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(54) Title: NON-INTEGRATING VIRAL DELIVERY SYSTEM AND METHODS OF USE THEREOF

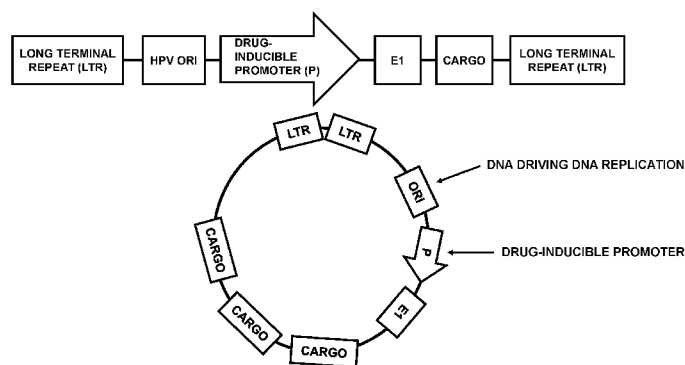


FIG 1

(57) Abstract: The present invention relates generally to non-integrating viral delivery system and methods of using the same. The viral delivery systems includes a viral carrier, a heterologous viral episomal origin of replication, a sequence encoding at least one initiator protein specific for the heterologous viral episomal origin of replication, and at least one gene, shRNA, siRNA, miRNA, or other gene-silencing RNA of interest. In certain embodiments, the disclosed system can be used for gene therapy.

## PRIORITY AND INCORPORATION BY REFERENCE

FIELD

## BACKGROUND

In view of the wide variety of potential genes available for gene therapy, an efficient means of delivering these genes is desired. Several viral systems including murine retrovirus, adenovirus, and parvovirus (adeno-associated virus) have been developed as therapeutic gene transfer vectors. Many factors must be considered when developing viral vectors, including stability and control of expression, genome packaging capacity, and construct-dependent

5 vector stability. In addition, *in vivo* application of viral vectors is often limited by host immune responses against viral structural proteins and/or transduced gene products.

An approach for the production of recombinant polypeptides or gene regulatory molecules, including small RNA, is the use of stable expression systems. These systems are based upon chromosomal integration of a transduced retrovirus genome (or at least a portion  
10 thereof) into the genome of the host cell, short-duration plasmid transfection, or non-integrating viral vectors also with limited half-life. The sites of gene integration are generally random, and the number and ratio of genes integrating at any particular site are often unpredictable; likewise, non-integrating plasmids or viral vectors also generate nuclear DNA but these species usually lack sequences required for DNA replication and continuous  
15 maintenance. Thus, vectors that rely on chromosomal integration result in permanent maintenance of the recombinant gene that may exceed the therapeutic interval, and plasmid or other non-replicating DNA is poorly controlled and may decay before completing a desired therapeutic interval.

Alternatives to stable expression systems for gene expression are transient expression  
20 systems. The expression of the latter gene expression systems is based on non-integrated plasmids, and hence the expression is typically lost as the cell undergoes division or the plasmid vectors are destroyed by endogenous nucleases. Thus, transient gene expression systems typically do not lead to sufficient expression over time and would traditionally require repeated treatments, which are generally undesirable.

25

## SUMMARY

In one embodiment, the invention is directed to a novel non-integrating viral delivery system and methods of using the same.

In one aspect, the invention is directed to a viral delivery system comprising a viral  
30 carrier, a heterologous viral episomal origin of replication, a sequence encoding at least one

5 initiator protein specific for the heterologous viral episomal origin of replication, and at least one gene, shRNA, siRNA, miRNA, and/or other gene-regulating RNA of interest, wherein the viral carrier has a defective integrase gene, and wherein expression of the sequence encoding the at least one initiator protein specific for the heterologous viral episomal origin of replication is under the control of an inducible promoter.

10 In some embodiments, the viral carrier is a lentivirus, although other viral carriers may also be suitable. In some embodiment, the heterologous viral episomal origin of replication is from a papillomavirus, such as a bovine papillomavirus or a human papillomavirus, and the at least one initiator protein specific for the heterologous viral episomal origin of replication is the papillomavirus E1 and/or E2. In certain embodiments both E1 and E2 are present in the  
15 systems described herein.

Alternatively, in some embodiments, the heterologous viral episomal origin of replication is from Epstein-Barr virus or any of the related mammalian herpesviruses, and the at least one initiator protein specific for the heterologous viral episomal origin of replication is EBNA-1 or a specific initiator protein found in each individual herpesvirus.

20 In another aspect, the disclosed invention is directed to a pharmaceutical composition comprising the disclosed viral delivery system and a pharmaceutically acceptable carrier.

In another aspect, the disclosed invention is directed to a method of treating or preventing a disease, comprising identifying a subject in need of treatment or prevention of a disease and administering to the subject a therapeutically effective amount of a viral delivery system  
25 according to the invention. The viral delivery system comprises a viral carrier, a heterologous viral episomal origin of replication, a sequence encoding at least one initiator protein specific for the heterologous viral episomal origin of replication, and at least one gene, shRNA, siRNA, miRNA, and/or other gene-silencing RNA of interest, wherein the viral carrier has a defective integrase gene or integration is suppressed by pharmaceutical inhibitors of the viral  
30 integrase protein, and wherein expression of the sequence encoding the at least one initiator

5 protein specific for the heterologous viral episomal origin of replication is under the control of an inducible promoter.

In some embodiments, the disease can be an infectious disease, such as Ebola virus, Lassa Fever virus or any viral agents that can be protected by specific antibodies, and in those embodiments, the gene, shRNA, siRNA, miRNA, and/or other gene-silencing RNA of interest  
10 can encode an antibody specific for Ebola virus or Lassa Fever virus or any other specific virus target.

In some embodiments, the disease can be an infectious disease such as *Staphylococcus aureus* or *Escherichia coli* or other bacterial, fungal, or protozoan pathogens, wherein the gene, shRNA, siRNA, miRNA, and/or other gene-silencing RNA of interest encodes an  
15 antibody targeted to reduce pathogen replication or block the activity of an exogenous pathogen-produced toxin.

In some embodiments, the disease treated or prevented using the disclosed methods is a genetic disease or disorder. In some embodiments, the genetic disease is alcohol abuse, and the gene, shRNA, siRNA, miRNA, and/or other gene-silencing RNA of interest encodes brain-  
20 derived growth factor.

In another aspect, the disclosed invention is directed to a method of treating nerve damage, comprising identifying a subject with nerve damage and administering to the subject a therapeutically effective amount of a viral delivery system according to the invention. The viral delivery system comprises a viral carrier, a heterologous viral episomal origin of  
25 replication, a sequence encoding at least one initiator protein specific for the heterologous viral episomal origin of replication, and at least one gene, shRNA, siRNA, miRNA, and/or other gene-silencing RNA of interest, wherein the viral carrier has a defective integrase gene, and wherein expression of the sequence encoding the at least one initiator protein specific for the heterologous viral episomal origin of replication is under the control of an inducible promoter.

5 In some embodiment, the gene, shRNA, siRNA, miRNA, and/or other gene-silencing RNA of interest is nerve growth factor.

In yet another embodiment, the disclosed invention is directed to a method of enhancing wound healing, comprising identifying a subject with a wound and administering to the subject a therapeutically effective amount of a viral delivery system according to the invention. The viral delivery system comprises a viral carrier, a heterologous viral episomal origin of replication, a sequence encoding at least one initiator protein specific for the heterologous viral episomal origin of replication, and at least one gene, shRNA, siRNA, miRNA, and/or other gene-silencing RNA of interest, wherein the viral carrier has a defective integrase gene, and wherein expression of the sequence encoding the at least one initiator protein specific for the heterologous viral episomal origin of replication is under the control of an inducible promoter. The wound can be, for example, resulting from an accident, injury, or surgery. In some embodiments, the gene, shRNA, siRNA, miRNA, and/or other gene-silencing RNA of interest is platelet-derived growth factor or vascular endothelial growth factor.

20 In another embodiment, the disclosed invention is directed to a method of enhancing recovery from traumatic skin burns, comprising identifying a subject with traumatic burns and administering to the subject a therapeutically effective amount of a viral deliver system according to the invention. The viral delivery system may comprise a viral carrier, a heterologous viral episomal origin of replication, a sequence encoding at least one initiator protein specific for the heterologous viral episomal origin of replication, and at least one gene, shRNA, siRNA, miRNA, and/or other gene-silencing RNA of interest, wherein the viral carrier has a defective integrase gene, and wherein expression of the sequence encoding the at least one initiator protein specific for the heterologous viral episomal origin of replication is under the control of an inducible promoter. Skin trauma may result from chemical or physical (heat or ultraviolet light or radiation) injury. In some embodiments, the gene, shRNA, siRNA,

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5 miRNA, and/or other gene-silencing RNA of interest encodes epidermal or keratinocyte growth factors, or other promoters of epidermal cell growth and differentiation.

In another embodiment, the disclosed invention is directed to a method of treating cancer or neoplastic disease comprising identifying a subject with cancer or neoplastic disease and administering to the subject a therapeutically effective amount of a viral delivery system according to the invention. The viral delivery system may comprise a viral carrier, a heterologous viral episomal origin of replication, a sequence encoding at least one initiator protein specific for the heterologous viral episomal origin of replication, and at least one gene, shRNA, siRNA, miRNA, and/or other gene-silencing RNA of interest, wherein the gene, shRNA, siRNA, miRNA, and/or other gene-silencing RNA of interest encodes an antibody  
10 targeted at tumor antigens, or polypeptides and/or regulatory RNA intended to modify tumor susceptibility to immunotherapy, surgery, radiation, or chemotherapy.  
15

In another embodiment, the disclosed invention is a system for delivering proteins and/or nucleic acids that comprise a system for gene deletion, modification, or re-sequencing. Current technology for modifying chromosomal genes lacks appropriate safety factors to prevent off- target effects. The proposed invention will ensure that genes encoding the DNA  
20 modifying systems of the current invention are depleted after a required therapeutic interval.

In another embodiment, the disclosed invention is directed to a method of enhancing bone healing, comprising identifying a subject with a wound and administering to the subject a therapeutically effective amount of a viral delivery system according to the invention. The viral delivery system comprises a viral carrier, a heterologous viral episomal origin of replication, a sequence encoding at least one initiator protein specific for the heterologous viral episomal origin of replication, and at least one gene, shRNA, siRNA, miRNA, and/or other gene-silencing RNA of interest, wherein the viral carrier has a defective integrase gene, and wherein expression of the sequence encoding the at least one initiator protein specific for the  
25 heterologous viral episomal origin of replication is under the control of an inducible promoter.  
30

- 5     The bone fracture can be, for example, resulting from an accident, injury, trauma or surgery and may be bone nonunion, or acute fracture or required spinal fusion. In some embodiments, the gene, shRNA, siRNA, miRNA, and/or other gene-silencing RNA of interest encodes bone morphogenetic proteins 1-4 or cyclooxygenase-2 or vascular endothelial growth factor.

10     The foregoing general description and following detailed description are exemplary and explanatory and are intended to provide further explanation of the invention as claimed. Other objects, advantages, and novel features will be readily apparent to those skilled in the art from the following brief description of the drawings and detailed description of the invention.

### BRIEF DESCRIPTION OF THE DRAWINGS

15     **Figure 1** depicts an exemplary virus within a virus vector according to an embodiment of the invention.

**Figure 2** depicts an exemplary vector-in-vector (VIV) embodiment (Vector 1) also containing an E1 initiator protein.

**Figure 3** depicts transduction results in 293 T cells using Vector 1.

20     **Figure 4** depicts an exemplary vector-in-vector embodiment also containing both E1 and E2 initiator proteins.

### DETAILED DESCRIPTION

Disclosed herein are non-integrating, episomally replicating viral vectors (*e.g.*,  
25     lentiviral vectors) and methods of using the same. Episomally replicating vectors like the present invention can contain viral components from viruses like Papovaviridae (*e.g.*, bovine papillomavirus or BPV) or Herpesviridae (*e.g.*, Epstein Barr Virus or EBV) or Hepadnaviridae (*e.g.*, Hepatitis B Virus or HBV). Episomal replicating vectors derived from these viruses may contain a replication origin and at least one viral trans-acting factor, *e.g.*, an initiator protein,  
30     such as E1 for BPV and EBNA-1 for EBV or HBV polymerase or terminus binding protein of



5     Adenovirus. The process of episomal replication typically incorporates both host cell replication machinery and viral trans-acting factors.

By using heterogeneous viral origins of replication, novel vectors can be engineered with an “off” switch for expression of viral proteins required to recognize the origin of replication. Switching off DNA replication of the vector will prevent further expression of the transduced genes, and the viral vector will disappear over time such that the transduced genes  
10     will no longer be present in the host cell. The disclosed systems and methods provide numerous improvements over the prior art, including the ability to prevent any toxic effects from over-expression or prolonged expression of transduced genes. Eliminating the gene once DNA replication ceases, prevents unwanted gene expression or knockdown in the future.  
15     Likewise, combining the benefits of episomal replication into a heterogeneous viral system provides for a platform that can safely and efficiently transduce genes of interest into a variety of cell types.

### **Papillomavirus**

20     Papillomaviruses replicate primarily as episomes in mammalian cells. Action of the viral E1 protein, which functions as a DNA helicase, on the viral origin of replication (ori) drives the production of hundreds to thousands of copies per cells depending on differentiation status of infected epithelial cells. Recognizing this property, several laboratories have tried to develop papillomavirus-based gene delivery systems using what became known as “shuttle  
25     plasmids.” With a bacterial origin of replication to allow production of DNA in *E. coli* and a papillomavirus ori to allow episomal replication in mammalian cells, a number of studies were performed to demonstrate safety and durability of gene expression. In most cases, the ori came from bovine papillomavirus.

Papillomaviruses are evolved to infect epidermal and epithelial cells. As infected cells  
30     differentiate from basal to luminal surfaces, papillomaviruses increase DNA replication and

5 copy number becomes very high until a tremendous dose of virus is released at the luminal surface. This makes papillomaviruses highly contagious as is apparent from human papillomavirus. The surge in copy number is due primarily to host factors. However, this feature of papillomavirus can be exploited to target transient gene therapy to epidermal and epithelial surfaces.

10 Thus, certain features of papillomavirus can be used for driving expression and replication of an episomal vector, as well as targeting expression of the vector to specific cell types.

### **Epstein Barr Virus (EBV)**

15 Epstein-Barr virus (EBV), also known as human herpesvirus 4, is a member of the herpes virus family. It is one of the most common human viruses, and most people become infected with EBV at some point in their lives.

EBV is a double stranded DNA virus that contains approximately 85 genes, and that is known to infect B cells and epithelial cells. EBV is capable of both lytic and latent replication, the latter of which results in a circularized version of the EBV genome translocating to the host cell nucleus where it may be replicated by host cell DNA polymerases.

EBV can undergo latent replication via at least three distinct pathways, but each one involves the expression of Epstein-Barr virus nuclear antigen 1 (EBNA-1), a protein that binds the episomal replication origin and mediates partitioning of the episome during division of the host cell. EBNA-1 plays an integral role in EBV gene regulation, replication, and episomal maintenance.

Due to its natural tropism and ability to replicate extrachromosomally, EBV is of interest for use as a viral vector. Such vectors will often be mutated to delete EBV's viral oncogenes and/or the essential lytic genes. However, EBV's utility as a viral vector has thus far been limited to *ex vivo* and research purposes.

## 5 **Hepatitis B virus (HBV)**

Hepatitis B virus (HBV) is a member of the hepadna virus family. It is a common human virus associated with progressive liver fibrosis, hepatitis and hepatocellular carcinoma.

HBV is a double stranded DNA virus that replicates through an RNA intermediate and depends on a viral polymerase. Stable maintenance of HBV in liver cells is due to the presence  
10 of covalently-closed viral DNA circular forms that are difficult to eradicate.

Due to its natural tropism and ability to replicate extrachromosomally, HBV as a vector provides significant potential advantages for targeting liver cells and delivering therapeutic genes. The unique life cycle for HBV provides a unique method for regulating episomal DNA levels through controlled expression of the viral polymerase required to  
15 convert an RNA intermediate into viral DNA.

## **Retrovirus**

Retrovirus is a virus family characterized by encoding a reverse transcriptase capable of generating DNA copies from RNA templates and integration of proviruses into the host cell  
20 chromosome. Lentivirus is a genus of retroviruses that can deliver a significant amount of viral nucleic acid into a host cell. Lentiviruses are characterized as having a unique ability to infect/transduce non- dividing cells, and following transduction, lentiviruses integrate their nucleic acid into the host cell's chromosomes.

Infectious lentiviruses have three main genes coding for the virulence proteins *gag*,  
25 *pol*, and *env*, and two regulatory genes including *tat* and *rev*. Depending on the specific serotype and virus, there may be additional accessory genes that code for proteins involved in regulation, synthesis, and/or processing viral nucleic acids and other replicative functions.

Moreover, lentiviruses contain long terminal repeat (LTR) regions, which may be approximately 600 nt long. LTRs may be segmented into U3, R, and U5 regions. LTRs can  
30 mediate integration of retroviral DNA into the host chromosome via the action of integrase.

5 Alternatively, without functioning integrase, the LTRs may be used to circularize the viral nucleic acid.

Viral proteins involved in early stages of lentivirus replication include reverse transcriptase and integrase. Reverse transcriptase is the virally encoded, RNA-dependent DNA polymerase. The enzyme uses a viral RNA genome as a template for the synthesis of a  
10 complementary DNA copy. Reverse transcriptase also has RNaseH activity for destruction of the RNA-template. Integrase binds both the viral cDNA generated by reverse transcriptase and the host DNA. Integrase processes the LTR before inserting the viral genome into the host DNA. *Tat* acts as a trans-activator during transcription to enhance the initiation and elongation of RNA copies made from viral DNA. The *rev* responsive element acts post-transcriptionally,  
15 regulating mRNA splicing and transport to the cytoplasm.

#### **Vector-in-Vector Technology**

Disclosed herein is a novel vector-in-vector (VIV) system that can precisely regulate the delivery and expression of genes by combining desirable features from various viral  
20 species. Many viral vectors, including lentivirus (LV) platforms, are known in the art. LV benefits from ease of manufacturing and flexible targeting characteristics, but lentiviral transduction, like most other forms of stable transduction, results in chromosomal integration of the LV payload. The property of chromosomal integration can be abolished through mutations that inactivate the viral integrase gene.

25 Avoiding chromosomal integration reduces the barrier to *in vivo* gene delivery. Even integration-defective LV can have a background frequency of integration, and any DNA molecule can find rare homologies to recombine with host sequences; but these rates of integration are exceptionally rare and generally not clinically significant.

As examples, the papillomavirus ori plus E1 protein, or the EBV ori plus EBNA-1 or  
30 the Hepadnavirus termini plus viral polymerase can be added as part of the genetic cargo of a

5 heterologous virus that would not ordinarily be able to be maintained episomally. Incorporating this heterogeneous viral replication machinery into a lentiviral vector leaves approximately 5 kb of additional cargo space available to accommodate therapeutic genes of interest.

Additionally, other control elements can be incorporated into the disclosed VIV  
10 system. For instance, the expression of E1 or EBNA-1 or HBV polymerase can be driven by an inducible promoter. Numerous types of inducible promoters are known in the art, and for the purposes of this invention, inducible promoters can include but are not limited to promoters that respond to antibiotics (*i.e.*, tetracyclines, aminoglycosides, penicillins, cephalosporins, polymyxins, *etc.*) or other drugs, copper and other metals, alcohol, steroids,  
15 light, oxygen, heat, cold, or other physical or chemical stimulation. For example, a method of using the disclosed viral system could employ tetracycline-inducible gene expression that depends upon a constant supply of the drug for expression of the cargo genes. A compound used to induce the inducible promoter may be added once or repeatedly depending on the duration of episomal replication and timing of cargo delivery that is desired. DNA replication  
20 and maintenance of the episome depends on E1 and possibly E2 or EBNA-1 induction, which in turn depends upon an inducer of gene expression (*i.e.*, tetracycline). An exemplary diagram of a VIV system appears in Figure 1. Another exemplary diagram of a VIV system appears in Figure 2. As shown in Figure 2, an E1 initiator protein is present and the cargo is GFP under an EF1-HTLV promoter. Another exemplary diagram of a VIV system appears in Figure 4  
25 where the genetic cargo is represented by a CMV/GFP expression cassette. Cargo gene sequences can be amplified by polymerase chain reaction (PCR) using synthetic oligonucleotide primers identical to the 5' end of the cargo gene and complementary to the 3' end of the cargo gene. The 5' primer can be extended from its 5' end with a recognition site for an endonuclease. The 3' primer can also be extended at its 3' end with the complement for  
30 an endonuclease recognition. The resulting amplified cargo gene sequences can be annealed

5 into a suitable vector, such as a lentiviral vector. Non-limiting examples of the genetic cargo include CMV/VEGF, CMV/anti-epidermal growth factor receptor (EGFR), or miRNA suppressing C-C chemokine receptor type 5 (CCR5).

Suitable expression of the cargo may be determined by an appropriate assay. For example, DNA copy numbers can be measured by quantitative PCR. Protein products  
10 translated from any of Vector 1 or Vector 19 (described herein) can be measured, for example, by analytical flow cytometry. An ELISA assay may be used to detect the presence of certain cargo, such as a secreted protein, such as VEGF. A Western blot technique may also be used to detect certain cargo such as an antibody, such as anti-EGFR. Further, monitoring a reduction in cell surface expression of a cargo protein, such as a chemokine receptor such as  
15 CCR5, can also be employed.

As shown in Figure 1, the disclosed VIV can comprise at least one gene or sequence of interest. The genes or sequences incorporated into the VIV will depend upon the purpose of the VIV. Referring to Figure 1, lentivirus is packaged with an integrase-defective system or transduction is performed in the presence of clinical drugs used to block integrase activity  
20 (*e.g.*, Dolutegravir or Raltegravir). Failing to integrate, the linear double strand vector DNA will generally circularize using host enzymatic machinery. A drug inducible promoter can be activated to express E1 and/or E2 protein if desired, which will in turn drive DNA replication. Therapeutic cargo will be expressed from cassettes. In various embodiments, the compound that induces the inducible promoter (also referred to herein as an “inducer”) is withdrawn or  
25 terminated. Termination of the inducer will down regulate E1 and/or E2 synthesis. In further embodiments, E1 and/or E2 production is effectively terminated. In either event, this will lead to declining levels of episomal DNA and eventually elimination of the vector construct. Figures 1 and 2 depict a VIV system containing E1. Figure 4 depicts a VIV system containing both E1 and E2 on a single viral vector. In a preferred embodiment, in order to express both

5 E1 and E2 from the same mRNA, an internal ribosome entry site (IRES) is added to allow for re-initiation of protein translation.

For instance, the gene encoding platelet-derived growth factor (PDGF) could be incorporated as a gene along with shRNA, siRNA, miRNA, and/or other gene-silencing RNA of interest into a VIV used to promote wound healing. The disclosed VIV system is not  
10 limited to a type of gene or sequence that can be expressed. Thus, the disclosed VIV can incorporate numerous therapeutic or prophylactic genes or sequences, for instance, sequences that encode antibodies directed to an antigen associated with an infectious disease or cancer (including antigens on replicating pathogens and antigens that are exogenous toxins and antigens on tumor cells), platelet derived growth factor, vascular endothelial growth factor,  
15 brain derived growth factor, nerve growth factor, human growth factor, human chorionic gonadotropin, cystic fibrosis transmembrane conductance regulator (CFTR), dystrophin or dystrophin-associated complex, phenylalanine hydroxylase, lipoprotein lipases,  $\alpha$ - and/or  $\beta$ -thalassemias, factor VIII, bone morphogenetic proteins 1-4, cyclooxygenase 2, vascular endothelial growth factor, chemokine receptor CCR5, chemokine receptor CXCR4, chemokine  
20 receptor CXCR5, antisense DNA or RNA against autoimmune antigens involved in colitis, inflammatory bowel disease or Crohn's disease, small interfering RNA that are involved in addiction including miRNA regulating neural attenuation to opiates or alcohol, tumor suppressor genes, genes regulating cell survival including pro- or anti-apoptosis genes and pro- or anti-autophagy genes, genes encoding radiation resistance factors, genes encoding light  
25 emitting proteins used for tracking tumor cell metastasis or other cell trafficking phenomena, or a variety of other therapeutically useful sequences that may be used to condition the body for maximum effect of radiation, surgical or chemotherapeutics or to protect tissues against radiation, surgical or chemotherapeutics, to modify the host or graft tissues to improve organ transplantation or to suppress hyperreactivity especially in the airway.

5 Maintaining genes in episomal form in a VIV system builds in a safety switch. If the gene product is toxic, withdrawal of the inducer molecule will terminate DNA replication, episome numbers will subsequently decline, and the gene and vector will disappear. Unlike traditional regulated gene expression as the safety switch, the disclosed expression construct is degraded by endogenous nucleases and diluted by cell division until it has effectively  
10 disappeared, thereby preventing any short- or long-term breakthrough expression.

Maintaining a gene, shRNA, siRNA, miRNA, and/or other gene-silencing RNA of interest in episomal form also allows for regulating the copy number over a broad range and at much higher levels than is achieved by traditional lentivirus transduction.

The disclosed VIV system presents numerous benefits. For instance, episomal DNA is  
15 less susceptible to chromosomal modification, which can lead to gene silencing of traditional transduction vectors. Likewise, VIV episomal DNA vectors support active gene delivery at least over short- to medium-range time intervals of about 1 to about 4 months, and possibly longer. In other embodiments of the invention, episomal DNA vectors support active gene delivery over a period of about 1, about 2, about 3, about 4, about 5, about 6, about 7, about 8,  
20 about 9, about 10, about 11, or about 12 weeks. In other embodiments, episomal DNA vectors support active gene delivery over a period of about 1 month, 2 months, 3 months, 4 months, 5 months, 6 months, 7 months, 8 months, 9 months, 10 months, 11 months, 12 months, or longer. Any combination of these time periods can also be used in the methods of the invention, *e.g.*, 1 month and 1 week, or 3 months and 2 weeks.

25 While there are benefits specifically associated with the use of a lentiviral carrier for incorporation of the disclosed VIV system, the disclosed system is not limited to a single type of viral vector. Any DNA virus or virus that uses a DNA intermediate can be used as a carrier for incorporating the VIV replication strategy, including but not limited to lentivirus, adeno-associated virus (AAV), adenovirus, vaccinia, herpes virus, measles virus, hepadnavirus,  
30 parvovirus and murine viruses.



5

**Methods**

In some embodiments, the invention is directed to methods of administering a VIV vector to a patient in need thereof, wherein the VIV vector encodes at least one, at least two, at least three, at least four, or at least five genes of interest. Given the versatility and therapeutic potential and the disclosed VIV system, a VIV system of the invention may encode genes or nucleic acid sequences that include but are not limited to an antibody directed to an antigen associated with an infectious disease or a toxin produced by the infectious pathogen, platelet derived growth factor, vascular endothelial growth factor, brain derived growth factor, nerve growth factor, human growth factor, human chorionic gonadotropin, cystic fibrosis transmembrane conductance regulator (CFTR), dystrophin or dystrophin-associated complex, lipoprotein lipases,  $\alpha$ - and/or  $\beta$ -thalassemias, factor VIII, bone morphogenetic proteins 1-4, cyclooxygenase 2, vascular endothelial growth factor, chemokine receptor CCR5, chemokine receptor CXCR4, chemokine receptor CXCR5, antisense DNA or RNA against autoimmune antigens involved in colitis, inflammatory bowel disease or Crohn's disease, small interfering RNA that are involved in addiction including miRNA regulating neural attenuation to opiates or alcohol, tumor suppressor genes, genes regulating cell survival including pro- or anti-apoptosis genes and pro- or anti-autophagy genes, genes encoding radiation resistance factors, genes encoding light emitting proteins used for tracking tumor cell metastasis or other cell trafficking phenomena, or a variety of other therapeutically useful sequences that may be used to condition the body for maximum effect of radiation, surgical or chemotherapeutics or to protect tissues against radiation, surgical or chemotherapeutics, to modify the host or graft tissues to improve organ transplantation or to suppress hyperreactivity especially in the airway.

30 **Infectious Disease**

5           The disclosed compositions and methods can provide protection against disease, particularly to individuals temporarily residing in a high-risk region. Several key targets for genetic therapy involve a great deal of risk if the gene product is chronically expressed. One exemplary application is the prophylactic delivery of monoclonal antibodies, including protective antibodies against a lethal viral agent, which are necessary to protect individuals  
10 moving through an endemic region (*e.g.*, military and aid workers entering an Ebola-infected region). Vaccines are largely untested for diseases such as Ebola or Lassa Fever virus or Dengue fever or Chikungunya virus or Plasmodium spp. causing malaria, and chronic expression of prophylactic antibody genes through the use of integrating vectors carries unknown health risks. Thus, there is a significant medical need for effective antibody  
15 expression that must be high but transient. The disclosed VIV system and methods of delivering high copy numbers of a gene, shRNA, siRNA, miRNA, and/or other gene-silencing RNA of interest for a limited period satisfy this medical need.

          In one embodiment, the invention is directed to methods of treating, preventing, or minimizing conditions, symptoms, or side effects associated with infectious disease. In some  
20 embodiments, the infectious disease can be human immunodeficiency virus (HIV), human T cell leukemia virus, Ebola virus, Lassa fever virus, dengue fever, Zika virus, malaria, tuberculosis, rabies, vaccinia virus or other infectious diseases. In some embodiments, a VIV vector can be administered prophylactically or following infection with an infectious disease.

## 25   **Wound Healing**

          In another embodiment, the invention is directed to methods of treating, preventing, or minimizing conditions, symptoms, or side effects associated with wound healing. The disclosed composition can be administered systemically or directly to a wound after an accident, injury, or surgery. In the case of surgery, a VIV vector may be administered  
30 prophylactically in order to expedite healing. In the case of a wound from an accident, injury,

5 or surgery, a VIV vector may be administered sometime after the formation of the wound. For instance, the VIV vector may be administered within about 1, about 2, about 3, about 4, about 5, about 10, about 12, about 24, about 36, about 48, about 60, about 72, about 84, about 96, about 108, about 120, or about 168 hours of the formation of a wound.

Another application of the methods and compositions of the invention is transient  
10 delivery of VIV constructs capable of expressing platelet growth factor that would accelerate wound healing. A high dose of platelet-derived growth factor (PDGF) is required very quickly but transiently. The disclosed system and methods are ideal for this type of application.

Additional short-term applications include expression of brain-derived growth factor for intermittent treatment of alcohol abuse, nerve growth factor for spinal cord regeneration,  
15 and topical applications for skin conditions.

### **Bone Disease or Injury**

In one embodiment, the disclosed invention is directed to a method of enhancing bone healing, comprising identifying a subject with a bone injury and administering to the subject a  
20 therapeutically effective amount of a viral delivery system according to the invention. The viral delivery system comprises a viral carrier, a heterologous viral episomal origin of replication, a sequence encoding an initiator protein specific for the heterologous viral episomal origin of replication, and at least one gene, shRNA, siRNA, miRNA, and/or other gene-silencing RNA of interest, wherein the viral carrier has a defective integrase gene, and  
25 wherein expression of the sequence encoding the initiator protein specific for the heterologous viral episomal origin of replication is under the control of an inducible promoter. The bone injury can be, for example, resulting from an accident, injury, or surgery and may be bone nonunion, or acute fracture or required spinal fusion. In some embodiments, the gene, shRNA, siRNA, miRNA, and/or other gene-silencing RNA of interest encodes bone morphogenetic  
30 proteins 1-4 or cyclooxygenase-2 or vascular endothelial growth factor.

5           In one embodiment, the disclosed invention is directed to a method of enhancing bone healing, comprising identifying a subject with a bone disease and administering to the subject a therapeutically effective amount of a viral delivery system according to the invention. The viral delivery system comprises a viral carrier, a heterologous viral episomal origin of replication, a sequence encoding an initiator protein specific for the heterologous viral episomal origin of replication, and at least one gene, shRNA, siRNA, miRNA, and/or other gene-silencing RNA of interest, wherein the viral carrier has a defective integrase gene, and wherein expression of the sequence encoding the initiator protein specific for the heterologous viral episomal origin of replication is under the control of an inducible promoter. The bone disease can be, for example, resulting from an accident, injury, or surgery and may be bone nonunion, or acute fracture or required spinal fusion. Additionally, the bone disease may be from low bone density, low blood flow to the bone, aging, hereditary conditions, and the like. In some embodiments, the gene, shRNA, siRNA, miRNA, and/or other gene-silencing RNA of interest encodes bone morphogenetic proteins 1-4 or cyclooxygenase-2 or vascular endothelial growth factor.

20

## 5 Hereditary Genetic Disease

Table 1		
Disorder	Mutation	Chromosome
22q11.2 deletion syndrome	D	22q
Alpha-1-anti-trypsin disorder	P	14q32
14q32 Angelman syndrome		10
Canavan disease		17p
Charcot–Marie–Tooth disease		
Color blindness	P	X
Cri du chat	D	5
Cystic fibrosis	P	7q
Down syndrome	C	21
Duchenne muscular dystrophy	D	Xp
Haemochromatosis	P	6
Haemophilia	P	X
Klinefelter syndrome	C	X
Neurofibromatosis		17q/22q/?
Phenylketonuria	P	12q
Polycystic kidney disease	P	16 (PKD1) or 4 (PKD2)
Prader–Willi syndrome	DC	15
Sickle-cell disease	P	11p
Tay–Sachs disease	P	15
Turner syndrome	C	X

5           In another embodiment, the invention is directed to methods of treating, preventing, or minimizing conditions, symptoms, or side effects associated with a hereditary genetic disease. Several examples of such hereditary genetic diseases are given in Table 1, along with the causal type of mutation and chromosome involved using the nomenclature below:

P – Point mutation, or any insertion/deletion entirely inside one gene

10           D – Deletion of a gene or genes

C – Whole chromosome extra, missing, or both (see Chromosome abnormality)

T – Trinucleotide repeat disorders: gene is extended in length

          Current gene therapy includes efforts to edit genomic DNA through gene deletion, replacement, or re-sequencing. Various gene therapy systems known in the art, including  
15    Talen, CRISPR-Cas9, zinc finger endonuclease and others, rely on delivery of genetic material by lentivirus transduction. But, unlike the disclosed invention, these systems may have unexpected consequences if left active in cells for extended periods because active chromosome modification systems may alter unexpected sites, leading to new genetic diseases including cancer. Truly practical systems for modification of host DNA require transient,  
20    well-regulated expression through methods such as the method disclosed herein.

          Thus, in one embodiment, the disclosed invention is directed to a method of treating a hereditary genetic disease, comprising identifying a subject with a hereditary genetic disease and administering to the subject a therapeutically effective amount of a viral delivery system according to the invention. The viral delivery system comprises a viral carrier, a heterologous  
25    viral episomal origin of replication, a sequence encoding an initiator protein specific for the heterologous viral episomal origin of replication, and at least one gene, shRNA, siRNA, miRNA, and/or other gene-silencing RNA of interest, wherein the viral carrier has a defective integrase gene, and wherein expression of the sequence encoding the initiator protein specific for the heterologous viral episomal origin of replication is under the control of an inducible  
30    promoter. The hereditary genetic disease can be, for example, the diseases listed in Table 1,

5 and in some embodiments, the gene, shRNA, siRNA, miRNA, and/or other gene-silencing RNA of interest encodes non-mutated versions of the genes listed in Table 1.

It is also possible to incorporate a guide RNA target sequence into the disclosed VIV vectors. Guide RNA are sequences used to target gene editing machinery to specific sites within the host genome that are mutated or otherwise require correction. Inclusion of guide  
10 RNA within the cargo of a VIV vector allows for a modification of a section of a chromosome that requires correction, and the same modification will occur within VIV to accelerate degradation and/or dilution by the host. Thus, in certain embodiments of the invention, the disclosed viral delivery system comprises a viral carrier, a heterologous viral episomal origin of replication, a sequence encoding an initiator protein specific for the heterologous viral  
15 episomal origin of replication, at least one gene, shRNA, siRNA, miRNA, and/or other gene-silencing RNA of interest, and at least one guide RNA, wherein the viral carrier has a defective integrase gene, and wherein expression of the sequence encoding the initiator protein specific for the heterologous viral episomal origin of replication is under the control of an inducible promoter.

20

#### **Ex vivo modification of cells or tissues**

In another embodiment, VIV vectors may be used to modify cells or tissues that are used for disease therapy. Cells may include, without limitation, primary cells such as lymphocytes, stem cells, epithelial cells, neural cells and others. For example, VIV vectors  
25 may be used to modify lymphocytes that are redirected to specific disease including cancer, infectious disease or autoimmunity, and where long-term presence of genetically modified cells poses a health risk. For example, VIV vectors may also be used to program pluripotent stem cells that require high levels of transcript factors for a defined interval and where the long-term presence of an integrated viral vector is undesirable. Epithelial cells that may be  
30 used for synthetic skin or other applications may require the expression of trophic or growth

- 5 factors during the initial treatment that would be deleterious to function of the normal tissue after treatment and are best delivered by VIV vectors.

### **Doses and Dosage Forms**

The disclosed VIV vectors allow for short, medium, or long-term expression of genes  
10 or sequences of interest and episomal maintenance of the disclosed vectors. Accordingly, dosing regimens may vary based upon the condition being treated and the method of administration.

In one embodiment, VIVs may be administered to a subject in need in varying doses. Specifically, a subject may be administered  $\geq 10^6$  infectious doses (where 1 dose is needed on  
15 average to transduce 1 target cell). More specifically, a subject may be administered  $\geq 10^7$ ,  $\geq 10^8$ ,  $\geq 10^9$ , or  $\geq 10^{10}$  infectious doses. Upper limits of VIV dosing will be determined for each disease indication and will depend on toxicity/safety profiles for each individual product or product lot.

Additionally, a VIV of the present invention may be administered once or twice a day.  
20 Alternatively, a VIV may be administered to a subject in need once a week, once every other week, once every three weeks, once a month, every other month, every three months, every six months, every nine months, once a year, every eighteen months, every two years, every 36 months, or every three years.

In one embodiment, VIVs are administered as a pharmaceutical composition. In one  
25 embodiment, the pharmaceutical composition comprising VIV can be formulated in a wide variety of nasal, pulmonary, oral, topical, or parenteral dosage forms for clinical application. Each of the dosage forms can contain various disintegrating agents, surfactants, fillers, thickeners, binders, diluents such as wetting agents or other pharmaceutically acceptable excipients. The pharmaceutical composition comprising a VIV can also be formulated for  
30 injection.



5           The VIV composition can be administered using any pharmaceutically acceptable method, such as intranasal, buccal, sublingual, oral, rectal, ocular, parenteral (intravenously, intradermally, intramuscularly, subcutaneously, intracisternally, intraperitoneally), pulmonary, intravaginal, locally administered, topically administered, topically administered after scarification, mucosally administered, via an aerosol, or via a buccal or nasal spray  
10   formulation.

          Further, the VIV composition can be formulated into any pharmaceutically acceptable dosage form, such as a solid dosage form, tablet, pill, lozenge, capsule, liquid dispersion, gel, aerosol, pulmonary aerosol, nasal aerosol, ointment, cream, semi-solid dosage form, and a suspension. Further, the composition may be a controlled release formulation, sustained  
15   release formulation, immediate release formulation, or any combination thereof. Further, the composition may be a transdermal delivery system.

          In another embodiment, the pharmaceutical composition comprising a VIV can be formulated in a solid dosage form for oral administration, and the solid dosage form can be powders, granules, capsules, tablets or pills. In yet another embodiment, the solid dosage form  
20   can include one or more excipients such as calcium carbonate, starch, sucrose, lactose, microcrystalline cellulose or gelatin. In addition, the solid dosage form can include, in addition to the excipients, a lubricant such as talc or magnesium stearate. In some embodiments, the oral dosage form can be immediate release, or a modified release form. Modified release dosage forms include controlled or extended release, enteric release, and the  
25   like. The excipients used in the modified release dosage forms are commonly known to a person of ordinary skill in the art.

          In a further embodiment, the pharmaceutical composition comprising a VIV can be formulated as a sublingual or buccal dosage form. Such dosage forms comprise sublingual tablets or solution compositions that are administered under the tongue and buccal tablets that  
30   are placed between the cheek and gum.

5           In yet a further embodiment, the pharmaceutical composition comprising a VIV can be formulated as a nasal dosage form. Such dosage forms of the present invention comprise solution, suspension, and gel compositions for nasal delivery.

          In one embodiment, the pharmaceutical composition can be formulated in a liquid dosage form for oral administration, such as suspensions, emulsions or syrups. In other  
10   embodiments, the liquid dosage form can include, in addition to commonly used simple diluents such as water and liquid paraffin, various excipients such as humectants, sweeteners, aromatics or preservatives. In particular embodiments, the composition comprising VIV or a pharmaceutically acceptable salt thereof can be formulated to be suitable for administration to a pediatric patient.

15           In one embodiment, the pharmaceutical composition can be formulated in a dosage form for parenteral administration, such as sterile aqueous solutions, suspensions, emulsions, non- aqueous solutions or suppositories. In other embodiments, the non-aqueous solutions or suspensions can include propyleneglycol, polyethyleneglycol, vegetable oils such as olive oil or injectable esters such as ethyl oleate. As a base for suppositories, witepsol, macrogol,  
20   tween 61, cacao oil, laurin oil or glycerinated gelatin can be used.

          The dosage of the pharmaceutical composition can vary depending on the patient's weight, age, gender, administration time and mode, excretion rate, and the severity of disease.

### **Definitions**

25           Words that are not specifically defined herein will be understood to have a meaning consistent with that as understood by persons of ordinary skill in the art.

          As used herein, the term “about” will be understood by persons of ordinary skill in the art and will vary to some extent depending upon the context in which it is used. If there are uses of the term which are not clear to persons of ordinary skill in the art given the context in  
30   which it is used, “about” will mean up to plus or minus 10% of the particular term.

5           A “treatment” is intended to target the disease state and combat it, *i.e.*, ameliorate or prevent the disease state. The particular treatment thus will depend on the disease state to be targeted and the current or future state of medicinal therapies and therapeutic approaches. A treatment may have associated toxicities.

          The terms “administration of” or “administering” an active agent should be understood  
10       to mean providing an active agent of the invention to the subject in need of treatment in a form that can be introduced into that individual's body in a therapeutically useful form and therapeutically effective amount.

          The term “therapeutically effective amount” refers to a sufficient quantity of the active agents of the present invention, in a suitable composition, and in a suitable dosage form to  
15       treat or prevent the symptoms, progression, or onset of the complications seen in patients suffering from a given ailment, injury, disease, or condition. The therapeutically effective amount will vary depending on the state of the patient's condition or its severity, and the age, weight, *etc.*, of the subject to be treated. A therapeutically effective amount can vary, depending on any of a number of factors, including, *e.g.*, the route of administration, the  
20       condition of the subject, as well as other factors understood by those in the art.

          The term “treatment” or “treating” generally refers to an intervention in an attempt to alter the natural course of the subject being treated, and can be performed either for prophylaxis or during the course of clinical pathology. Desirable effects include, but are not limited to, preventing occurrence or recurrence of disease, alleviating symptoms, suppressing,  
25       diminishing or inhibiting any direct or indirect pathological consequences of the disease, ameliorating or palliating the disease state, and causing remission or improved prognosis.

          The terms “individual,” “host,” “subject,” and “patient” are used interchangeably herein.

          As used herein, “expression,” “expressed,” or “encodes” refers to the process by which  
30       polynucleotides are transcribed into mRNA and/or the process by which the transcribed

5 mRNA is subsequently being translated into peptides, polypeptides, or proteins. Expression may include splicing of the mRNA in a eukaryotic cell or other forms of post-transcriptional modification or post-translational modification.

The following examples are given to illustrate the present invention. It should be understood, however, that the invention is not to be limited to the specific conditions or details  
10 described in these examples. All printed publications referenced herein are specifically incorporated by reference.

## Examples

### Example 1 - Use of VIV in the Treatment of Infectious Disease

15 This example demonstrates the use of a disclosed VIV, as demonstrated by a non-limiting example exemplified in Figure 1, in the treatment of Ebola virus or other infectious diseases. In this example, at least one of the “cargo” regions shown in Figure 1 encodes an antibody that specifically targets Ebola virus.

Subjects suspected of having or diagnosed as having Ebola receive administrations of a  
20 therapeutically effective amount of a VIV encoding an antibody that specifically targets Ebola virus, either alone or in combination with one or more additional agents for the treatment or prevention of Ebola. VIV encoding an antibody that specifically targets Ebola virus and/or additional agents are administered orally, intranasally, intrathecally, intraocularly, intradermally, transmucosally, iontophoretically, topically, systemically, intravenously,  
25 subcutaneously, intraperitoneally, or intramuscularly according to methods known in the art or as described herein. Subjects will be evaluated daily for the presence and/or severity of signs and symptoms associated with Ebola, including, but not limited to, *e.g.*, fever, fatigue, malaise, weakness, reddened eyes, joint and muscle pain, headache, nausea, vomiting, hemorrhage, and death. Treatments are maintained until such a time as one or more signs or symptoms of Ebola  
30 are ameliorated or eliminated.

5           It is rationally predicted that subjects suspected of having or diagnosed as having Ebola and receiving therapeutically effective amounts of a VIV encoding an antibody that specifically targets Ebola virus, will display reduced severity or elimination of one or more symptoms associated with Ebola. It is further expected that administration of a VIV encoding an antibody that specifically targets Ebola virus in combination with one or more additional  
10       agents will have additive or synergistic effects.

          These results will show that VIV encoding an antibody that specifically targets Ebola virus are useful in the treatment of Ebola or other infectious disease. Accordingly, a VIV encoding an antibody that specifically targets an infectious antigen is useful in methods comprising administering the VIV to a subject in need thereof for the treatment of an  
15       infectious disease.

#### **Example 2 - Use of VIV in the Prevention of Infectious Disease**

          This example demonstrates the use of the disclosed VIV, as demonstrated by a non-limiting example exemplified in Figure 1, in the prevention of Ebola virus or other infectious  
20       diseases. In this example, at least one of the “cargo” regions shown in Figure 1 encodes an antibody that specifically targets Ebola virus.

          Subjects suspected of having an increased risk of contracting Ebola receive administrations of a prophylactically effective amount of a VIV encoding an antibody that specifically targets Ebola virus, either alone or in combination with one or more additional  
25       agents for the treatment or prevention of Ebola prior to entering an area in which risk of contracting Ebola is increased. VIV encoding an antibody that specifically targets Ebola virus and/or additional agents are administered orally, intranasally, intrathecally, intraocularly, intradermally, transmucosally, iontophoretically, topically, systemically, intravenously, subcutaneously, intraperitoneally, or intramuscularly according to methods known in the art or  
30       as described herein. Subjects will be evaluated daily for the presence and/or severity of signs

5 and symptoms associated with Ebola, including, but not limited to, *e.g.*, fever, fatigue, malaise, weakness, reddened eyes, joint and muscle pain, headache, nausea, vomiting, hemorrhage, and death. Treatments are maintained until such a time as one or more signs or symptoms of Ebola are prevented.

10 It is rationally predicted that subjects suspected of having or diagnosed as having Ebola and receiving prophylactically effective amounts of a VIV encoding an antibody that specifically targets Ebola virus, will have a reduced risk of contracting Ebola. It is further expected that administration of VIV encoding an antibody that specifically targets Ebola virus in combination with one or more additional agents will have additive or synergistic effects in this regard.

15 These results will show that VIV encoding an antibody that specifically targets Ebola virus are useful in the prevention of Ebola or other infectious disease. Accordingly, a VIV encoding an antibody that specifically targets an infectious antigen is useful in methods comprising administering the VIV to a subject in need thereof for the prevention of an infectious disease.

20

### **Example 3 - Use of VIV for Enhanced Wound Healing**

This example demonstrates the use of the disclosed VIV, as demonstrated by a non-limiting example exemplified in Figure 1, for enhanced wound healing. In this example, at least one of the “cargo” regions shown in Figure 1 encodes platelet-derived growth factor (PDGF).

25 Subjects with a wound (*e.g.*, from accident, injury, or surgery) receive administrations of a therapeutically effective amount of a VIV encoding platelet-derived growth factor (PDGF), alone or in combination with one or more additional agents for treating or sterilizing a wound. VIV PDGF and/or additional agents are administered orally, intranasally, intrathecally, 30 intraocularly, intradermally, transmucosally, iontophoretically, topically, systemically,

5 intravenously, subcutaneously, intraperitoneally, or intramuscularly according to methods known in the art or as described herein. Subjects will be evaluated daily to determine the status of the wound. Treatments are maintained until such a time as the wound is healed and scarring is minimized.

It is rationally predicted that subjects with a wound and receiving therapeutically effective amounts of a VIV PDGF will display enhanced wound healing. It is further expected that administration of VIV encoding PDGF in combination with one or more additional agents will have additive or synergistic effects.

These results will show that VIV encoding PDGF are useful for enhancing wound healing. Accordingly, a VIV encoding PDGF or a similar gene is useful in methods comprising administering the VIV to a subject in need thereof for the treatment of a wound.

#### **Example 4 - Use of VIV in the Treatment of Bone Injury**

This example demonstrates the use of the disclosed VIV, as demonstrated by a non-limiting example exemplified in Figure 1, in the treatment of a bone injury. In this example, at least one of the “cargo” regions shown in Figure 1 encodes vascular endothelial growth factor (VEGF).

Subjects suspected of having or diagnosed as having a bone injury receive administrations of a therapeutically effective amount of a VIV encoding vascular endothelial growth factor (VEGF), alone or in combination with one or more additional agents for the treatment of the bone injury. VIV encoding VEGF and/or additional agents are administered orally, intranasally, intrathecally, intraocularly, intradermally, transmucosally, iontophoretically, topically, systemically, intravenously, subcutaneously, intraperitoneally, or intramuscularly according to methods known in the art or as described herein. Subjects will be evaluated weekly for the presence and/or severity of signs and symptoms associated with the

5 bone injury to determine the rate and strength of healing. Treatments are maintained until such a time as the bone has healed.

It is rationally predicted that subjects suspected of having or diagnosed as having a bone injury and receiving therapeutically effective amounts of a VIV encoding VEGF will display reduced severity of injury and enhanced healing. It is further expected that  
10 administration of VIV encoding VEGF in combination with one or more additional agents will have additive or synergistic effects.

These results will show that VIV encoding VEGF are useful in the treatment of bone injuries or diseases. Accordingly, a VIV encoding VEGF or similar genes is useful in methods comprising administering a VIV to a subject in need thereof for the treatment of a bone injury  
15 or disease.

#### **Example 5 - Use of VIV in the Treatment of Hereditary Disease**

This example demonstrates the use of the disclosed VIV, as demonstrated by a non-limiting example exemplified in Figure 1, in the treatment of cystic fibrosis (CF) or other  
20 hereditary genetic diseases. In this example, at least one of the “cargo” regions shown in Figure 1 encodes cystic fibrosis transmembrane conductance regulator (CFTR).

Subjects suspected of having or diagnosed as having (CF) receive administrations of a therapeutically effective amount of a VIV encoding cystic fibrosis transmembrane conductance regulator (CFTR), alone or in combination with one or more additional agents for  
25 the treatment of CF. VIV encoding CFTR and/or additional agents are administered orally, intranasally, intrathecally, intraocularly, intradermally, transmucosally, iontophoretically, topically, systemically, intravenously, subcutaneously, intraperitoneally, or intramuscularly according to methods known in the art or as described herein. Subjects will be evaluated weekly for the presence and/or severity of signs and symptoms associated with CF, including,  
30 but not limited to, *e.g.*, poor growth, persistent cough, thick sputum and mucus, wheezing,



5     breathlessness, decreased ability to exercise, repeated lung infections, inflamed nasal passage, greasy stools, intestinal blockage, and poor weight gain. Treatments are maintained until such a time as one or more signs or symptoms of CF are ameliorated or eliminated.

It is rationally predicted that subjects suspected of having or diagnosed as having CF and receiving therapeutically effective amounts of a VIV encoding CFTR will display reduced severity or elimination of one or more symptoms associated with CF. It is further expected that administration of VIV encoding CFTR in combination with one or more additional agents will have additive or synergistic effects.

These results will show that VIV encoding CFTR are useful in the treatment of CF or other hereditary genetic diseases. Accordingly, a VIV encoding a mutated or otherwise defective gene related to a genetic disease is useful in methods comprising administering the VIV to a subject in need thereof for the treatment of the genetic disease.

#### **Example 6 - Use of VIV Containing E1 to Express Cargo**

A vector according to Figure 2 was produced containing green fluorescent protein gene (GFP) as the cargo. DNA containing the complete Locus Control Region and E1 protein from human papillomavirus type 16 (NCBI accession number U89348) was chemically synthesized. Individual segments and/or coding sequences were initially synthesized. These were amplified by polymerase chain reaction (PCR) using synthetic oligonucleotide primers identical to the 5' end of green fluorescent protein gene and complementary to the 3' end of green fluorescent protein. The 5' primer was extended from its 5' end with the recognition site for BamHI or EcoRI endonucleases. The 3' primer was extended at its 3' end with the complement of BamHI, or EcoRI endonuclease recognition sites. The resulting amplified green fluorescent protein gene sequences were then digested with BamHI and EcoRI restriction endonucleases.

5           A lentiviral vector was obtained from System Biosciences, Inc. The plasmid was cleaved with BamHI and EcoRI enzymes, and mixed with excess amplified green fluorescent protein gene sequences in a 1:3 ratio of insert to vector.

Enzymatic activity was then stopped by heat inactivation at 70 degrees Celsius for 20 minutes. The above mixture was cooled to room temperature to allow annealing.

10           The annealing reactions were performed with bacteriophage T4 DNA ligase for 30 minutes at room temperature. 2.5 microliters of the resulting ligation mix were added to 25 microliters of STBL3 competent bacterial cells.

Transfection was then carried out by a brief (1 minute) heat-shock at 42 degrees Celsius.

15           Bacterial cells were streaked onto agar plates containing ampicillin to obtain bacterial cultures. These cultures were expanded in Luria broth.

To check for insertion of amplified green fluorescent protein gene sequences into the lentivirus vector packaging plasmid, DNA was extracted from the above bacterial cultures and purified by standard methods. Purified DNA was digested with the same endonucleases used  
20           to make the construct. Fragment lengths were analyzed by agarose gel electrophoresis, and the amplified green fluorescent protein gene sequences were verified by DNA sequencing using specific primers obtained from Eurofins MWG Operon LLC.

Lentivirus vector stocks were produced as follows. At least two lentiviral packaging plasmids plus the cargo plasmid were co-transfected into HEK cells where viral genes and  
25           genomic RNA are expressed, assembled into integrase-deficient lentivirus particles, and released into the culture medium. Cell-free supernatants were produced and collected during the interval of 3-10 days after transfection. Lentivirus particles were purified by standard procedures including a combination of methods that could include centrifugation, transient flow filtration, size exclusion chromatography, size exclusion filtration or ion exchange

5 chromatography. The concentration and biological activity (transducing units per ml) for each stock were determined.

Mammalian cells, including 293 T cells, were used to test for lentivirus-derived episome formation, copy number and expression. The 293 T cells were transduced with integrase deficient lentivirus particles at a multiplicity of infection ranging from 1 to 10 in the presence of polybrene. Unabsorbed virus was removed by washing cells 3 hours after application, and cells were cultured for 3 days. Cells were observed in a fluorescence microscope and cells expressing GFP are counted. Untransduced 293 T cells were used as a negative control. Data was reported as GFP-positive cells per 100 viable cells in culture. A minimum of 300 cells were counted per microscope field and 5-10 fields are counted for each replicate experiment. Four independent transduction experiments comprising one negative control and three replicate experiments were performed to determine the frequency of transduced cells. The data is depicted in Figure 3, and shows expression of GFP across three replicate experiments.

#### 20 **Example 7 - Use of VIV Containing E1 and E2 to Express Cargo**

Vector 19, as shown in Figure 4, is constructed to contain both E1 and E2 initiator proteins. In this Example, genetic cargo is represented by a CMV/GFP expression cassette, and a suitable inducible promoter is also selected such that external addition of a compound that can induce the inducible promoter.

25 293 T cells are transduced with Vector 19 at a multiplicity of infection ranging between 1 and 20 transducing units per cell. 3 hours later, cells are washed with medium to remove unadsorbed virions and returned to culture. 12-24 hours after transduction, cells are treated with at least one dosage of a compound that can induce the inducible promoter. Upon addition of a compound that can induce the inducible promoter, E1 and E2 mRNA are transcribed from the episome and combine and assemble on the Locus Control Region

30

- 5      Fragment 2 (LCR/F2) to trigger DNA replication. Lentivirus-derived episomes decay starting approximately 24-36 hours after the cessation of promoter induction. Protein products from the cargo in Vector 19 are measured by analytical flow cytometry.

While certain preferred embodiments of the present invention have been described and specifically exemplified herein, it is not intended that the invention be limited to such  
10      embodiments. Various modifications may be made thereto without departing from the scope and spirit of the present invention.

## 5 CLAIMS

What is claimed is:

1. A viral delivery system comprising:
  - (a) a viral carrier, wherein the viral carrier has a defective integrase gene;
  - (b) a heterologous viral episomal origin of replication;
  - 10 (c) a sequence encoding at least one initiator protein specific for the heterologous viral episomal origin of replication, wherein expression of the sequence encoding the at least one initiator protein specific for the heterologous viral episomal origin of replication is under the control of an inducible promoter; and
  - 15 (d) at least one gene, shRNA, siRNA, miRNA, or other gene-silencing RNA of interest.
2. The viral delivery system of claim 1, wherein the viral carrier is a lentivirus.
3. The viral delivery system of claim 1, wherein the heterologous viral episomal origin of replication is from a papillomavirus.
- 20 4. The viral delivery system of claim 3, wherein the heterologous viral episomal origin of replication is from bovine papillomavirus.
5. The viral delivery system of claim 3, wherein the heterologous viral episomal origin of replication is from human papillomavirus.
6. The viral delivery system of claim 1, wherein the at least one initiator protein specific  
25 for the heterologous viral episomal origin of replication is E1.
7. The viral delivery system of claim 1, wherein the at least one initiator protein specific for the heterologous viral episomal origin of replication is E2.
8. The viral delivery system of claim 1, wherein the system comprises two initiator proteins specific for the heterologous viral episomal origin of replication.

- 5     9.     The viral delivery system of claim 8, wherein the two initiator proteins specific for the heterologous viral episomal origin of replication are E1 and E2.
10.    The viral delivery system of claim 1, wherein the heterologous viral episomal origin of replication is from Epstein-Barr virus.
11.    The viral delivery system of claim 10, wherein the initiator protein specific for the  
10 heterologous viral episomal origin of replication is EBNA-1.
12.    A pharmaceutical composition comprising the viral delivery system of claim 1 and at least one pharmaceutically acceptable carrier.
13.    A method of treating or preventing a disease, comprising:
- 15     (a)    identifying a subject in need of treatment or prevention of the disease; and
- 16     (b)    administering to the subject a therapeutically effective amount of a viral delivery system comprising:
- 17        (i)    a viral carrier, wherein the viral carrier has a defective integrase gene;
- 18        (ii)   a heterologous viral episomal origin of replication;
- 19        (iii)   a sequence encoding at least one initiator protein specific for the  
20 heterologous viral episomal origin of replication, wherein expression of the sequence encoding the at least one initiator protein is under the control of an inducible promoter; and
- 21        (iv)   at least one gene, shRNA, siRNA, miRNA, or other gene-silencing RNA of interest.
- 22    14.    The method of claim 13, wherein the disease is an infectious disease.
- 23    15.    The method of claim 14, wherein the infectious disease is Ebola virus or Lassa fever virus.
- 24    16.    The method of claim 15, wherein the gene, shRNA, siRNA, miRNA, or other gene-silencing RNA of interest encodes an antibody specific for Ebola virus or Lassa fever virus.
- 25    17.    The method of claim 13, wherein the disease is a genetic disease or disorder.
- 30

- 5      18.      The method of claim 13, wherein the genetic disease is alcohol abuse.
19.      The method of claim 13, wherein the gene, shRNA, siRNA, miRNA, or other gene-silencing RNA of interest encodes brain-derived growth factor.
20.      A method of treating nerve damage, comprising:
- 10            (a)      identifying a subject with nerve damage; and
- (b)      administering to the subject a therapeutically effective amount of a viral delivery system comprising:
- (i)      a viral carrier, wherein the viral carrier has a defective integrase gene;
- (ii)      a heterologous viral episomal origin of replication;
- 15            (iii)      a sequence encoding at least one initiator protein specific for the heterologous viral episomal origin of replication, wherein expression of the sequence encoding the at least one initiator protein is under the control of an inducible promoter; and
- (iv)      at least one gene, shRNA, siRNA, miRNA, or other gene-silencing RNA of interest.
- 20      21.      The method of claim 20, wherein the gene, shRNA, siRNA, miRNA, or other gene-silencing RNA of interest is nerve growth factor.
22.      A method of enhancing wound healing, comprising:
- 25            (a)      identifying a subject with a wound; and
- (b)      administering to the subject a therapeutically effective amount of a viral delivery system comprising:
- (i)      a viral carrier, wherein the viral carrier has a defective integrase gene;
- (ii)      a heterologous viral episomal origin of replication;
- (iii)      a sequence encoding at least one initiator protein specific for the heterologous viral episomal origin of replication, wherein expression of

5                                   the sequence encoding the at least one initiator protein is under the  
control of an inducible promoter; and

(iv)    at least one gene, shRNA, siRNA, miRNA, or other gene-silencing  
RNA of interest.

23.    The method of claim 22, wherein the gene, shRNA, siRNA, miRNA, or other gene-  
10   silencing RNA of interest is platelet-derived growth factor or vascular endothelial growth  
factor.

24.    The method of claim 22, wherein the wound is from an accident or injury.

25.    The method of claim 22, wherein the wound is from surgery.

26.    The method of claim 22, wherein the wound is a burn.

15   27.    The method of claim 22, wherein the wound is a bone nonunion.



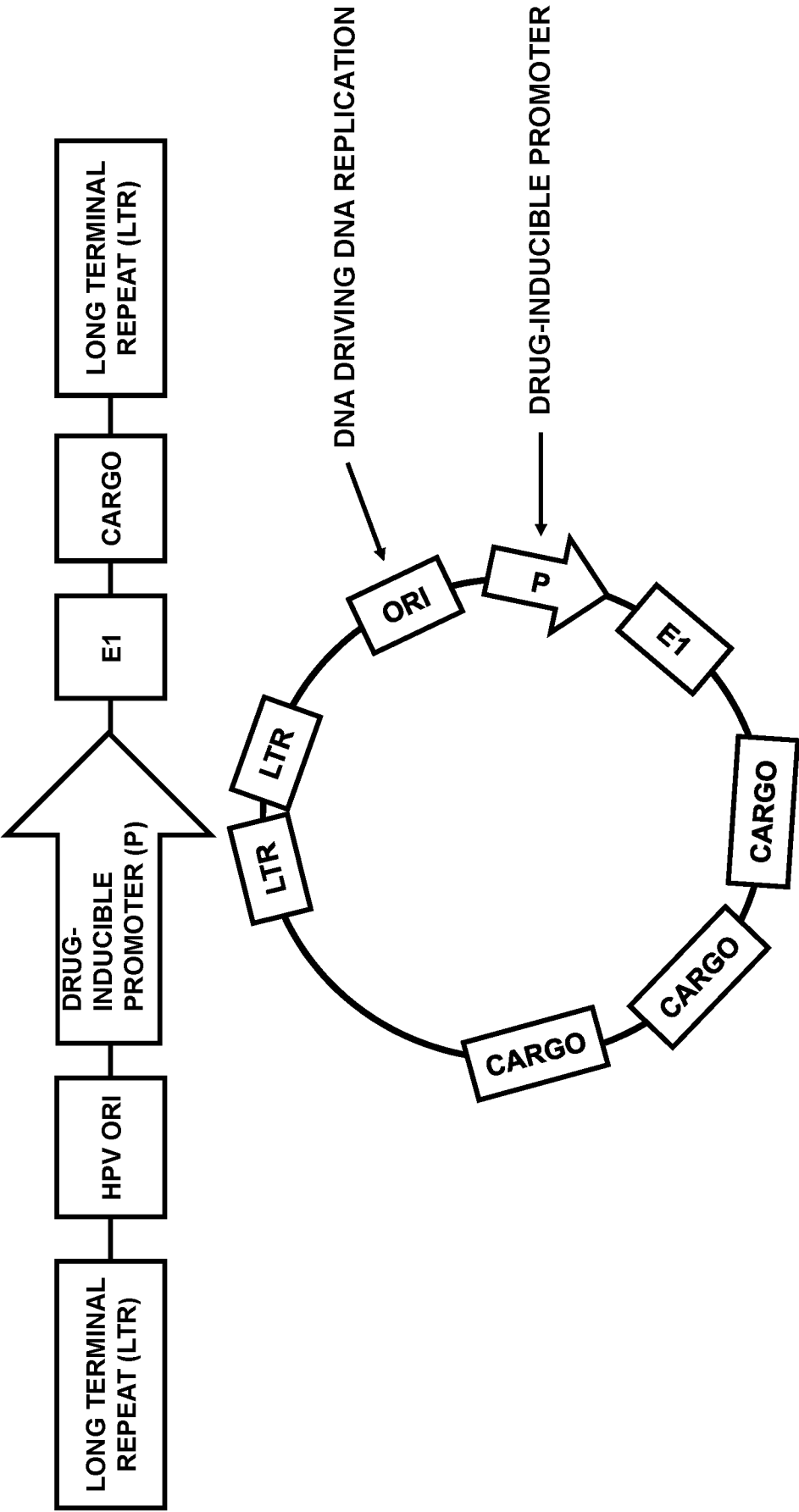
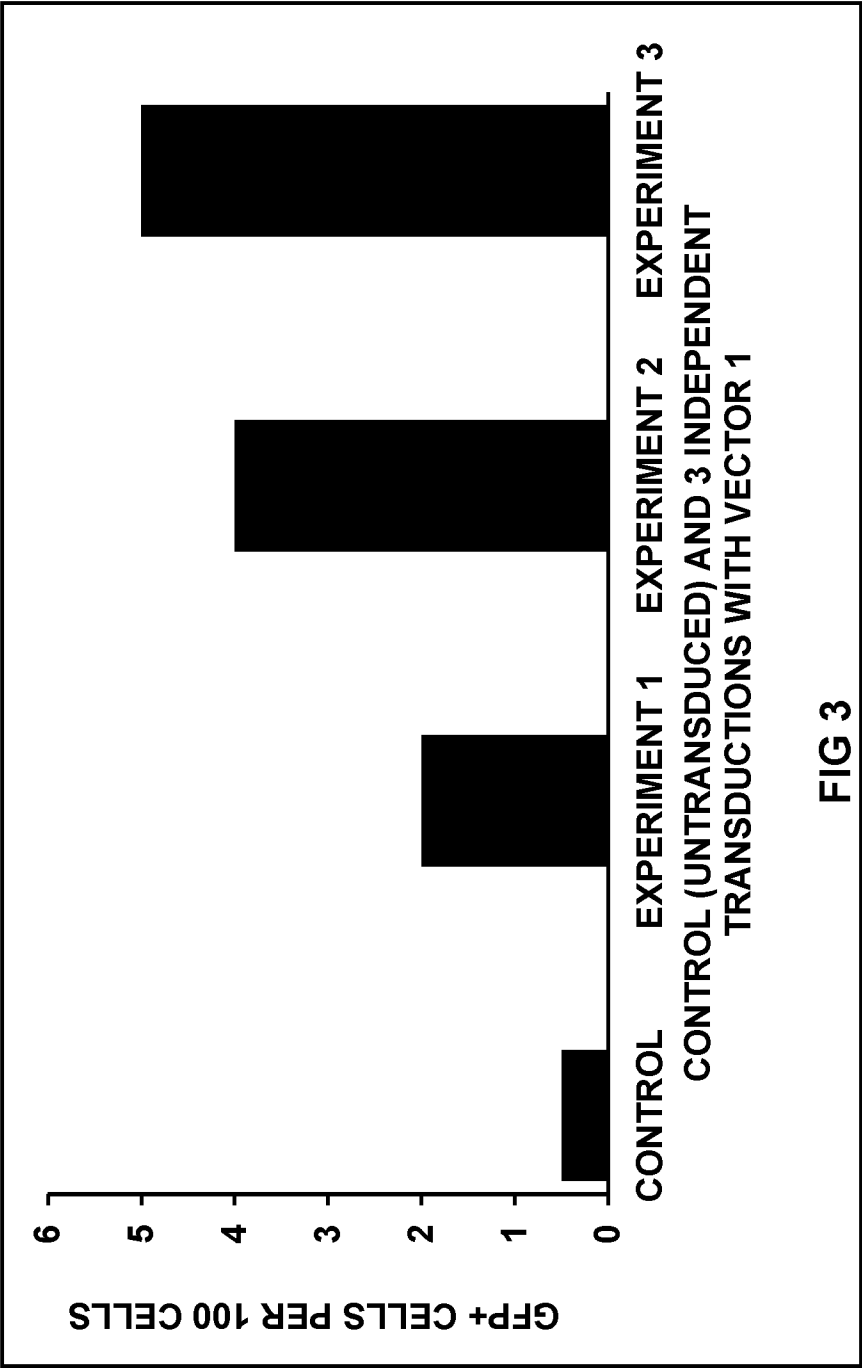


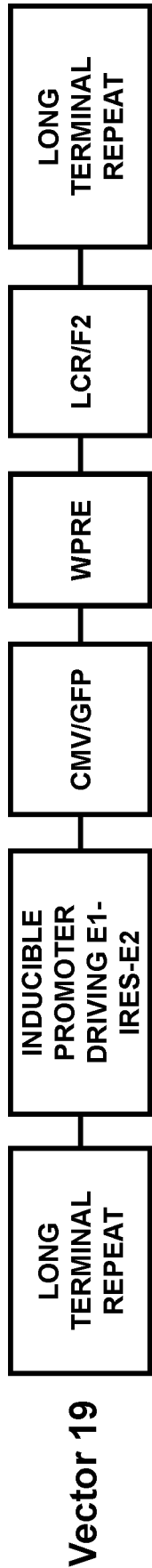
FIG 1



Vector 1

FIG 2





**FIG 4**

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 16/36519

## Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
  
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
  
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:  
-----go to Extra Sheet for continuation-----

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:  
1-4, 6, 12 limited to bovine papillomavirus origin of replication and initiator E1

### Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 16/36519

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - C12N 15/86, 15/867, 15/113; A61K 48/00 (2016.01)

CPC - C12N 2800/108, 15/8613, 15/867, 15/113

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC(8): C12N 15/86, 15/867, 15/113; A61K 48/00 (2016.01)

CPC: C12N 2800/108, 15/8613, 15/867, 15/113

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

CPC: C12N 2800/108, 15/8613, 15/867, 15/113 (text search)

USPC: 435/320.1; 514/44A, 536/23.72

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Electronic data bases: PatBase; Google Patents; Google Scholar

Search terms: Integration deficient lentiviral vector (IDLV), chimeric viral vector, viral episomal origin or replication, papillomavirus, initiator E1 and/or E2, gene silencing payload (RNAi, miRNA, siRNA, etc.)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	VARGAS et al. Conditionally replicating lentiviral-hybrid episomal vectors for suicide gene therapy. Antiviral Res. December 2008 Vol 80 No 3 Pages 288-294. Especially abstract.	1-4, 6, 12
Y	WO 2015/078999 A1 (Fundacion Centro Nacional De Investigaciones Cardiovasculares Carlos III (CNIC)) 4 June 2015 (04.06.2015). Especially pg 20 ln 4-8	1-4, 6, 12
Y	US 5,674,703 A (WOO et al.) 7 October 1997 (07.10.1997). Especially col 2 ln 26-47.	3, 4, 6

☐ Further documents are listed in the continuation of Box C.

\* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&amp;" document member of the same patent family

Date of the actual completion of the international search

17 October 2016

Date of mailing of the international search report

07 NOV 2016

Name and mailing address of the ISA/US

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PCT OSP: 571-272-7774

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 16/36519

-----continuation of Box III (Lack of Unity of Invention)-----

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I+: Claims 1-12, drawn to a viral delivery system composition. The vector delivery system will be searched to the extent to encompass the first named heterologous episomal origin of replication, bovine papillomavirus origin of replication (claim 4) and the first named initiator for the origin of replication, E1 (claim 6). It is believed that claims 1-4, 6, 12 read on this first named invention and thus these claims will be searched without fee to the extent that they encompass bovine papillomavirus origin of replication and initiator E1. Additional heterologous origins of replication and initiators will be searched upon payment of additional fees. Applicant must specify the claims that encompass any additional elected origins of replication and initiators. Applicants must further indicate, if applicable, the claims which read on the first named invention if different than what was indicated above for this group. Failure to clearly identify how any paid additional invention fees are to be applied to the "+" group(s) will result in only the first claimed invention to be searched/examined. An exemplary election would be: Epstein-Barr origin of replication (claim 10) and initiator EBNA-1 (claim 11) (Claims 1,2, 10-12).

Group II: Groups 13-27, drawn to a method of treating or preventing a disease or medical condition.

The inventions listed as Groups I+ and II do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

## Special Technical Features:

Group I+ has the special technical feature of a viral delivery system composition, not required by Group II.

Group II has the special technical feature of administering a therapeutically effective amount of a viral delivery system, not required by Group I+.

Among the inventions listed as Groups I+ is the specific heterologous viral origins of replications and their initiators. The inventions do not share a special technical feature, because no significant structural similarities can readily be ascertained among the origins or replications or their initiators.

## Common Technical Feature:

Groups I+ and II share the common technical feature of a viral delivery system comprising:

- (a) a viral carrier, wherein the viral carrier has a defective integrase gene;
- (b) a heterologous viral episomal origin of replication;
- (c) a sequence encoding at least one initiator protein specific for the heterologous viral episomal origin of replication, wherein expression of the sequence encoding the at least one initiator protein specific for the heterologous viral episomal origin of replication is under the control of an inducible promoter; and
- (d) at least one gene, shRNA, siRNA, miRNA, or other gene-silencing RNA of interest.

However, said common technical feature does not represent a contribution over the prior art, and is obvious over the publication titled "Conditionally replicating lentiviral-hybrid episomal vectors for suicide gene therapy" by VARGAS et al. (hereinafter "Vargas") [published December 2008 in Antiviral Res Vol 80 No 3 Pages 288-294], in view of WO 2015/078999 A1 (Fundacion Centro Nacional De Investigaciones Cardiovasculares Carlos III (CNIC)) (hereinafter "CNIC") [published 4 June 2015].

As to the common technical feature (and claim 1), Vargas teaches a viral delivery system comprising:

- (a) a viral carrier, wherein the viral carrier has a defective integrase gene (abstract; "Here we report on the design of a conditionally replicating integrase (IN)-defective lentiviral-hybrid episomal vector")
- (b) a heterologous viral episomal origin of replication (abstract; "in which the insertion of the SV40 promoter/origin of replication provides long-term persistence of the extrachromosomal DNA in the presence of the corresponding trans-acting T antigen (Tag)"). Although Vargas teaches at least one initiator protein specific for the heterologous viral episomal origin of replication (abstract; T antigen), Vargas does not specifically teach (c) a sequence encoding at least one initiator protein specific for the heterologous viral episomal origin of replication, wherein expression of the sequence encoding the at least one initiator protein specific for the heterologous viral episomal origin of replication is under the control of an inducible promoter. However, it would have been obvious to one of ordinary skill in the art to have included the sequence encoding T-antigen into the vector construct under an inducible promoter, because it would have enabled use of the vector system beyond cells that do not include the T antigen expressed in trans (i.e. 293T cells), without undue effort, and with a high probability of success. Although Vargas does teach a payload (abstract; SV40-driven GFP expression), Vargas does not teach (d) at least one gene, shRNA, siRNA, miRNA, or other gene-silencing RNA of interest. However, inclusion of gene-silencing RNAi into integration defective lentiviral vectors (IDLV) viral vectors was well-known in the art, as exemplified by the teaching of CNIC (pg 20 In 4-8; The term "polynucleotide of interest", as used herein, refers to any polynucleotide whose expression in a cell is desirable. The polynucleotide of interest can be.....a microRNA, a siRNA or a shRNA")

As the common technical feature was known in the art at the time of the invention, this cannot be considered a common special technical feature that would otherwise unify the groups. The inventions lack unity with one another.

Therefore, Groups I+ and II lack unity of invention under PCT Rule 13 because they do not share a same or corresponding special technical feature.