operably connected to a second polynucleotide encoding the single guide RNA. This construct allows for the tRNA to direct RNA Polymerase III dependent production of a fusion RNA including the tRNA linked to the single guide RNA with a tRNase Z cleavage site. Action of the endogenous cellular tRNase Z enzyme cleaves the tRNA from the single guide RNA and allows for efficient production of the single guide RNA using a very small promoter element. Kits including these constructs are also included.

In one aspect, there is provided a recombinant construct for expression of a single guide RNA comprising a first polynucleotide encoding a mammalian or viral tRNA operably connected to a second polynucleotide encoding at least a second portion of a single guide RNA capable of interacting with a Cas9 polypeptide, wherein the mammalian or viral tRNA is selected from the group consisting of a Gln tRNA, Pro tRNA, Gly tRNA, Asn tRNA, Cys tRNA, Glu tRNA, and a mouse gamma herpesvirus-68 (MHV68) tRNA.

In another aspect, there is provided a construct comprising a first promoter operably connected to a first polynucleotide encoding a first single guide RNA and a second promoter operably connected to a second polynucleotide encoding a Cas9 polypeptide, the first single guide RNA comprising a first portion complementary to a strand of a target sequence of a DNA virus and a second portion capable of interacting with the Cas9 polypeptide.

In another aspect, there is provided a recombinant vector comprising the construct as described herein

In another aspect, there is provided a pharmaceutical composition comprising the recombinant vector of as disclosed herein and a pharmaceutical carrier.

In another aspect, there is provided the use of the recombinant vector of as disclosed herein or the construct of as disclosed herein in an amount effective to allow delivery of the recombinant vector to the cell and production of the single guide RNA and the Cas9 polypeptide in the manufacture of a medicament for inhibiting viral replication or target sequence expression in a cell infected with a DNA virus, wherein the medicament is to be contacted with the cell.

In another aspect, there is provided the use of an effective amount of the recombinant vector of as disclosed herein or the pharmaceutical composition as disclosed herein in the manufacture of a medicament for treating a viral infection in a subject, wherein the medicament is to be administered to the subject to treat the viral infection.

In another aspect, there is provided an *in vitro* method of inhibiting viral replication or target sequence expression in a cell infected with a DNA virus comprising contacting the cell with the

recombinant vector as disclosed herein or the construct as disclosed herein in an amount effective to allow delivery of the recombinant vector to the cell and production of the single guide RNA and the Cas9 polypeptide.

In another aspect, there is provided a recombinant Sau Cas9 polypeptide of SEQ ID NO: 57.

In another aspect, there is provided a recombinant Sau Cas9 construct comprising a promoter operably connected to a polynucleotide encoding a recombinant Sau Cas9 polypeptide, the polynucleotide comprising SEQ ID NO: 55.

In another aspect, there is provided a recombinant construct for expression of a single guide RNA comprising a first polynucleotide encoding a mammalian or viral tRNA operably connected to a second polynucleotide encoding at least a second portion of a single guide RNA capable of interacting with a Cas9 polypeptide.

In another aspect, there is provided a kit comprising the recombinant Sau Cas9 construct as disclosed herein.

In another aspect, there is provided a kit comprising the recombinant construct as disclosed herein.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a set of figures showing an Spy Cas9 HPV-18-specific sgRNA screen in which two sgRNAs were designed to target DNA sequences encoding the amino terminus of the HPV-18 E6 or E7 proteins, and screened to identify the most effective candidate. Fig. 1A is a schematic depicting the fusion protein-based reporter assay, which includes an amino-terminal HIV-1 Rev fragment that acts as an epitope tag, an in-frame HPV-18 derived target sequence, and a carboxy-terminal eGFP open reading frame (ORF). In other assays a firefly luciferase (FLuc) ORF was used in place of GFP. Fig. 1B is a graph showing eGFP expression data from 293T cells co-transfected with plasmids expressing the FLAG-tagged Spy Cas9 protein and sgRNAs specific for the HPV-18 E6 gene, or a control construct, and their cognate indicator plasmids. Transfected cells were processed for flow cytometry at 72 hours. The number of GFP-positive cells and the mean fluorescence intensity (MFI) of these cells is indicated. Average of three independent experiments with SD indicated. Fig. 1C is similar to panel B but using two HPV-18 E7-specific sgRNAs. Fig. 1D depicts a Western blot using an HIV-1 Rev specific antiserum to detect expression of the Rev-GFP indicator fusion protein, thus demonstrating sgRNA efficacy and specificity.

Figure 2 is a photograph of SURVEYOR assay showing HPV-18 E6 and E7-specific Spy Cas9 sgRNAs induce mutagenesis at the predicted cleavage site in the HPV-18 genome. E6 and E7 sgRNA and Spy Cas9 expression constructs were transfected and the SURVEYOR assay performed.

The predicted size of the SURVEYOR cleavage product is indicated by an arrow. DNA markers (left lane) are indicated by base pairs.

Figure 3 is a set of photographs showing that HPV-18 E6- and E7-specific RNA guided DNA endonucleases (RGNs) induce tumor suppressor gene expression in HeLa cells. HeLa cells were transfected with vectors expressing Spy Cas9 and HPV-18-specific sgRNAs, as indicated,

CLAIMS

We claim:

1. A recombinant construct for expression of a single guide RNA comprising a first polynucleotide encoding a mammalian or viral tRNA operably connected to a second polynucleotide encoding at least a second portion of a single guide RNA capable of interacting with a Cas9 polypeptide, wherein the mammalian or viral tRNA is selected from the group consisting of a Gln tRNA, Pro tRNA, Gly tRNA, Asn tRNA, Cys tRNA, Glu tRNA, and a mouse gamma herpesvirus-68 (MHV68) tRNA.

2. The construct of claim 1, wherein the first polynucleotide is selected from SEQ ID NO: 41-43, 45-48 and 50.

3. The construct of claim 1 or 2, wherein the second polynucleotide further encodes a first portion of the single guide RNA complementary to a target sequence 5' to the second portion capable of interacting with the Cas9 polypeptide.

4. The construct of any one of claims 1-3, wherein the second polynucleotide further encodes a first portion of the single guide RNA having one or more recognition site for a restriction endonuclease.

5. The construct of any one of claims 1-4, further comprising a promoter operably connected to a third polynucleotide encoding a Cas9 polypeptide.

6. The construct of any one of claims 1-5, wherein the single guide RNA comprises a first portion complementary to a strand of a target sequence of a DNA virus or a virus with a DNA intermediate.

7. The construct of claim 6, wherein the virus is selected from the group consisting of herpes simplex virus type 1 (HSV-1), herpes simplex virus type 2 (HSV-2), Epstein Barr Virus (EBV), human cytomegalovirus (hCMV), Varicella zoster virus (VZV), Kaposi's sarcoma associated herpesvirus (KSHV), hepatitis B virus (HBV), human papilloma virus (HPV) and human immunodeficiency virus (HIV-1).

8. The construct of claim 6 or 7, wherein the target sequence is selected from the group consisting of HSV ICP0, HSV ICP4, the HSV viral latency associated transcript, EBV FR (family of repeats), EBV DS (direct symmetry), EBV nuclear antigen (EBNA1), EBV Qp promoter, EBV latent membrane protein-1 (LMP-1) gene, HBV reverse transcriptase,

HBV surface antigen, HBV core, HPV E6 gene, HPV E7 gene, HIV-1 Tat, and HIV-1 TAR or adjacent promoter sequences.

9. The construct of claim 8, wherein the first portion of the single guide RNA is selected from SEQ ID NOs: 1-27, 30-35 and 63.

10. The construct of any one of claims 5-9, wherein the Cas9 polypeptide is selected from the Cas9 polypeptides from *S. pyogenes*, *S. aureus*, or nickase Cas9.

11. The construct of claim 10, wherein the Cas9 polypeptide comprises SEQ ID NO: 57 or is encoded by a polynucleotide comprising SEQ ID NO: 55.

12. The construct of claim 11, further comprising at least one of a nuclear localization signal, an intron or a poly(A) addition site.

13. The construct of any one of claims 5-12, wherein the promoter operably connected to the Cas9 polypeptide is an RNA Polymerase II dependent promoter/enhancer selected from the group consisting of EFS, hCMV or mCMV immediate early, CBA, hSynapsin, HSV TK, SV40 early and LSP.

14. The construct of any one of the preceding claims, further comprising a first inverted terminal repeat and a second inverted terminal repeat, wherein the inverted terminal repeats flank the construct for packaging in an adeno-associated virus (AAV) vector.

15. The construct of any one of the preceding claims, further comprising polynucleotide encoding a second single guide RNA comprising a first portion complementary to a strand of a second target sequence of a virus, wherein the polynucleotide encoding the second single guide RNA is operably connected to an RNA polymerase III-dependent promoter.

16. The construct of claim 15, wherein the first target sequence and the second target sequence are located within 30 base pairs of each other on opposite strands of the virus.

17. A recombinant vector comprising the construct of any one of the preceding claims.

18. The recombinant vector of claim 17, wherein the vector is a viral vector and the viral vector is selected from the group consisting of a retrovirus, a lentivirus, an adenovirus or an adeno-associated virus.