used.

In some embodiments (TriLink) a linearized plasmid template is for "Run-off Transcription," which transcription stops when RNA polymerase falls off the DNA. The plasmid encodes an approximate poly(A)80 tail. The process co-transcriptionally adds methylated 5′ cap ("Cap1"). This process is offered under the proprietary name CleanCap, by TriLink. The process can use normal or modified NTPs, such as 5-methylcytosine (5mC), in any ratio. After the transcription reaction, mRNA is phosphatase treated to remove any 5′ tri-phosphates from uncapped mRNAs. HPLC purifies final mRNA product.

In certain embodiments, PCR primers are used to amplify double stranded DNA template from only the mRNA-encoding region of a plasmid. One PCR primer contains the poly(A)120 tail, to prevent issues of poly(A) tail loss in plasmid. This process can use normal or modified NTPs, such as 5mC, in any ratio, and can do capping co-

transcriptionally, or post-transcriptionally and enzymatically. After transcription, mRNA is phosphatase treated to remove any 5′ tri-phosphates from uncapped mRNAs. The mRNA is purified via spin columns to remove dsRNAs. This process is offered by Amp-Tec.

In general, plasmid vectors for transcription templates may be linearized by restriction enzyme digestion. Because transcription proceeds to the end of the DNA template, linearization ensures that RNA transcripts of a defined length and sequence are generated. PCR products can also function as templates for transcription. A promoter can be added to the PCR product by including the promoter sequence at the 5′ end of either the forward or reverse PCR primer.

The template DNA is then transcribed by a T7, T3 or SP6 RNA phage polymerase in the presence of ribonucleoside triphosphates (rNTPs). The polymerase traverses the template strand and uses base pairing with the DNA to synthesize a complementary RNA strand (using uracil in the place of thymine). The RNA polymerase travels from the 3′ → 5′ end of the DNA template strand, to produce an RNA molecule in the 5′ → 3′ direction. *See* Jani, 2012, *In vitro* Transcription and Capping of Gaussia Luciferase mRNA Followed by HeLa Cell Transfection, J Vis Exp 61:3702, incorporated by reference.

Encapsulating the mRNA and the guide RNA in a plurality of nanoparticles comprising a cationic lipid may proceed by any suitable method. Methods for preparation may include direct mixing between cationic liposomes and mRNA in solution, or rehydration of a thin-layer lipid membrane with mRNA in solution. In some embodiments, LNPs are prepared by mixing lipids in ethanol and RNAs in aqueous buffer with scalable microfluid mixing technology, followed by dilution, filtration, and concentration.

The specificity of a RNP may be determined by electroporating the mRNA encoding a Cas endonuclease and a guide RNA into target cells along with double-stranted oligo-deoxynucleoside (dsODN). The cells are incubated and breaks introduced by the Cas endonuclease capture the dsODN. After one day, genomic DNA (gDNA) is extracted and subject to sample preparation that includes adding adapters to create a sequencing library. Sequencing is used to identify the captured dsODN.

Thus, Off-target activity may be assessed by the genome-wide unbiased identification of double-stranded breaks enabled by sequencing (GUIDE-seq) method. *See* Tsai, 2015, GUIDE-seq enables genome-wide profiling of off-target cleavage by CRISPR-Cas nucleases. Nat Biotechnol 33:187-197, incorporated by reference. Those GUIDE-seq methods of Tsai, 2015, were used to determine off target effects of the composition. By such means, the compositions may be shown to have acceptable low off-target effects and high on-target specificity.

In addition to good on-target specificity via the disclosed mRNAs and the delivery methods, the disclosed compositions and particularly mRNAs encapsulated in lipid nanoparticles exhibit good penetration into and release within target tissues and cells, where release of the mRNA allows expression through, *e.g.*, translation into active protein.

EXAMPLES

Example 1: Generation of Guide RNAs Targeting HPV E6 or E7 Genes

Over 150 fully sequenced HPV16 genomes in addition to hundreds of sequenced E6 and E7 genes were aligned to computationally identify conserved regions within E6 and E7. Within these regions, every *Streptococcus pyogenes* (*Spy*) Cas9 recognition element (known as Protospacer Adjacent Motifs (PAMs)) was identified. Each possible CRISPR targeting sequence located within these regions was then screened using multiple *in silico* specificity prediction tools to generate a list of guide RNAs (gRNAs) that were predicted to have high specificity toward HPV16 E6 or E7 target DNA and minimal predicted specificity toward the human genome. These gRNAs were then chemically synthesized and tested in combination with *Spy* Cas9 mRNA (SEQ ID NO: 3) and *Spy* Cas9-HF2 mRNA (SEQ ID NO: 5) (*see* Slaymaker *et al.*, *Science* 351(6268):84-8, 2016 Jan 1) utilizing HPV16-positive and HPV16-negative cell models to determine DNA cleavage activity and cytotoxicity. To further profile on-target and off-target DNA cleavage activity, a genome-wide unbiased next generation sequencing (NGS) method, GUIDE-seq (Tsai *et al.*, *Nat Biotechnol.* 33(2):187–97, 2015), was utilized to profile specificity. Fig. 1 shows a schematic summarizing these methods.

Using these tools, the "E7-14" guide RNA (SEQ ID NO: 7) was identified.

Example 2: Targeting HPV16 with E7-14 Guide RNA

The ability of the E7-14 guide RNA ("E7-14 gRNA") to disrupt HPV16 DNA was tested using HPV16-positive SiHa cells derived from human cervical cancer tissue.

In a single dose exposure model, the E7-14 guide induced high indel activity in HPV16 E7 target DNA relative to a buffer-only treatment two days following drug treatment. SiHa cells were electroporated with mRNA encoding *Spy* Cas9 combined with either E7-14 gRNA or buffer only (negative control). A 1:1 mass ratio of mRNA and gRNA was used to dose cells at 4 nanograms (ng) total RNA per 1,000 cells. Cell DNA was harvested 48 hours post-treatment and used as template in a PCR reaction with primers flanking the E7 cleavage site. Standard Illumina sequencing methods were used to analyze the sequences of each sample. The fraction of wild-type E7 gene sequence is shown for cells receiving either E7-14 gRNA (*upper bar*) or buffer only ("neg ctrl", *lower bar*) in Fig. 2A. NGS on-target amplicon analysis of E7-14 gRNA-induced on-target activity was conducted. Repaired DNA was composed predominantly of small deletions and insertions out-of-frame with the E7 coding sequence two days post-treatment (Fig. 2B). Fig. 2B shows the percent of indels, including single nucleotide variants, within the 172 bp amplicon window for cells receiving the *Spy* Cas9 mRNA + E7-14 gRNA treatment. The peak of indels corresponds to the target site for the E7-14 gRNA. Large deletions are also observed when a larger amplicon of 5916 bp was analyzed. (Fig. 2C) Most deletions were less than 10 bp but deletions up to several thousand base pairs were observed. (Fig. 2D) Due to the size of the HPV genome and the location of the E7-14 target, even the largest deletions observed were still within the HPV genome and no deletions within the human portion of the amplicon were observed. The majority of indels were small deletions that would alter the downstream reading frame of the E7 protein. These results indicate potent and specific activity of Cas9 mRNA combined with E7-14 gRNA.

The cytotoxic activity of treatment with *Spy* Cas9 mRNA + E7-14 gRNA relative to treatment with control guide RNA was assessed in SiHa cells. SiHa cells

were electroporated with mRNA encoding *Spy* Cas9 combined with a 1:1 mass ratio of either E7-14 gRNA or a non-targeting negative control gRNA (SEQ ID NO: 9) having a sequence specific to HPV18, at the doses indicated in Fig. 3A. SiHa cells electroporated with buffer alone served as a normalization control. Cells were incubated at 37°C with 5% CO_2 in the presence of rich media. Viable cells were counted by flow cytometry three and seven days later, and were normalized to the cells that had been electroporated with buffer-only. Treatment with mRNA encoding *Spy* Cas9 combined with E7-14 gRNA induced E7 DNA cleavage and potently killed SiHa cells within 3 days post-treatment (data not shown). Treatment with mRNA encoding *Spy* Cas9 combined with E7-14 gRNA (*circles*) induced maximal cytotoxicity by 7 days post-single dose exposure with minimal background activity induced by non-targeting gRNA (*square*) (Fig. 3A). In contrast, in HPV16-negative cells, the *Spy* Cas9 mRNA + E7-14 gRNA treatment induced minimal cytotoxicity comparable to non-targeting gRNA (data not shown).

Cytotoxicity was also assessed in Cerv186 cells. Cerv186 were dosed with a 1:1 mass ratio of Cas9-HF2 mRNA and sgRNA at 5 ng total RNA per 1,000 cells using lipid transfection reagents. Dosing occurred on either day 1 (treated 1X) or on days 1 and 7 (treated 2X). Cells were incubated at 37 °C with 5% CO_2 in the presence of rich media. Total viable cells were counted by flow cytometry on day 15 and were normalized to cells treated with only lipid and buffer. In a repeat dose exposure model, administration of Cas9-HF2 mRNA + E7-14 gRNA induced cumulative HPV16-positive Cerv186 cell cytotoxicity by day 15 when treated at day 1 and again at day 7 (Fig. 3B; right two bars indicate Cas9-HF2 + E7-14 sgRNA and * indicates measured cell count was < 0.005 of control; left bar indicates Cas9-HF2 + a non-targeting guide with a sequence specific to HPV18).

To test drug delivery efficiency versus efficacy, SiHa cells were dosed with a combination of 1:1 mass ratio of Cas9 mRNA and E7-14 gRNA at 5 ng total RNA per 1,000 cells and a trace amount of either EGFP mRNA or a reporter plasmid (*see* Fig. 4A). Negative control samples were performed substituting the E7-14 gRNA for a non-targeting sgRNA. This allowed for SiHa cells to be sorted according to either presumed drug delivery or activity. The EGFP mRNA would be converted to detectable green

protein; cells expressing the green protein would be sorted into single cells aliquots of 96 well plates and were allowed to grow for 4 weeks. The reporter plasmid contained the RFP gene followed by an out-of-frame GFP gene with the E7-14 target site between the two genes. Cas9 editing events could shift the GFP gene into frame, resulting in expression of the green reporter protein. SiHa cells expressing both the red and green proteins were sorted into single cell aliquots and allowed to grow for 4 weeks. After this, all of the cells in each well of the plates were counted and binned according to the cell growth. The results are shown in Fig. 4B. Most cells that received drug died while most of the cells that had received the negative control gRNA began to grow and divide. This suggests that the limits of the cell killing activity are due to delivery of the drug rather than its efficacy.

Table I describes the different HPV positive and negative cell lines that were used to test E7-14 CRISPR specificity and potency. The first column indicates the name of the cell line. The second column describes the cell line's anatomical origins and HPV viral copy number, which are all integrated into the human genome unless otherwise indicated. The third column indicates if E7 target site cleavage activity was successfully measured, either by custom qPCR assay or through amplicon sequencing of the region surrounding the target site. The fourth column indicates if cytotoxicity was observed in the cells after Cas9 mRNA and E7-14 gRNA were delivered to the cells. The fifth column indicates if unbiased genome-wide DNA sequencing was performed in the cell line which showed no off target cleavage with Cas9-HF2 mRNA and E7-14 gRNA.

Table I

Cell Line	**Description**	**Target DNA Cleavage**	**Cytotoxicity**	**Cleavage Specificity** LOD = 99.998%
AKC2	Anal Few HPV copies	Yes qPCR, Amp Seq	Yes	Not tested
W12	Cervical 100's of HPV copies (episome & integrated)	Yes qPCR, Amp Seq	Yes	Not tested

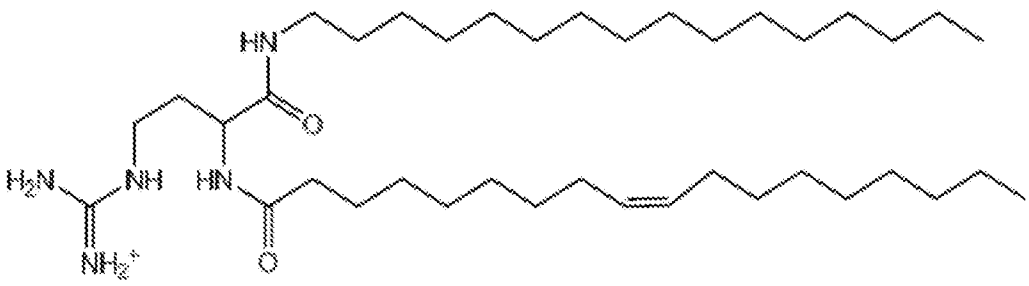
SiHa	Cervical 2 HPV copies	Yes qPCR, Amp Seq	Yes	No off-target cleavage detected
Cerv186	Cervical 26 HPV copies	Yes qPCR	Yes	No off-target cleavage detected
SCC-152	Hypopharynx 600 HPV copies	Yes qPCR	Yes	Not tested
HEK293	Kidney [hpv16 negative]	N/A	No	No off-target cleavage detected
C33a	Cervical [hpv16 negative]	N/A	No	No off-target cleavage detected

Taken together, these data demonstrate that the following series of events occur in relevant cells treated with Cas9 mRNA combined with E7-14 gRNA: (1) translation of Cas9 mRNA into Cas9 protein, (2) complexing of Cas9 protein with E7-14 gRNA to form CRISPR RNP, (3) nuclear localization of CRISPR RNP, (4) on-target CRISPR-mediated cleavage of HPV16 E7 DNA, and (5) specific cytotoxicity of HPV16-positive cells mediated by Cas9 mRNA combined with E7-14 gRNA. Furthermore, these findings demonstrate that HPV16-positive cells are not resistant to Cas9 mRNA combined with E7-14 gRNA and can be effectively eliminated following repeat exposure.

Example 3: Lipid Nanoparticles Comprising guide RNA Molecules

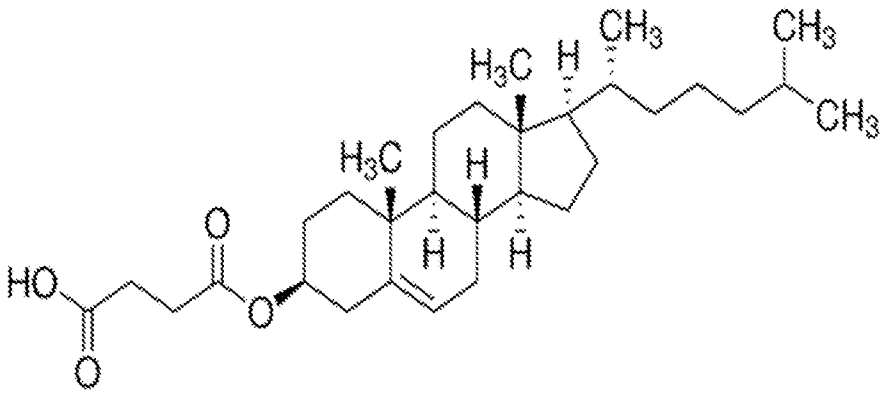
Guide RNA molecules were encapsulated in palmitoyl-oleoyl-nor-arginine (PONA)-based lipid nanoparticles (LNP). LNPs were prepared that included the following lipids:

Palmitoyl-oleoyl-nor-arginine (PONA)



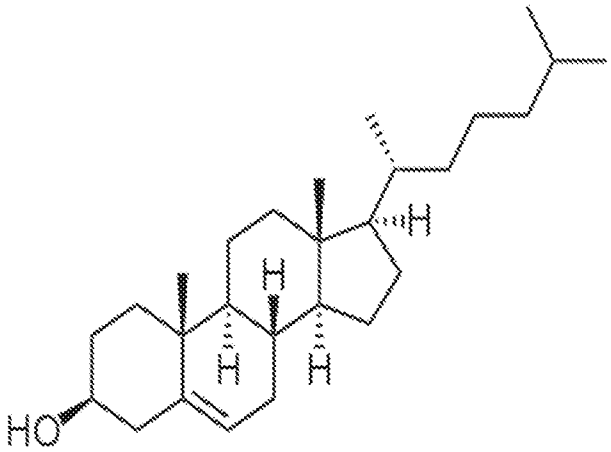
Formula I

Cholesteryl Hemisuccinate (CHEMS)



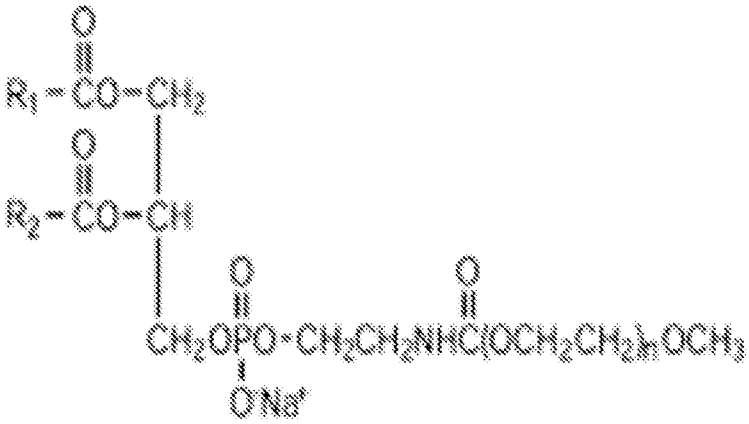
Formula II

Cholesterol



Formula III

DMPE-PEG2K



Formula IV

PONA LNP were prepared by mixing lipids in ethanol and RNAs in aqueous buffer with scalable microfluid mixing technology, followed by dilution, filtration, and concentration, as follows. The lipids solution was prepared by dissolving PONA, CHEMS, cholesterol, and DMPE-PEG2K in ethanol at a concentration of 0.22 mg/mL, 0.10 mg/mL, 0.07 mg/mL, and 0.04 mg/mL, respectively. The RNA solution was made by dissolving 112.5 micrograms (ug) of total RNA, either mRNA alone or mRNA combined with gRNA at an optimized mass ratio of 1:1, in formulation buffer (0.75 mL, pH 4.0). The RNA solution was co-injected with 0.25 mL of the lipid solution into a microfluids mixing cartridge using a Precision Nanosystems NanoAssemblr instrument. 0.6 mL of the resulting LNP formulation was diluted with 1.8 mL of PBS using the NanoAssemblr. This formulation was further diluted with 7.6 mL of PBS added to 2.4 mL of LNP solution. The resulting solution was passed through a 0.2 μm filter, and the filtrate was concentrated using a centrifugal filtration tube spun at 1500xg for 1 hr at 4 °C. The retentate was diluted with 7% sucrose, concentrated again, and frozen at -80 °C.

A dynamic light scattering (DLS) test was performed using a Zetasizer Nano ZS90. 2 microliters (uL) of concentrated LNP was mixed with 70 uL PBS and transferred to a disposable cuvette for size and zeta potential measurement. The test was repeated three times. Data presented are averages.

A RiboGreen assay was carried out by mixing LNP (with or without detergent disruption) and Quant-iT RiboGreen RNA reagent. The amount of RNA was quantified within a linear fluorescence detection under plate reader. The relative of RNA distribution percentage, either on the LNP surface or encapsulated inside LNP, can be calculated from the fluorescence strength using blank wells with media as controls. The concentration of RNA encapsulated within the LNP was measured with a standard curve by linear regression. Capillary gel electrophoresis of the LNP formulation was performed using an Agilent 2200 TapeStation System. With the control of pre-formulated RNA, the RNA inside LNP was electrophoresed after LNP structure disruption by detergent Triton X. At each channel, 1 uL of sample solution was loaded for electrophoresis.

The prepared EGFP mRNA - loaded PONA LNP was stored under -80 °C in a freezer. At different time points during storage, EGFP LNP was thawed and applied onto 293T cells at various dosages of LNP. 24 hours post-incubation, cells were harvested for flow cytometry to measure the relative proportion of viable EGFP+ cells, which are represented normalized to untreated cells.

The components, preparation, characterization, and cellular transfection application of PONA-based LNP formulations are shown in Fig. 5. The PONA-based LNPs have neutral PEG-lipids on the surface and PONA/CHEMS/cholesterol/RNA components inside (Fig. 5A). Upon dilution of the obtained PONA LNP in PBS for the DLS test, the typical physical properties included (i) the size less than 100 nm in diameter, (ii) the typical diagram shown on the right, (iii) low particle size distribution, and (iv) neutral or little negative charge on the surface (Figs. 5B and 5C). After disrupting the LNP structure with detergent Triton X, the RiboGreen assay suggested the high percentage of RNA encapsulation in the obtained LNP (Fig. 5B). Processing using TapeStation indicated the presence of integrated RNA (EGFP, mCherry, Cas9, gRNA) structures post-LNP encapsulation (Fig. 5D). EGFP mRNA encapsulated

PONA LNPs under -80 °C storage were sampled at multiple storage time points and applied for 293T cell transfection, and the EGFP florescence strength was recorded after drug treatment for 24 hrs with different dosages. No significant EGFP expression differentiation was observed among those samples following up to 3 months of storage under -80 °C (Fig. 5E).

Example 4: Delivery of Cas9 mRNA and E7-14 gRNA using PONA-Based Lipid Nanoparticles

Cas9 mRNA was encapsulated in palmitoyl-oleoyl-nor-arginine (PONA)-based lipid nanoparticles (LNPs). The LNP-encapsulated mRNA was transfected into HPV16 negative C33a cells at doses of 0.5 ng or 2 ng of RNA per 1,000 cells. After 24 hours of incubation at 37 °C with 5% CO_2, cells were harvested and lysed, and the protein contents were separated on a polyacrylamide gel. 10 ng of recombinant Cas9 protein from PNA Bio was loaded on the gel as a reference. The contents of the gel was transferred to a PVDF membrane and blotted with antibodies for Cas9 and beta-actin, which served as a loading control. The results are shown in Fig. 6A.

In a further experiment, LNP formulations were produced that contained either (i) a 1:1 mass ratio of Cas9 mRNA and E7-14 sgRNA or (ii) Cas9 mRNA alone (which served as a normalization control). LNPs were transfected into HPV16 positive SiHa cells at the doses indicated in Fig. 6B. After 24 hours of incubation at 37 °C with 5% CO_2, cells were harvested and genomic DNA was extracted and used as template in a custom qPCR reaction. Primers flank the E7 cleavage site and a chemically modified probe spans the cleavage site. The relative fraction of disrupted E7 target normalized to sample from Cas9 mRNA alone LNPs is shown in Fig. 6B as the average of 4 independent replicates, with error bars representing the standard error of the mean.

These data, along with those described in Example 3, demonstrate that PONA-based LNP formulations efficiently encapsulate mRNA and gRNA, protect RNA from degradation, and facilitate a high level of cell transfection, while maintaining high cell viability, a differentiating property of PONA compared to alternative cationic lipid LNP formulations. PONA LNP-RNA formulations enable highly efficient entry into multiple human cell types with minimal evidence of cytotoxicity. Furthermore, PONA

LNP-RNA formulations facilitate intracellular endosome escape of functional RNA and translation of mRNA-encoded full-length Cas9 protein in human cells. Moreover, PONA LNP formulations of Cas9 mRNA and E7-14 gRNA demonstrate potent and specific activity in HPV16-positive cells.

Example 5: Preclinical Models for LNP Delivery to Mucosal Epithelial Cells

Preclinical models and *in vivo* protocols were developed for the topical application of LNP to mucosal epithelial cells in the vagina and anus of mice and rats (Fig. 7A). Briefly, anal or vaginal tissue was cleaned using a small brush with water through the distal opening prior to dosing. After single or multiple doses of LNP, functional delivery of LNP cargo is measured via luminometry in case of luciferase mRNA, or tissue is harvested and embedded in paraffin for further analysis (Haematoxylin and Eosin Staining, H&E; *in situ* hybridization, ISH; or Immunohistochemistry, IHC).

To determine the effects that particle size of PONA LNP-formulated RNA may have on *in vivo* delivery efficiency, PONA LNP formulations containing luciferase mRNA were prepared with varying sizes by altering the relative percentage of DMPE-PEG2K to obtain LNP with DLS-determined diameters of 110 nm, 75 nm, or 57 nm. 10 ug of total RNA of each LNP formulation was administered topically to the anal canals of mice. 6 hours post-dose, animals were IP treated with luciferin and luminometry was performed 12 minutes later using an IVIS instrument. The results indicate a wide signal to noise ratio, the ability of PONA LNP to deliver luciferase mRNA cargo topically, and that decreasing the size of PONA LNP can increase *in vivo* topical delivery efficiency of functional RNA (Fig. 7B).

Example 6: PONA-Based LNPs for Delivery of mRNA to Mucosal Epithelial Cells in Mice

To determine whether topical delivery of PONA-formulated mRNA can result in ectopic protein expression in vaginal stratified epithelial cells, EGFP mRNA or Luciferase mRNA was formulated into PONA-based LNPs. Luciferase mRNA was used as the negative control. LNP-mRNA (20 ug of mRNA in 20 uL volume) was

delivered to the mouse vaginal cavity. 6 hours post-delivery, vaginal tissues were harvested, and 5-micron sections were prepared. Immunohistochemistry for EGFP was performed to determine where functional mRNA delivery occurred with cell-level resolution. When control luciferase mRNA-containing LNPs were delivered, no EGFP protein expression was detected (Fig. 8A, *upper left micrograph*, n=3 animals). Upon delivery of EGFP mRNA-containing LNPs, ectopic expression of EGFP protein was detected in the stratified epithelial cells of the mouse vagina (Fig. 8A, *dark staining in apical portion of lower left micrograph*, n=6 animals).

To determine if repeated exposure to PONA-formulated mRNA enhances ectopic protein expression in vaginal stratified epithelial cells, EGFP mRNA or Luciferase mRNA was formulated into PONA-based LNPs and delivered over multiple days. Luciferase mRNA was used as the negative control. LNP-mRNA (20 ug of mRNA in 20 uL volume) was delivered to the mouse vaginal cavity once per day for 3 days. 6 hours after the third LNP dose, vaginal tissues were harvested, and 5-micron sections were prepared. Immunohistochemistry for EGFP was performed to determine where functional mRNA delivery were delivered, no EGFP protein expression was detected (Fig. 8A, *upper right micrograph*, n=5 animals). Upon delivery of three doses of EGFP mRNA-containing LNPs, ectopic expression of EGFP protein was detected in the stratified epithelial cells of the mouse vagina (Fig. 8A, *areas of dark staining in lower right micrograph*, n=5 animals) and this expression is apparent in more total epithelial cells and basal epithelial cells compared to single dose exposure (as shown in Fig. 8A).

The results presented in Fig. 7 and Fig. 8 demonstrate that PONA-based LNP formulation protects mRNA from degradation *in vivo* for topical mucosal delivery, enables entry into multiple layers of stratified epithelial cells, facilitates escape from endosomes, and provides functional transcript for translation into the mRNA-encoded protein. Furthermore, repeat drug exposure with multiple topical applications of PONA LNP-formulated mRNA enhances the topical local delivery efficiency and coverage of encoded protein to the full thickness of the epithelium including the basal layer, the site of the persistent HPV reservoir, without microscopic evidence of sub-epithelial drug penetration or local histopathology.

Example 7: gRNA PONA LNP Activity in Ai9 Transgenic Mouse Model

Relevant animal models of HPV16 infection and disease have significant biological limitations and are not readily available. Therefore, an animal model with an alternative DNA target was used to demonstrate topical delivery of Cas9 mRNA combined with gRNA formulated in PONA LNP to target epithelial cells and induce local *in situ* CRISPR activity as well as *in vivo* CRISPR activity.

The Ai9 transgenic mouse model has been used extensively in *in vivo* cell lineage studies, as permanent Cre-Lox recombination allows for detection of single allelic events, *i.e.*, CRISPR/Cas9-mediated cleavage of surrogate target DNA. Ai9 transgenic mice encode an exogenous Tdtomato reporter gene engineered immediately downstream of a loxP-flanked repeat stop codon cassette. As a benchmark in this model, treatment with Cre efficiently causes recombination and removal of the stop codons, thereby inducing Tdtomato mRNA transcription and protein expression. Alternatively, treatment with Cas9 mRNA combined with gRNA targeting the upstream stop codons, g290 and/or g298 gRNA (Staahl *et al.*, *Nat Biotechnol.* 35(5):431-34, 2017), can induce Tdtomato expression following cell-mediated repair of CRISPR-mediated DNA cleavage. *See* Fig. 9A. The Ai9 model thus provides a sensitive and specific readout for CRISPR activity that can accumulate in surviving cells and be detected in cells and *in vivo*.

To demonstrate activity of CRISPR active molecules in Ai9 cells, primary Ai9 mouse fibroblasts were isolated from Ai9 mouse ear tissue and cultivated in rich media. Lipid transfection reagents were used to transfect either Cre mRNA or Cas9 mRNA and gRNA. Cre mRNA was transfected at 2 ng or 10 ng per 1,000 cells. Cas9 mRNA combined with stop codon-specific surrogate gRNA at a 1:1 mass ratio were transfected at 2 ng or 10 ng of total RNA per 1,000 cells. 24 hours after transfection, cells were harvested and the fraction of Tdtomato expressing cells relative to the total Ai9 cell population were quantified using flow cytometry with Tdtomato-specific antibody. The results indicate that comparable levels of Tdtomato are induced by Cre mRNA and CRISPR active molecules at the higher total RNA dose (Fig. 9B).

To demonstrate *in vivo* activity of CRISPR active molecules formulated in PONA LNP, female Ai9 mice were treated with Depo-Provera prior to topical intravaginal delivery of PONA LNP-formulated Cas9 mRNA combined with either surrogate gRNA or a non-targeting negative control gRNA. Mice were given 3 serial doses with 20 ug of total RNA per dose at days 1, 3, and 5. At day 12, vaginal tissue was harvested, fixed, embedded, and sectioned, and Tdtomato mRNA was detected with a sequence specific probe set using a branched probe *in situ* hybridization (ISH) method. The presence of Tdtomato mRNA signal (red color) indicates effective local *in vivo* delivery of functional CRISPR active molecules formulated in PONA LNP (Fig. 10, *areas of darker staining in right micrographs*).

The data in Fig. 9 and Fig. 10 demonstrate that co-formulation of Cas9 mRNA and gRNA into PONA LNP creates a therapeutic composition that safely and effectively induces target-specific CRISPR activity when delivered topically to the mucosal epithelium. Repeat exposure to drug product induces broad and local coverage of CRISPR activity that is present in and limited to the stratified epithelium including the basal layer, the site of the persistent HPV reservoir, without *in situ* evidence of sub-epithelial drug penetration to deeper tissues and without evidence of local histopathology.

The formulations enable repeat topical delivery of functional RNA, including Cas9 mRNA and gRNA, to human cells and to mucosal epithelium *in vivo*. Topical local delivery of a CRISPR-based drug offers significant potency and safety advantages compared to systemic delivery for targets that are solely present in the epithelium, such as foreign viral DNA in infected cells. For example, topical delivery has the potential to: reduce the amount of drug needed, limit drug exposure to local tissues, minimize the risk for systemic toxicity, and minimize the risk for immunogenicity induced against drug product. HPV is one example of a human viral pathogen that is localized to epithelium that is accessible by local delivery. Other relevant viral targets that may be amenable to this strategy include: respiratory viruses, such as influenza and RSV, gastrointestinal viruses, such as rotavirus and norovirus, and viruses in the keratinized skin, such as MCV.

The various embodiments described above can be combined to provide further embodiments. All of the U.S. patents, U.S. patent application publications, U.S. patent applications, foreign patents, foreign patent applications, and non-patent publications referred to in this specification and/or listed in the Application Data Sheet, including U.S. Provisional Patent Application 62/797029 filed January 25, 2019, are incorporated herein by reference, in their entirety. Aspects of the embodiments can be modified, if necessary to employ concepts of the various patents, applications and publications to provide yet further embodiments.

These and other changes can be made to the embodiments in light of the above-detailed description. In general, in the following claims, the terms used should not be construed to limit the claims to the specific embodiments disclosed in the specification and the claims, but should be construed to include all possible embodiments along with the full scope of equivalents to which such claims are entitled. Accordingly, the claims are not limited by the disclosure.

SEQUENCE LISTING

In the following sequences, all mRNAs possess a Cap1 structure on their 5' end. Abbreviations for nucleotide modifications used in the sequences are shown in Table 2.

Table 2. Abbreviations for nucleotide modifications.

Abbreviation	Modification
mC	2'-O-methylCytidine
mG	2'-O-methylGuanadine
mU	2'-O-methylUridine
mA	2'-O-methylAdenine
*	phosphorothioate backbone
5	5-methoxyUridine

SEQ ID NO: 1 – Wild-type *Spy* Cas9 protein, UniProt Q99ZW2

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SEQ ID NO: 2 – Wild-type Spy Cas9 mRNA

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SEQ ID NO: 3 – Wild-type *Spy* Cas9 mRNA with modified nucleotides

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SEQ ID NO: 4 - *Spy* Cas9 HF2 mRNA

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AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA

SEQ ID NO: 5 - *Spy* Cas9 HF2 mRNA with modified nucleotides

AGAGCCACCA5GGCCCCCAAGAAGAAGCGGAAGG5GGGCA5CCACGGCG5G
CCCGCCGCCGACAAGAAG5ACAGCA5CGGCC5GGACA5CGGCACCAACAGC
G5GGGC5GGGCCG5GA5CACCGACGAG5ACAAGG5GCCCAGCAAGAAG55CA
AGG5GC5GGGCAACACCGACCGGCACAGCA5CAAGAAGAACC5GA5CGGCG
CCC5GC5G55CGACAGCGGCGAGACCGCCGAGGCCACCCGGC5GAAGCGGA
CCGCCCGGCGGCGG5ACACCCGGCGGAAGAACCGGA5C5GC5ACC5GCAGG
AGA5C55CAGCAACGAGA5GGCCAAGG5GGACGACAGC55C55CCACCGGC5G
GAGGAGAGC55CC5GG5GGAGGAGGACAAGAAGCACGAGCGGCACCCCA5C
55CGGCAACA5CG5GGACGAGG5GGCC5ACCACGAGAAG5ACCCCACCA5C5
ACCACC5GCGGAAGAAGC5GG5GGACAGCACCGACAAGGCCGACC5GCGGC
5GA5C5ACC5GGCCC5GGCCCACA5GA5CAAG55CCGGGGCCAC55CC5GA5CG
AGGGCGACC5GAACCCCGACAACAGCGACG5GGACAAGC5G55CA5CCAGC5
GG5GCAGACC5ACAACCAGC5G55CGAGGAGAACCCCA5CAACGCCAGCGGC
G5GGACGCCAAGGCCA5CC5GAGCGCCCGGC5GAGCAAGAGCCGGCGGC5G
GAGAACC5GA5CGCCCAGC5GCCCGGCGAGAAGAAGAACGGCC5G55CGGC

AACC5GA5CGCCC5GAGCC5GGGCC5GACCCCCAAC55CAAGAGCAAC55CGA
CC5GGCCGAGGACGCCAAGC5GCAGC5GAGCAAGGACACC5ACGACGACGA
CC5GGACAACC5GC5GGCCCAGA5CGGCGACCAG5ACGCCGACC5G55CC5GG
CCGCCAAGAACC5GAGCGACGCCA5CC5GC5GAGCGACA5CC5GCGGG5GAA
CACCGAGA5CACCAAGGCCCCCC5GAGCGCCAGCA5GA5CAAGCGG5ACGA
CGAGCACCACCAGGACC5GACCC5GC5GAAGGCCC5GG5GCGGCAGCAGC5G
CCCGAGAAG5ACAAGGAGA5C55C55CGACCAGAGCAAGAACGGC5ACGCCG
GC5ACA5CGACGGCGGCGCCAGCCAGGAGGAG55C5ACAAG55CA5CAAGCC
CA5CC5GGAGAAGA5GGACGGCACCGAGGAGC5GC5GG5GAAGC5GAACCG
GGAGGACC5GC5GCGGAAGCAGCGGACC55CGACAACGGCAGCA5CCCCCA
CCAGA5CCACC5GGGCGAGC5GCACGCCA5CC5GCGGCGGCAGGAGGAC55C
5ACCCC55CC5GAAGGACAACCGGGAGAAGA5CGAGAAGA5CC5GACC55CC
GGA5CCCC5AC5ACG5GGGCCCCC5GGCCCGGGGCAACAGCCGG55CGCC5G
GA5GACCCGGAAGAGCGAGGAGACCA5CACCCCC5GGAAC55CGAGGAGG5
GG5GGACAAGGGCGCCAGCGCCCAGAGC55CA5CGAGCGGA5GACCAAC55C
GACAAGAACC5GCCCAACGAGAAGG5GC5GCCCAAGCACAGCC5GC5G5ACG
AG5AC55CACCG5G5ACAACGAGC5GACCAAGG5GAAG5ACG5GACCGAGGG
CA5GCGGAAGCCCGCC55CC5GAGCGGCGAGCAGAAGAAGGCCA5CG5GGA
CC5GC5G55CAAGACCAACCGGAAGG5GACCG5GAAGCAGC5GAAGGAGGAC
5AC55CAAGAAGA5CGAG5GC55CGACAGCG5GGAGA5CAGCGGCG5GGAGG
ACCGG55CAACGCCAGCC5GGGCACC5ACCACGACC5GC5GAAGA5CA5CAA
GGACAAGGAC55CC5GGACAACGAGGAGAACGAGGACA5CC5GGAGGACA5
CG5GC5GACCC5GACCC5G55CGAGGACCGGGAGA5GA5CGAGGAGCGGC5G
AAGACC5ACGCCCACC5G55CGACGACAAGG5GA5GAAGCAGC5GAAGCGGC
GGCGG5ACACCGGC5GGGGCCGGC5GAGCCGGAAGC5GA5CAACGGCA5CC
GGGACAAGCAGAGCGGCAAGACCA5CC5GGAC55CC5GAAGAGCGACGGC55
CGCCAACCGGAAC55CA5GCAGC5GA5CCACGACGACAGCC5GACC55CAAG
GAGGACA5CCAGAAGGCCCAGG5GAGCGGCCAGGGCGACAGCC5GCACGA
GCACA5CGCCAACC5GGCCGGCAGCCCCGCCA5CAAGAAGGGCA5CC5GCAG
ACCG5GAAGG5GG5GGACGAGC5GG5GAAGG5GA5GGGCCGGCACAAGCCC
GAGAACA5CG5GA5CGAGA5GGCCCGGGAGAACCAGACCACCCAGAAGGGC
CAGAAGAACAGCCGGGAGCGGA5GAAGCGGA5CGAGGAGGGCA5CAAGGA
GC5GGGCAGCCAGA5CC5GAAGGAGCACCCCG5GGAGAACACCCAGC5GCA
GAACGAGAAGC5G5ACC5G5AC5ACC5GCAGAACGGCCGGGACA5G5ACG5G
GACCAGGAGC5GGACA5CAACCGGC5GAGCGAC5ACGACG5GGACCACA5C
G5GCCCCAGAGC55CC5GGCCGACGACAGCA5CGACAACAAGG5GC5GACCC
GGAGCGACAAGAACCGGGGCAAGAGCGACAACG5GCCCAGCGAGGAGG5G
G5GAAGAAGA5GAAGAAC5AC5GGCGGCAGC5GC5GAACGCCAAGC5GA5CA
CCCAGCGGAAG55CGACAACC5GACCAAGGCCGAGCGGGGCGGCC5GAGCG
AGC5GGACAAGGCCGGC55CA5CAAGCGGCAGC5GG5GGAGACCCGGCAGA5
CACCAAGCACG5GGCCCAGA5CC5GGACAGCCGGA5GAACACCAAG5ACGA
CGAGAACGACAAGC5GA5CCGGGAGG5GAAGG5GA5CACCC5GAAGAGCAA
GC5GG5GAGCGAC55CCGGAAGGAC55CCAG55C5ACAAGG5GCGGGAGA5CA
ACAAC5ACCACCACGCCCACGACGCC5ACC5GAACGCCG5GG5GGGCACCGC
CC5GA5CAAGAAG5ACCCCGCCC5GGAGAGCGAG55CG5G5ACGGCGAC5AC
AAGG5G5ACGACG5GCGGAAGA5GA5CGCCAAGAGCGAGCAGGAGA5CGGC
AAGGCCACCGCCAAG5AC55C55C5ACAGCAACA5CA5GAAC55C55CAAGAC
CGAGA5CACCC5GGCCAACGGCGAGA5CCGGAAGGCCCCCC5GA5CGAGAC
CAACGGCGAGACCGGCGAGA5CG5G5GGGACAAGGGCCGGGAC55CGCCAC

CG5GCGGAAGG5GC5GAGCA5GCCCCAGG5GAACA5CG5GAAGAAGACCGA
GG5GCAGACCGGCGGC55CAGCAAGGAGAGCA5CC5GCCCAAGCGGAACAG
CGACAAGC5GA5CGCCCGGAAGAAGGAC5GGGACCCCAAGAAG5ACGGCGG
C55CGACAGCCCCACCG5GGCC5ACAGCG5GC5GG5GG5GGCCAAGG5GGAG
AAGGGCAAGAGCAAGAAGC5GAAGAGCG5GAAGGAGC5GC5GGGCA5CACC
A5CA5GGAGCGGAGCAGC55CGAGAAGAACCCCA5CGAC55CC5GGAGGCCA
AGGGC5ACAAGGAGG5GAAGAAGGACC5GA5CA5CAAGC5GCCCAAG5ACA
GCC5G55CGAGC5GGAGAACGGCCGGAAGCGGA5GC5GGCCAGCGCCGGCG
AGC5GCAGAAGGGCAACGAGC5GGCCC5GCCCAGCAAG5ACG5GAAC55CC5
G5ACC5GGCCAGCCAC5ACGAGAAGC5GAAGGGCAGCCCCGAGGACAACGA
GCAGAAGCAGC5G55CG5GGAGCAGCACAAGCAC5ACC5GGACGAGA5CA5C
GAGCAGA5CAGCGAG55CAGCAAGCGGG5GA5CC5GGCCGACGCCAACC5GG
ACAAGG5GC5GAGCGCC5ACAACAAGCACCGGGACAAGCCCA5CCGGGAGC
AGGCCGAGAACA5CA5CCACC5G55CACCC5GACCAACC5GGGCGCCCCCGC
CGCC55CAAG5AC55CGACACCACCA5CGACCGGAAGCGG5ACACCAGCACC
AAGGAGG5GC5GGACGCCACCC5GA5CCACCAGAGCA5CACCGGCC5G5ACG
AGACCCGGA5CGACC5GAGCCAGC5GGGCGGCGACAGCGGCGGCAAGCGGC
CCGCCGCCACCAAGAAGGCCGGCCAGGCCAAGAAGAAGAAGGGCAGC5GA
GCGGCCGC55AA55AAGC5GCC55C5GCGGGGC55GCC55C5GGCCA5GCCC55C
55C5C5CCC55GCACC5G5ACC5C55GG5C555GAA5AAAGCC5GAG5AGGAAGA
AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
AAAAAAAAAAAAAAAAAAAAA

SEQ ID NO: 6 – HPV16-specific gRNA, E7-14
5'-GCA AGU GUG ACU CUA CGC UUG UUU UAG AGC UAG AAA UAG CAA GUU AAA AUA AGG CUA GUC CGU UAU CAA CUU GAA AAA GUG GCA CCG AGU CGG UGC UUU UUU -3'

SEQ ID NO: 7 - HPV16-specific gRNA, E7-14 with modified nucleotides

5'- mG*mC*mA* AGU GUG ACU CUA CGC UUG UUU UAG AGC UAG AAA UAG CAA GUU AAA AUA AGG CUA GUC CGU UAU CAA CUU GAA AAA GUG GCA CCG AGU CGG UGC UU mU* mU*mU*U -3'

SEQ ID NO: 8 - HPV18-specific guide RNA (non-targeting for human cells or HPV16, negative control gRNA)

5'- CAA GCU ACC UGA UCU GUG CAG UUU UAG AGC UAG AAA UAG CAA GUU AAA AUA AGG CUA GUC CGU UAU CAA CUU GAA AAA GUG GCA CCG AGU CGG UGC UUU UUU -3'

SEQ ID NO: 9 – HPV18-specific guide RNA with modified nucleotides (non-targeting for human cells or HPV16, negative control gRNA)

5'- mC*mA*mA* GCU ACC UGA UCU GUG CAG UUU UAG AGC UAG AAA UAG CAA GUU AAA AUA AGG CUA GUC CGU UAU CAA CUU GAA AAA GUG GCA CCG AGU CGG UGC UU mU* mU*mU*U -3'

CLAIMS

What is claimed is:

1. A synthetic guide RNA comprising a nucleotide sequence having at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO: 6 or SEQ ID NO: 7.

2. The guide RNA according to claim 1, wherein the guide RNA comprises the nucleotide sequence as set forth in SEQ ID NO: 6 or SEQ ID NO: 7.

3. The guide RNA according to claim 1, wherein the guide RNA consists of the nucleotide sequence as set forth in SEQ ID NO: 6 or SEQ ID NO: 7.

4. The guide RNA according to any of claims 1-3, wherein the guide RNA comprises one or more modified bases.

5. The guide RNA according to claim 4, wherein the guide RNA comprises one or more modified bases, wherein the one or more modified bases is 2′-O-methylcytidine; 2′-O-methylguanadine; 2′-O-methyluridine; or 2′-O-methyladenine.

6. The guide RNA according to claim 4, wherein the guide RNA comprises a plurality of modified bases, wherein each of the modified bases is independently selected from the group consisting of 2′-O-methylcytidine; 2′-O-methylguanadine; 2′-O-methyluridine; and 2′-O-methyladenine.

7. The guide RNA according to any of claims 1-6, wherein one or more of the internucleoside bonds in the guide RNA is a phosphorothioate bond.

8. The guide RNA according to claim 7, wherein one or more of the internucleoside bonds within the ten terminal nucleotides at each of a 5′ end and at a 3′ end of the guide RNA is a phosphorothioate bond.

9. The guide RNA according to claim 1, wherein the guide RNA comprises the nucleotide sequence as set forth in SEQ ID NO: 7.

10. A pharmaceutical composition comprising:

an mRNA encoding a Cas endonuclease;

a guide RNA according to any one of claims 1-9;

a plurality of nanoparticles comprising a cationic lipid and encapsulating the mRNA and the guide RNA;

and a carrier formulation.

11. The pharmaceutical composition according to claim 10, wherein the cationic lipid comprises PONA.

12. The pharmaceutical composition according to any one of claims 10-11, wherein the nanoparticles further comprise one or more of CHEMS, cholesterol, and DMPE-PEG2K.

13. The pharmaceutical composition according to any one of claims 10-12, wherein the plurality of nanoparticles are dispersed within the carrier formulation, and the carrier formulation comprises a carrier liquid, oil, or gel.

14. The pharmaceutical composition according to any one of claims 10-13, wherein the Cas endonuclease is a Cas9 endonuclease.

15. The pharmaceutical composition according to claim 14, wherein the Cas9 endonuclease comprises between one and twenty-five amino acid substitutions relative to wild type Cas9 endonuclease.

16. The pharmaceutical composition according to any one of claims 14-15, wherein the mRNA encoding a Cas9 endonuclease comprises 5-methoxyuridine.

17. The pharmaceutical composition of claim 16, wherein the mRNA encoding a Cas9 endonuclease comprises a 5-methoxyuridine in place of each uridine present in the wild-type Cas9 endonuclease mRNA sequence.

18. The pharmaceutical composition according to claim 14, wherein the mRNA encoding a Cas endonuclease comprises the nucleotide sequence as set forth in any one of SEQ ID NO: 2 to SEQ ID NO: 5.

19. The pharmaceutical composition according to claim 17, wherein the mRNA encoding a Cas endonuclease comprises the nucleotide sequence as set forth in SEQ ID NO: 3 or SEQ ID NO: 5.

20. The pharmaceutical composition according to claim 10, wherein the guide RNA comprises the nucleotide sequence as set forth in SEQ ID NO: 7 and the mRNA encoding a Cas endonuclease comprises the nucleotide sequence as set forth in SEQ ID NO: 3 or SEQ ID NO: 5.

21. The pharmaceutical composition according to any one of claims 10-20 for use in a method for treating HPV infection.

22. The pharmaceutical composition according to any one of claims 10-20 for use in the manufacture of a medicament for the treatment of HPV infection.

23. A method of treating a viral infection in a subject, comprising administering to the subject a therapeutically effective amount of a guide RNA according to any one of claims 1-9, a pharmaceutical composition of any one of claims 10-20, the pharmaceutical composition for use of claim 21, or the pharmaceutical composition for use in the manufacture of a medicament of claim 22.

24. A method of treating a viral infection in a subject, comprising multiple sequential administration to the subject of a therapeutically effective amount of a guide RNA according to any one of claims 1-9, a pharmaceutical composition of any one of claims 10-20, the pharmaceutical composition for use of claim 21, or the pharmaceutical composition for use in the manufacture of a medicament of claim 22.

25. The method according to any one of claims 23-24, wherein administering comprises topical application to epithelium of the subject.

26. The method according to claim 25, wherein the epithelium comprises mucosal epithelium.

27. The method according to claim 26, wherein the mucosal epithelium comprises vaginal or anal epithelium.

28. The method according to any one of claims 23-27, wherein the subject is human.

29. The method according to any one of claims 23-28, wherein the viral infection is a human papillomavirus (HPV) infection.

30. The method according to any one of claims 23-29, wherein the subject has an HPV infection and has been diagnosed with HPV-positive pre-cancerous low-grade or high-grade lesions, HPV-positive squamous cell carcinoma *in situ*, or HPV-positive invasive cancer.

31. The method according to any one of claims 23-29, wherein the subject has an HPV infection and has HPV-positive pre-cancerous low-grade or high-grade lesions, HPV-positive squamous cell carcinoma *in situ*, or HPV-positive invasive cancer.

32. The method according to any one of claims 23-29, wherein the subject has an HPV infection and is at risk of HPV-positive pre-cancerous low-grade or high-grade lesions, HPV-positive squamous cell carcinoma *in situ*, or HPV-positive invasive cancer.

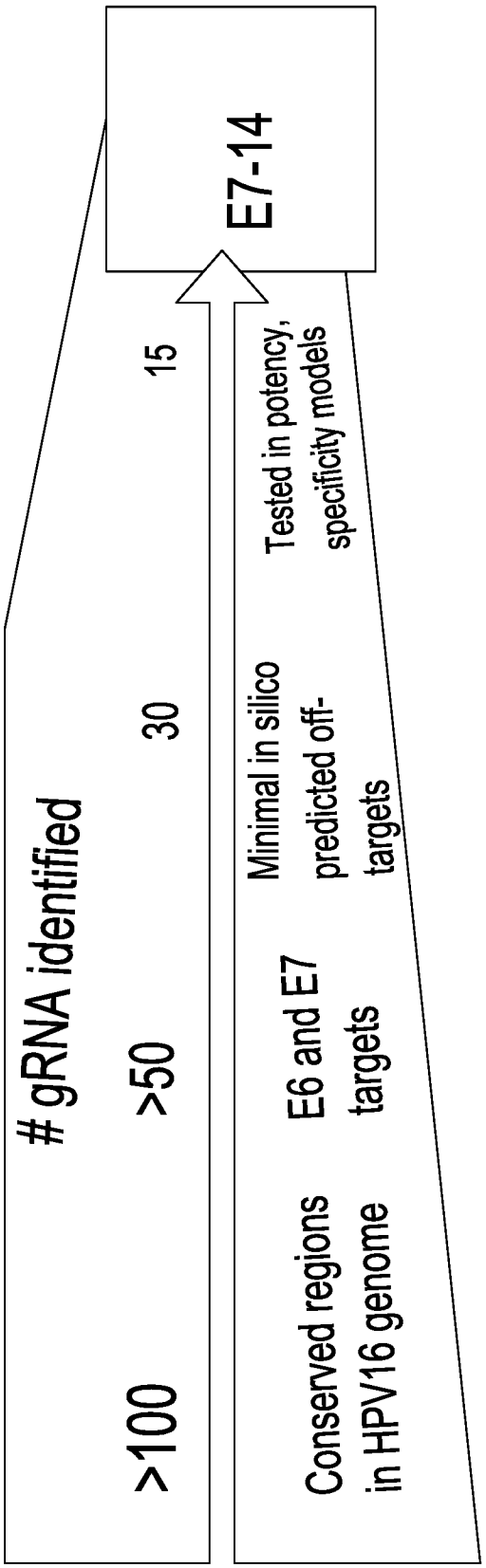
E7-14
15
30
>50
>100
gRNA identified
Conserved regions in HPV16 genome
E6 and E7 targets
Minimal in silico predicted off-targets
Tested in potency, specificity models

FIG. 1

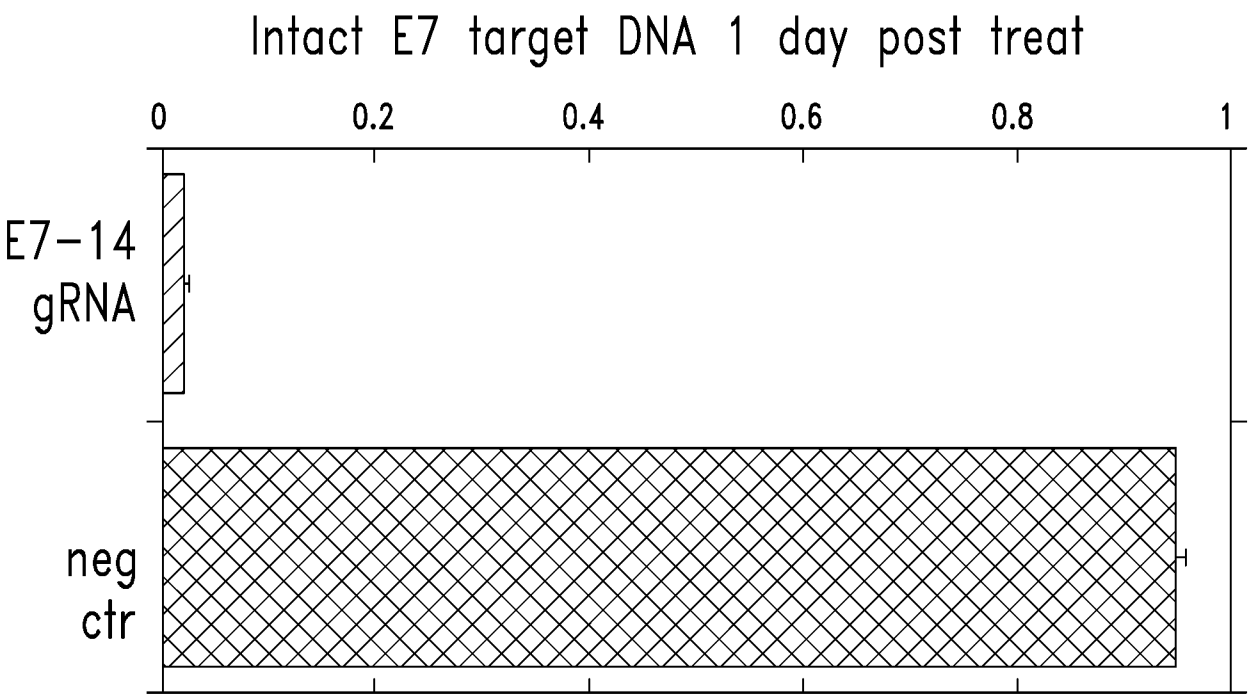
Intact E7 target DNA 1 day post treat
0
0.2
0.4
0.6
0.8
1
E7-14 gRNA
neg ctr

FIG. 2A

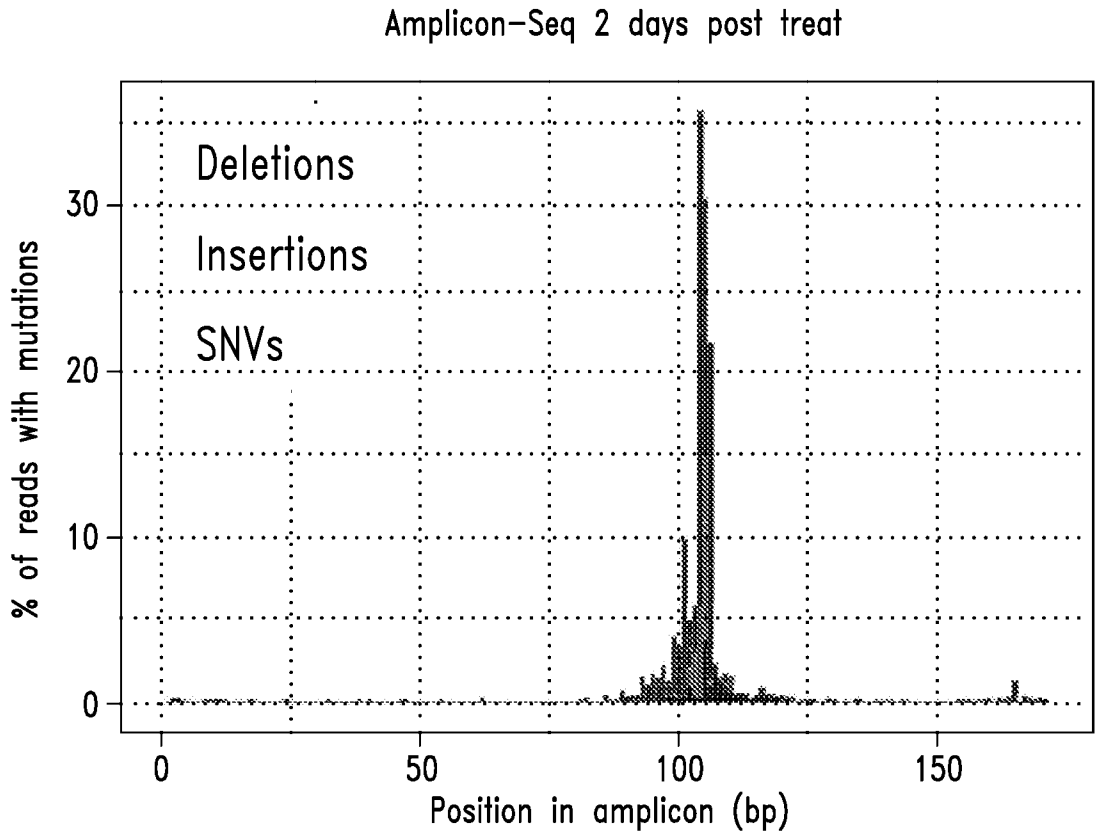
Amplicon-Seq 2 days post treat
Deletions
Insertions
SNVs
% of reads with mutations
30
20
10
0
0
50
100
150
Position in amplicon (bp)

FIG. 2B

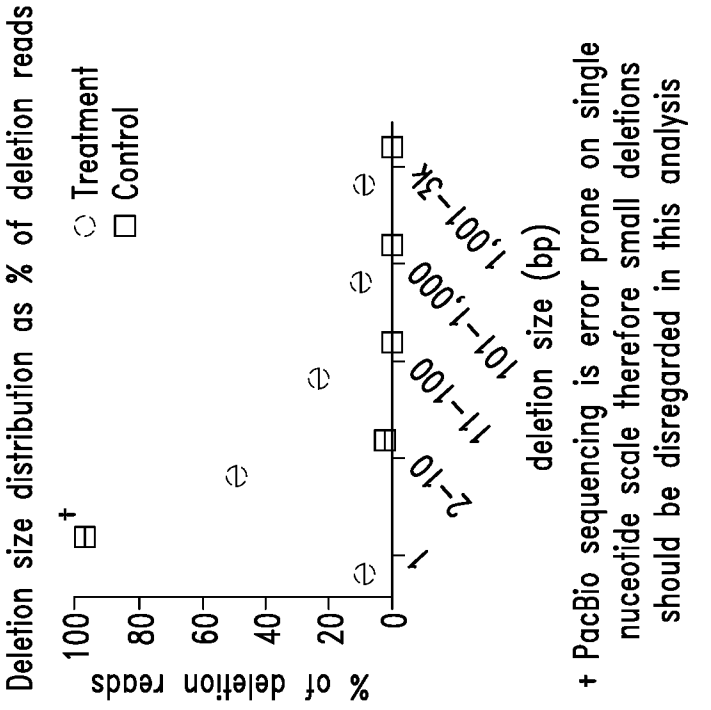
Deletion size distribution as % of deletion reads
Treatment
Control
% of deletion reads
100
80
60
40
20
0
1
2-10
11-100
101-1,000
1,001-2k
deletion size (bp)
+ PacBio sequencing is error prone on single nuceotide scale therefore small deletions should be disregarded in this analysis

FIG. 2D

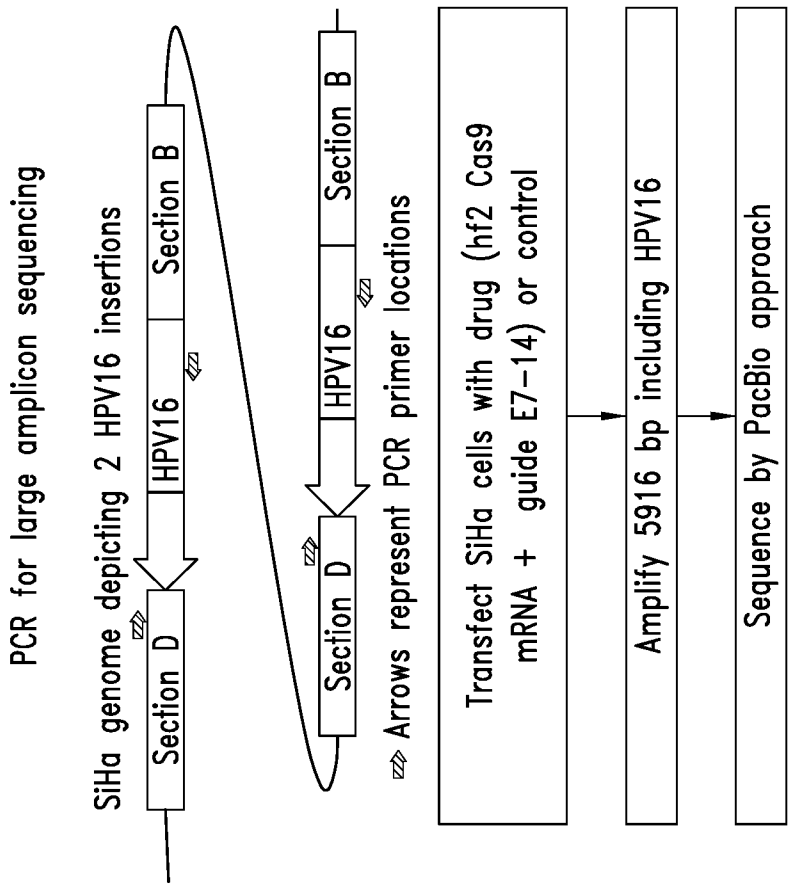
PCR for large amplicon sequencing
SiHa genome depicting 2 HPV16 insertions
Section D
HPV16
Section B
Section D
HPV16
Section B
Arrows represent PCR primer locations
Transfect SiHa cells with drug (hf2 Cas9 mRNA + guide E7-14) or control
Amplify 5916 bp including HPV16
Sequence by PacBio approach

FIG. 2C

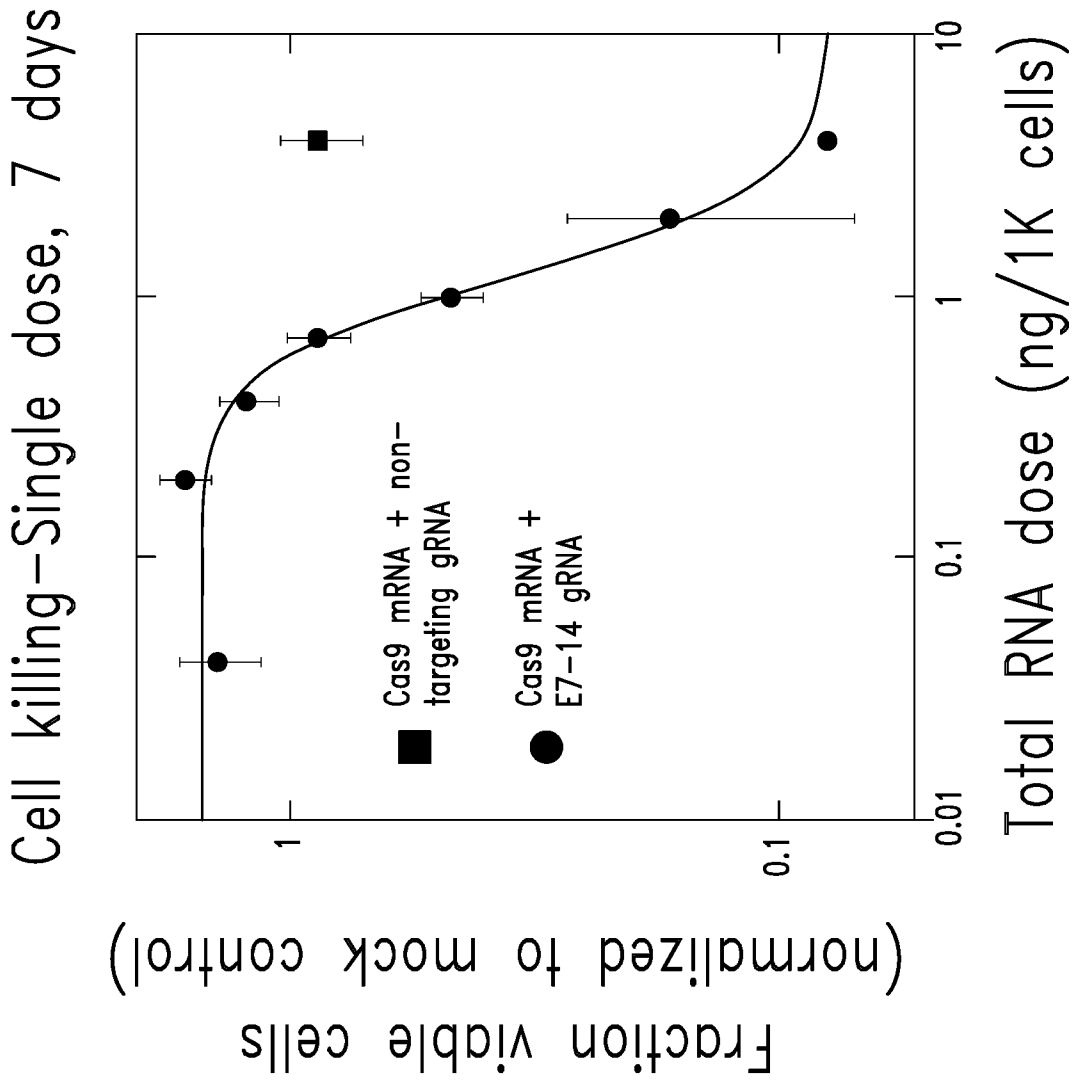
Cell killing–Single dose, 7 days
Fraction viable cells
(normalized to mock control)
1
0.1
Cas9 mRNA + non–targeting gRNA
Cas9 mRNA + E7–14 gRNA
0.01
0.1
1
10
Total RNA dose (ng/1K cells)

FIG. 3A

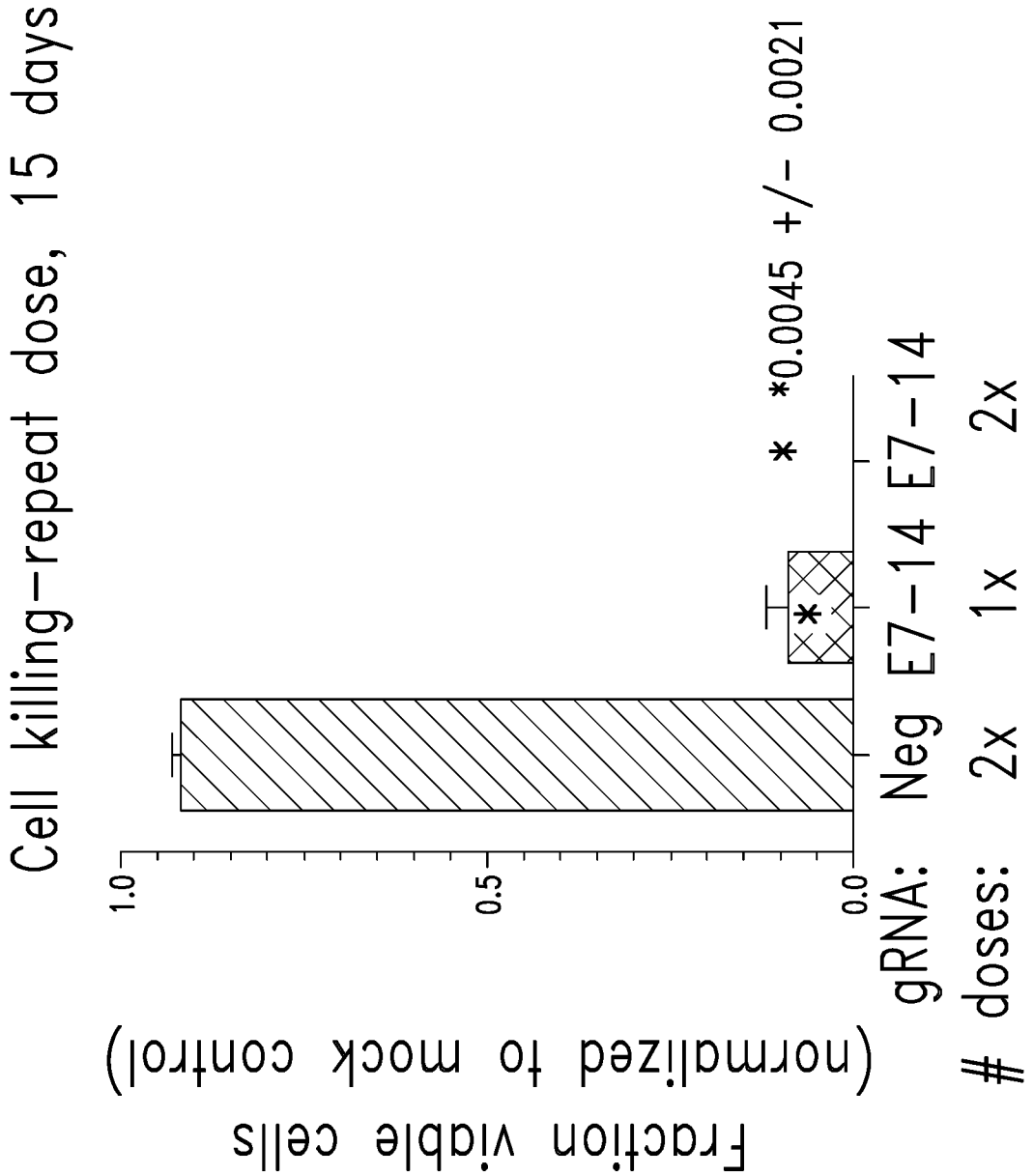
Cell killing–repeat dose, 15 days
Fraction viable cells
(normalized to mock control)
1.0
0.5
0.0
* *0.0045 +/– 0.0021
gRNA: Neg E7–14 E7–14
doses: 2x 1x 2x

FIG. 3B

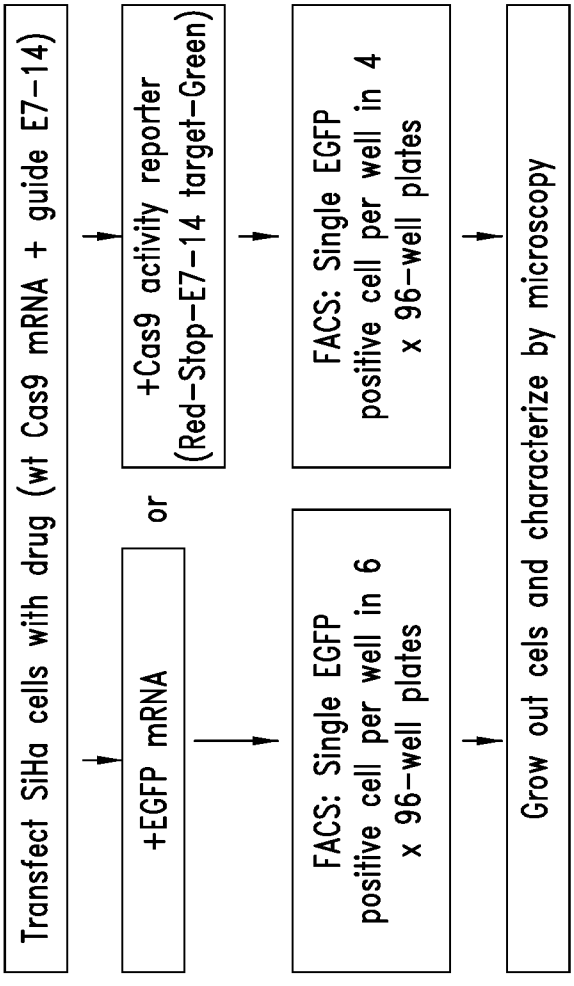
Transfect SiHa cells with drug (wt Cas9 mRNA + guide E7-14)
+EGFP mRNA
or
+Cas9 activity reporter (Red-Stop-E7-14 target-Green)
FACS: Single EGFP positive cell per well in 6 x 96-well plates
FACS: Single EGFP positive cell per well in 4 x 96-well plates
Grow out cels and characterize by microscopy

FIG. 4A

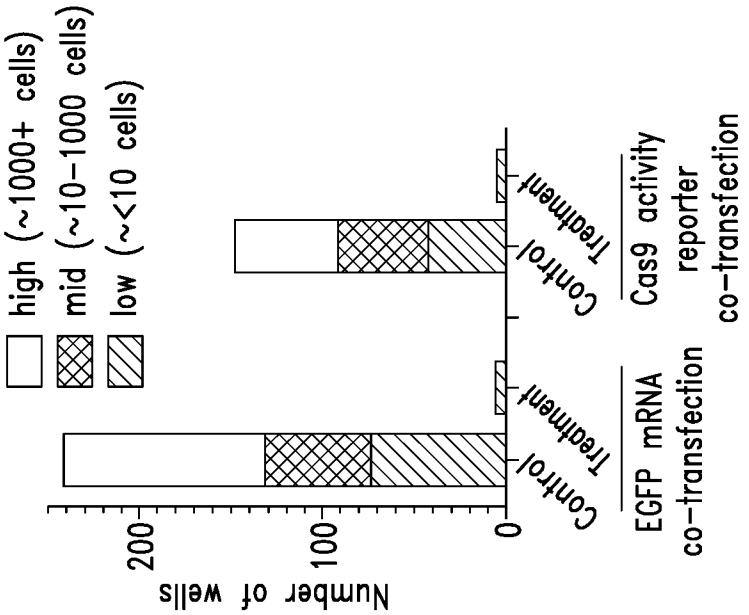
high (~1000+ cells)
mid (~10-1000 cells)
low (~<10 cells)
Number of wells
200
100
0
Control
Treatment
Control
Treatment
EGFP mRNA co-transfection
Cas9 activity reporter co-transfection

FIG. 4B

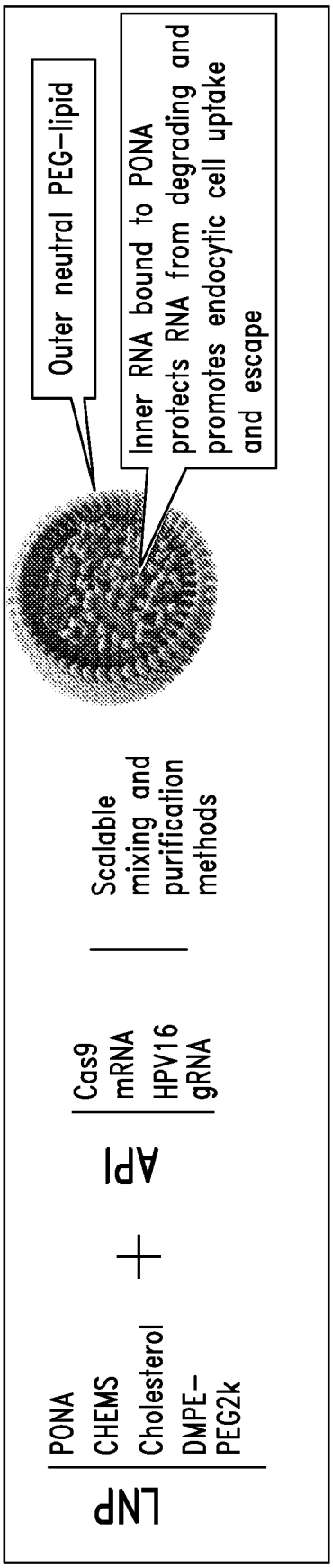
LNP
PONA
CHEMS
Cholesterol
DMPE-PEG2k
+
API
Cas9 mRNA
HPV16 gRNA
Scalable mixing and purification methods
Outer neutral PEG-lipid
Inner RNA bound to PONA protects RNA from degrading and promotes endocytic cell uptake and escape

FIG. 5A

Z-average	PDI	Zeta Potential	Final RNA	RNA in LNP
85 nm	0.10	-0.5 mV	> 1.0 mg/ml	> 95%

FIG. 5B

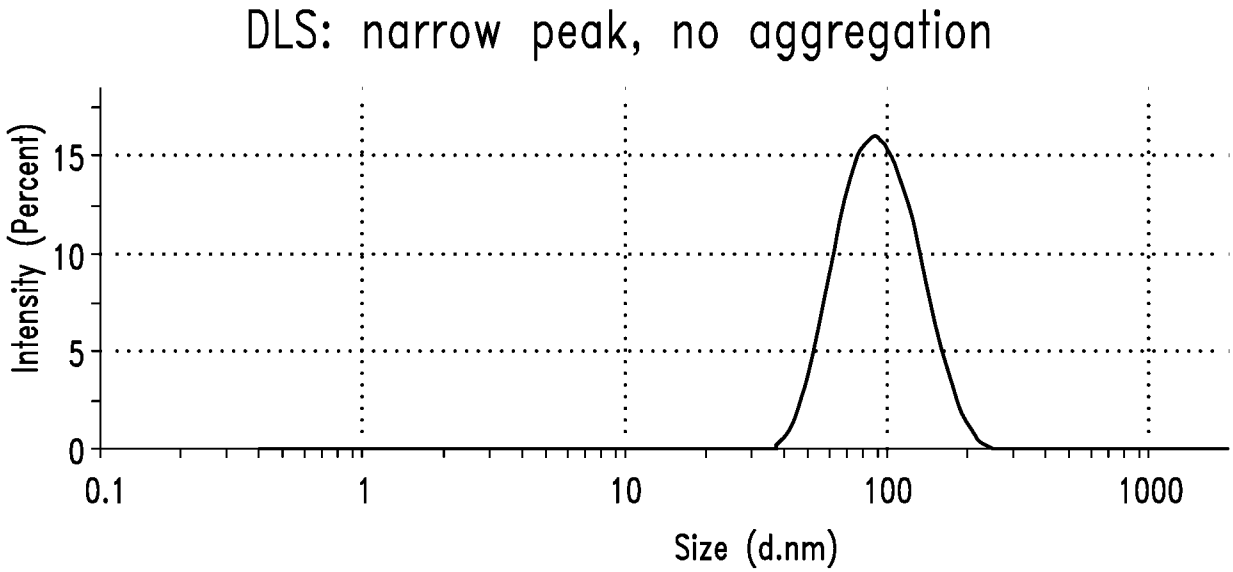

FIG. 5C

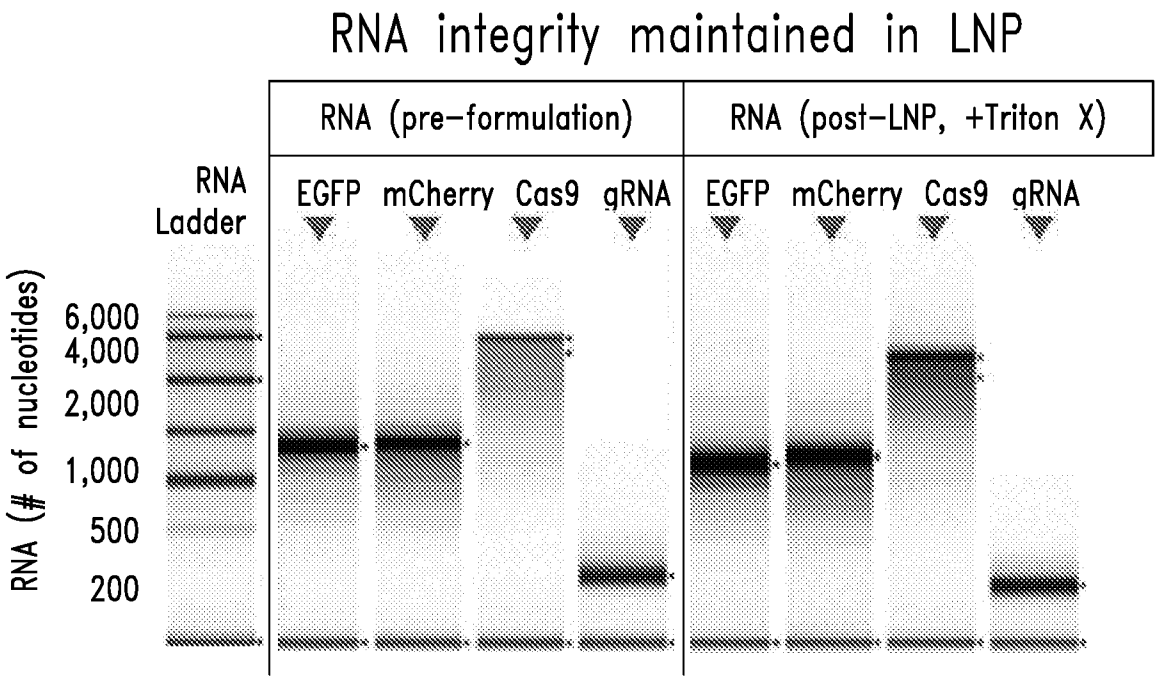
RNA integrity maintained in LNP
RNA (pre-formulation)
RNA (post-LNP, +Triton X)
RNA Ladder
EGFP
mCherry
Cas9
gRNA
EGFP
mCherry
Cas9
gRNA
RNA (# of nucleotides)
6,000
4,000
2,000
1,000
500
200

FIG. 5D

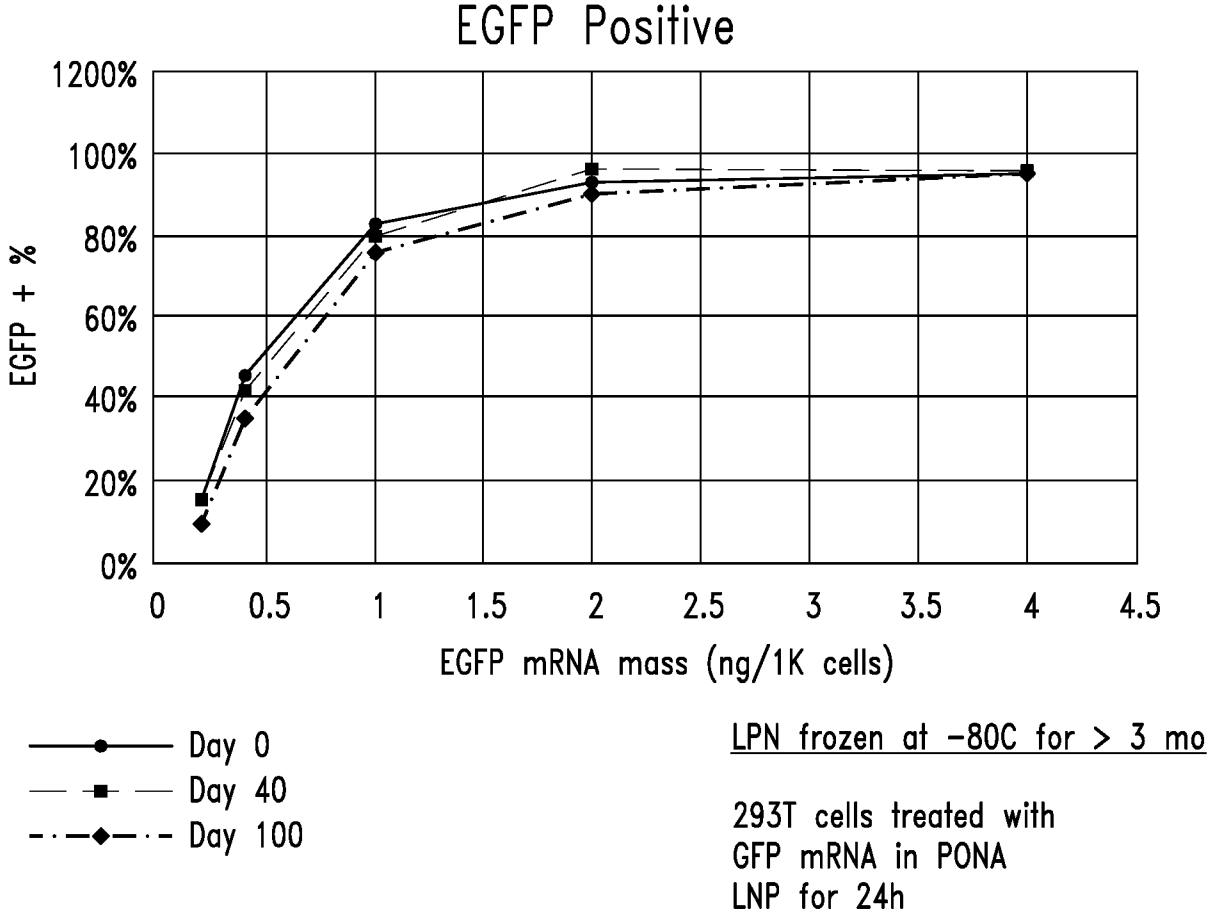
EGFP Positive
1200%
100%
80%
60%
40%
20%
0%
EGFP + %
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
EGFP mRNA mass (ng/1K cells)
Day 0
Day 40
Day 100
LPN frozen at -80C for > 3 mo
293T cells treated with
GFP mRNA in PONA
LNP for 24h

FIG. 5E

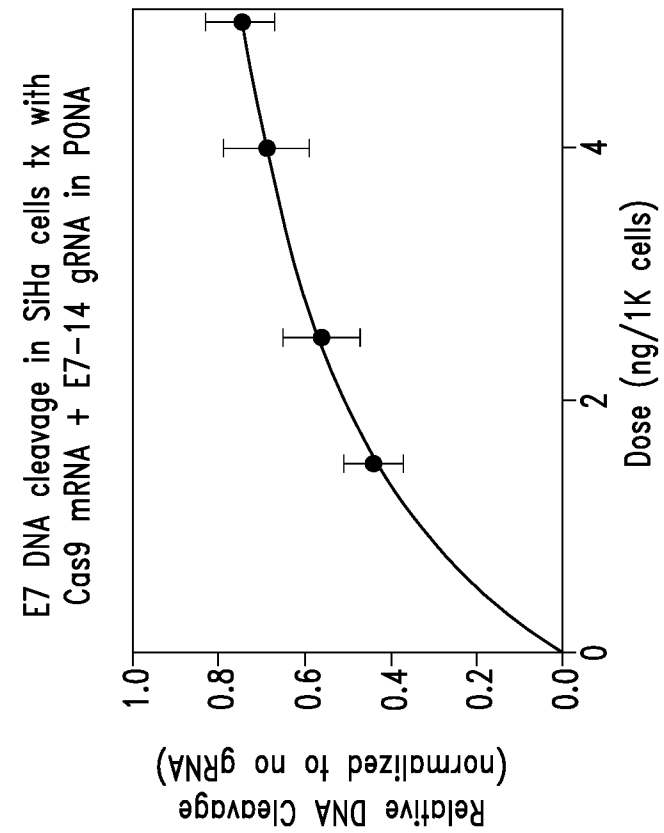
E7 DNA cleavage in SiHa cells tx with
Cas9 mRNA + E7-14 gRNA in PONA
Relative DNA Cleavage
(normalized to no gRNA)
1.0
0.8
0.6
0.4
0.2
0.0
0
2
4
Dose (ng/1K cells)

FIG. 6B

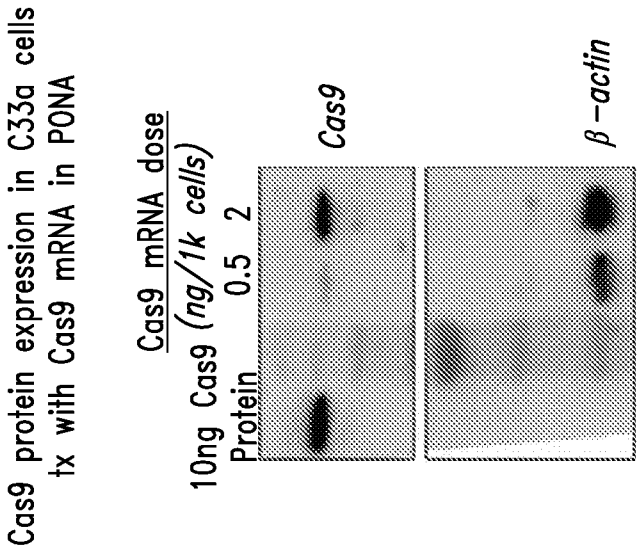
Cas9 protein expression in C33a cells
tx with Cas9 mRNA in PONA
Cas9 mRNA dose
10ng Cas9 (ng/1k cells)
Protein 0.5 2
Cas9
β-actin

FIG. 6A

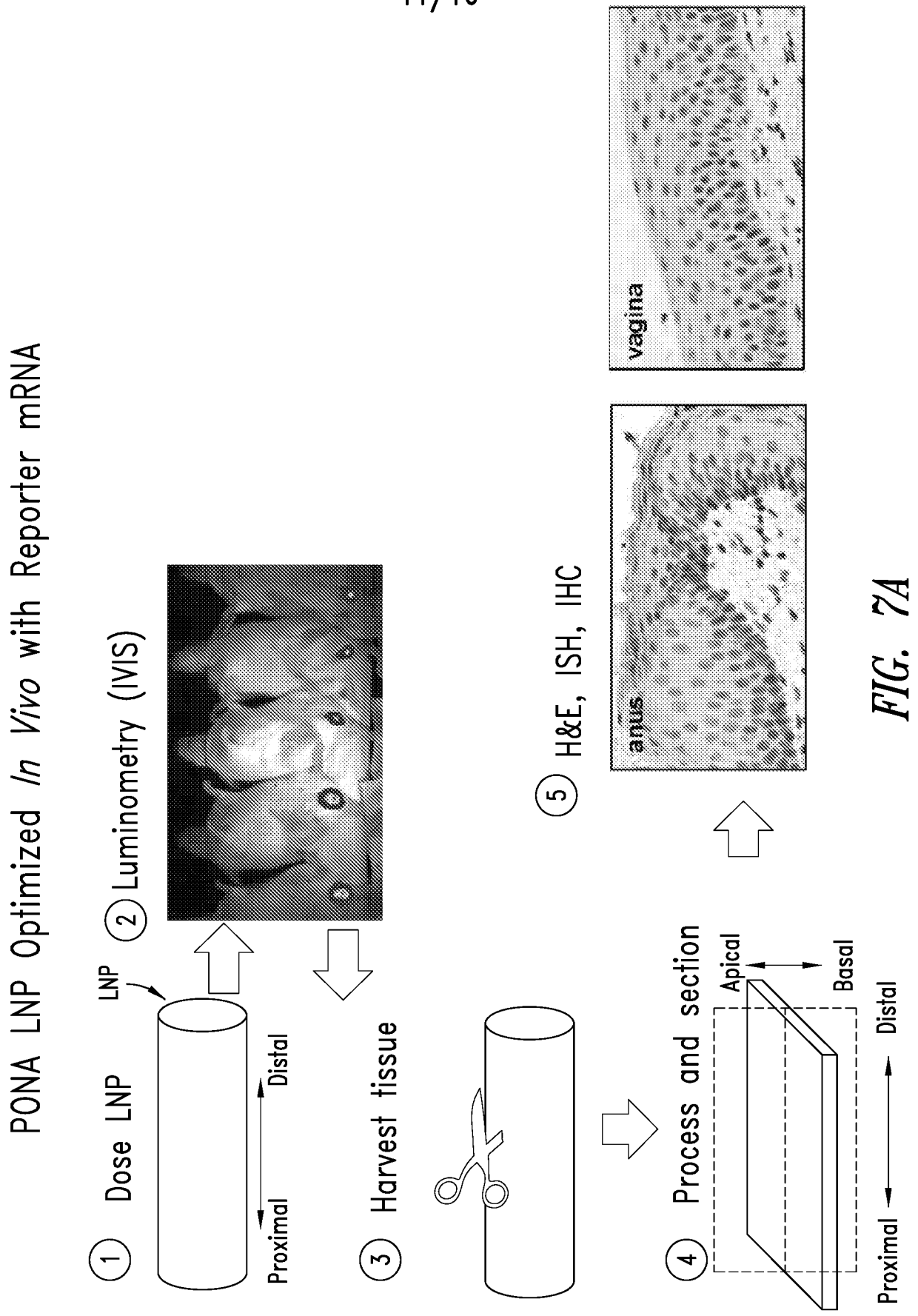
PONA LNP Optimized *In Vivo* with Reporter mRNA
1 Dose LNP
LNP
Proximal
Distal
2 Luminometry (IVIS)
3 Harvest tissue
4 Process and section
Apical
Basal
Proximal
Distal
5 H&E, ISH, IHC
anus
vagina

FIG. 7A

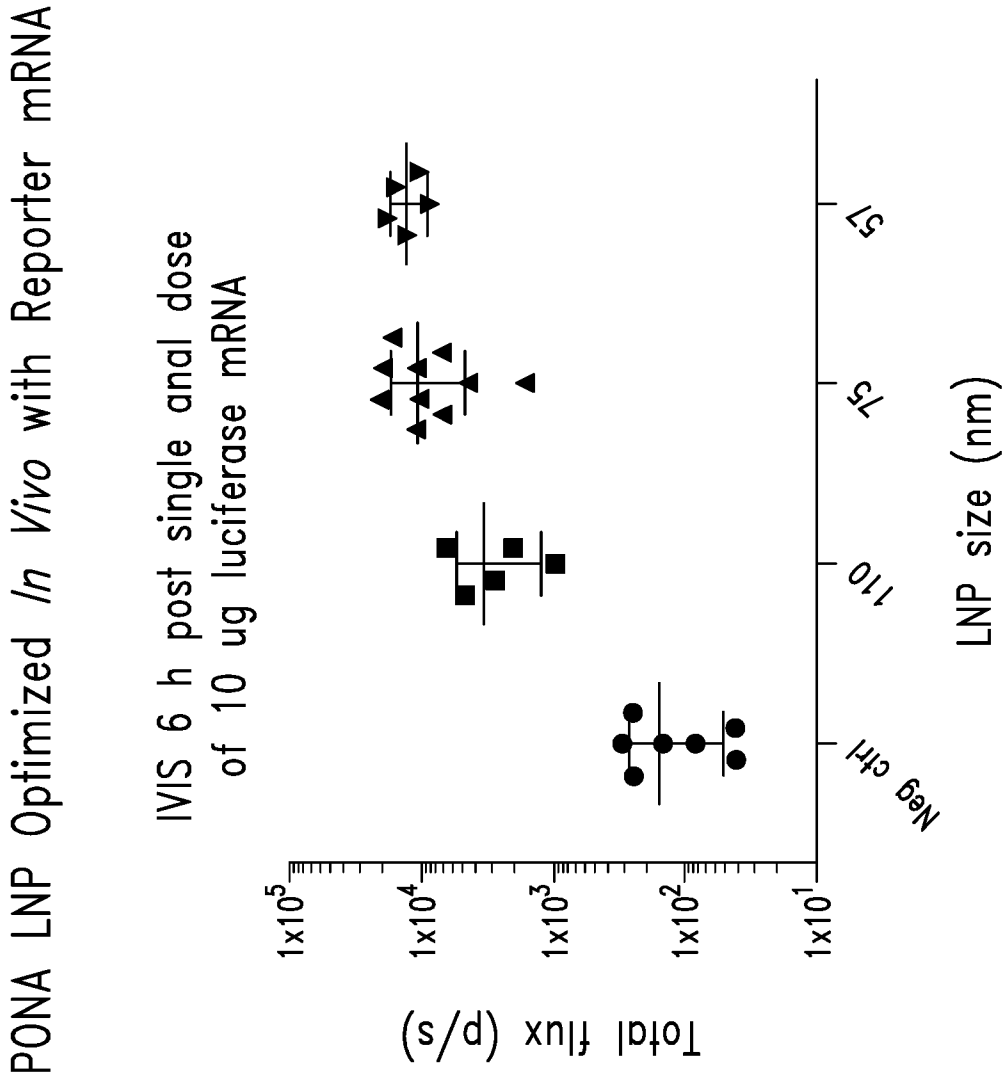
PONA LNP Optimized *In Vivo* with Reporter mRNA
IVIS 6 h post single anal dose
of 10 ug luciferase mRNA
Total flux (p/s)
1x10^5
1x10^4
1x10^3
1x10^2
1x10^1
Neg ctrl
110
75
57
LNP size (nm)

FIG. 7B

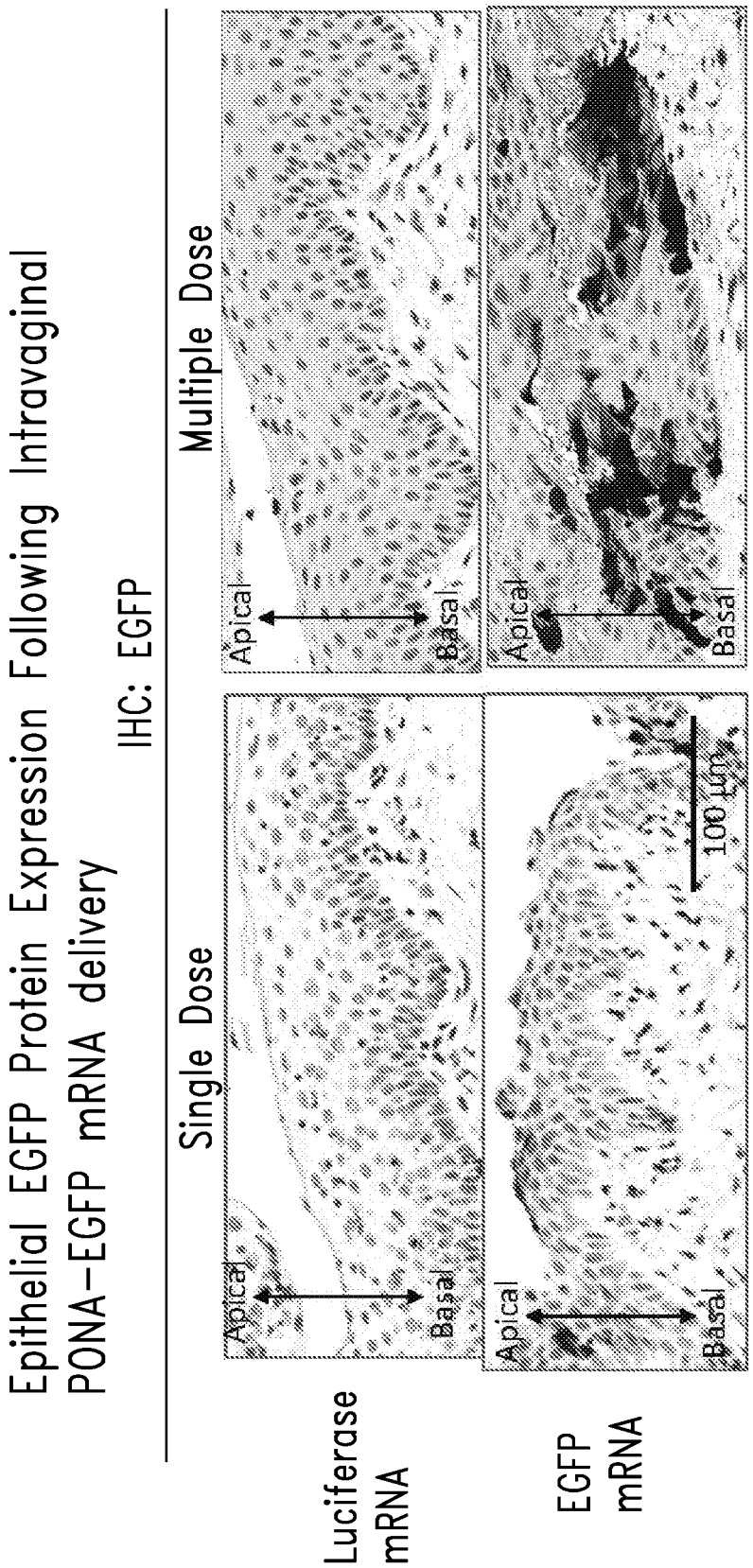
Epithelial EGFP Protein Expression Following Intravaginal PONA-EGFP mRNA delivery
IHC: EGFP
Single Dose
Multiple Dose
Luciferase mRNA
EGFP mRNA
Apical
Basal
100 µm

FIG. 8A

Number of Doses	mRNA Payload	mRNA Conc.	Form. no. (Particle Size)	GFP Positive Animals
1	Luciferase + EGFP	1 μg/μl	46-2 (57.2 nm)	6/6
1	Luciferase	1 μg/μl	43-3 (74.2 nm)	0/3
3	EGFP	1 μg/μl	66-2 (53.3 nm)	5/5
3	Luciferase	1 μg/μl	43-3 (74.2 nm)	0/5

FIG. 8B

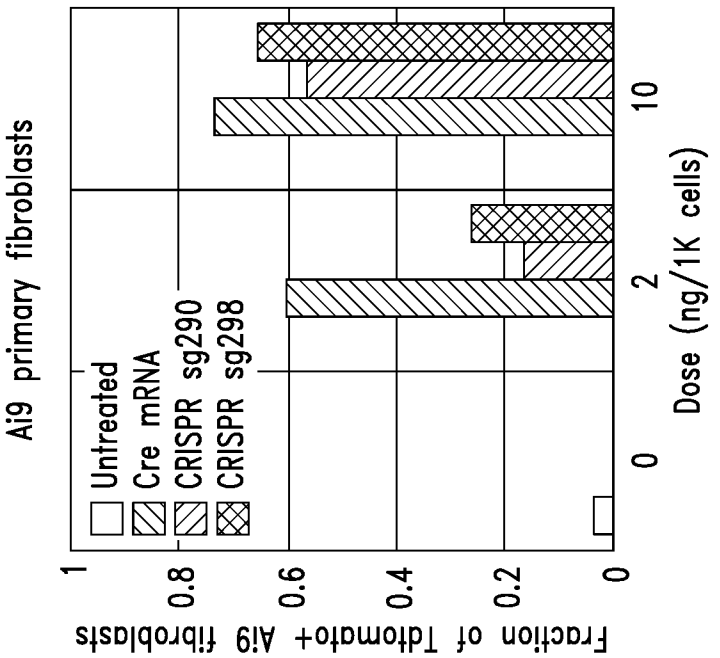
Ai9 primary fibroblasts
Untreated
Cre mRNA
CRISPR sg290
CRISPR sg298
Dose (ng/1K cells)
0
2
10
Fraction of Tdtomato+ Ai9 fibroblasts
0
0.2
0.4
0.6
0.8
1

FIG. 9B

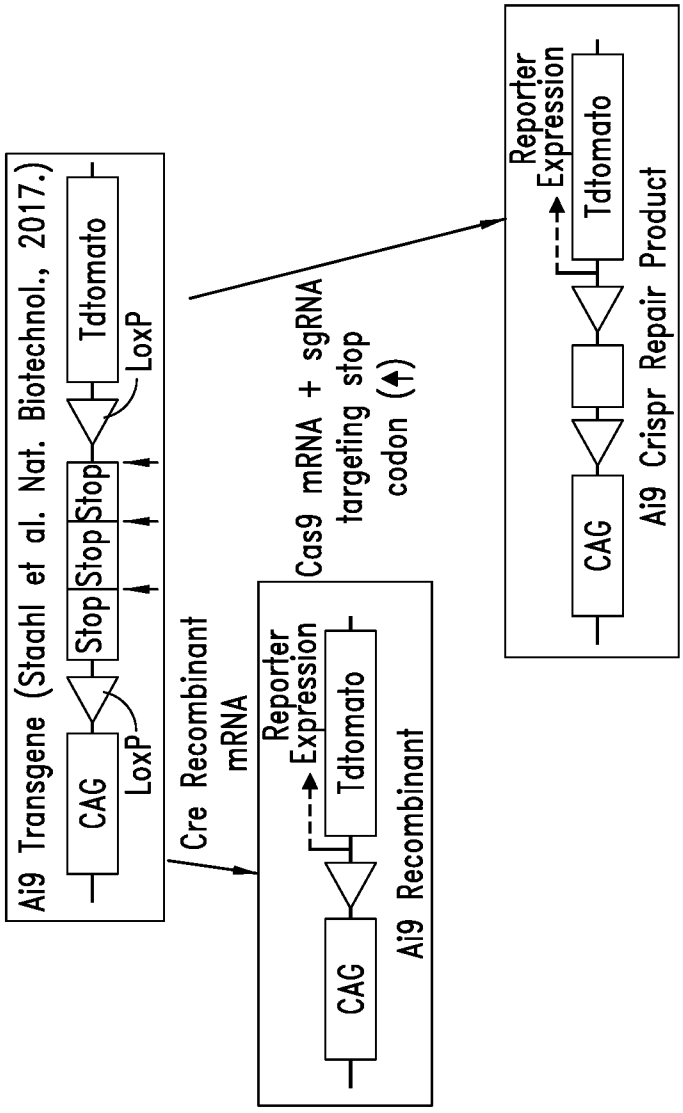
Ai9 Transgene (Staahl et al. Nat. Biotechnol., 2017.)
CAG
LoxP
Stop
Stop
Stop
LoxP
Tdtomato
Cre Recombinant mRNA
Ai9 Recombinant
CAG
Tdtomato
Reporter Expression
Cas9 mRNA + sgRNA targeting stop codon (↑)
Ai9 Crispr Repair Product
CAG
Tdtomato
Reporter Expression

FIG. 9A

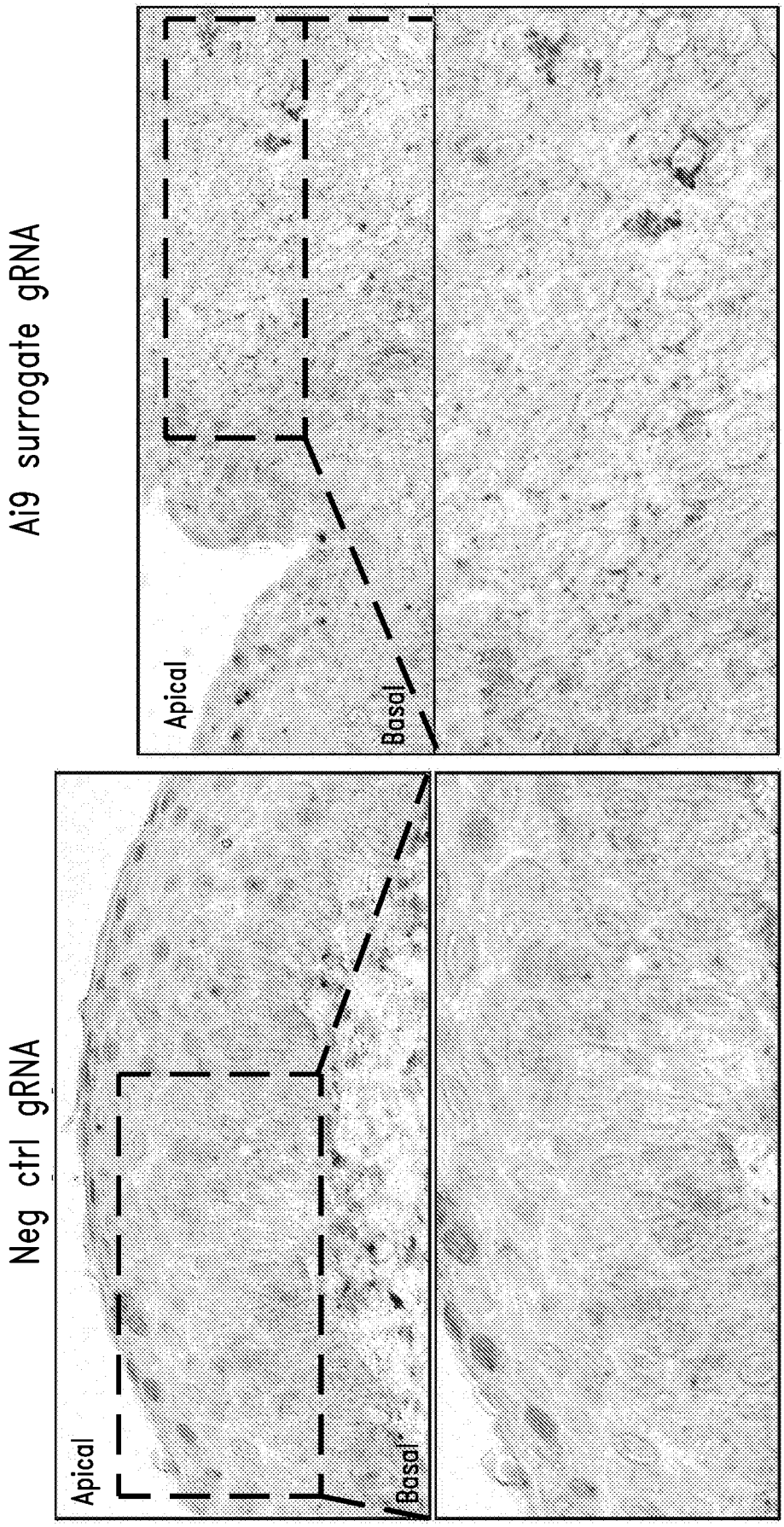

FIG. 10