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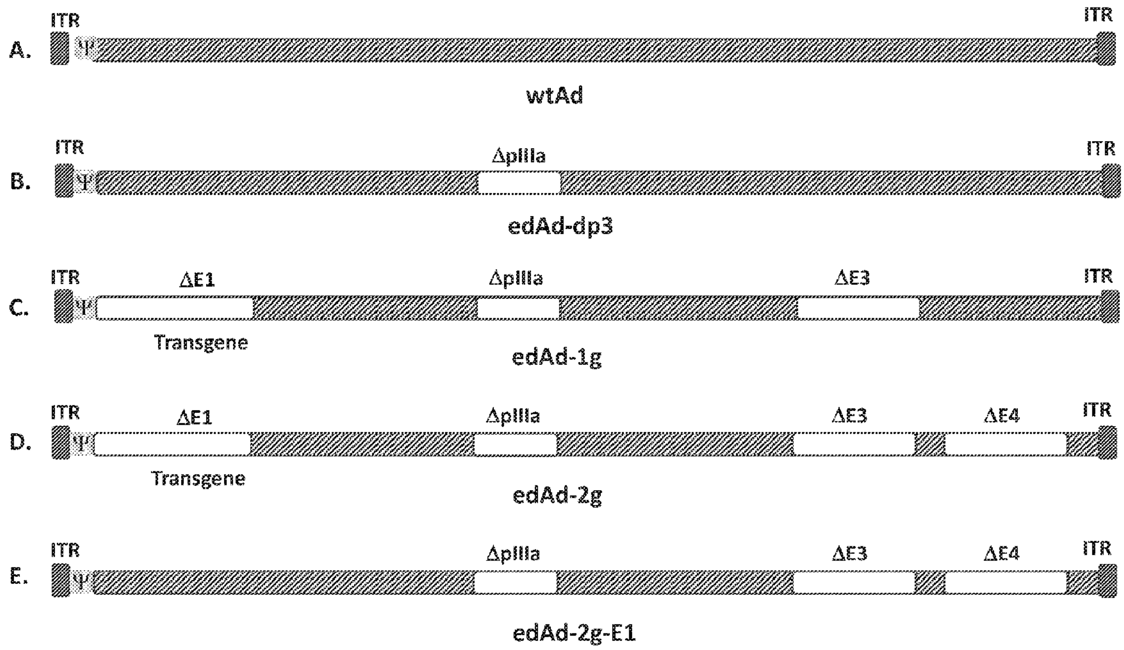

Figure 3

(57) Abstract: A method for preparing an infectious, recombinant virus vector comprises the steps of: (a) infecting host cells with a first virus comprising an encapsidation defective adenovirus (edAd), the edAd comprising a first defective virus genome; (b) incubating the infected host cells in a culture medium for a period of time sufficient for producing infectious virus particles; and (c) recovering infectious virus particles secreted into a culture supernatant, wherein the edAd or the host cells comprise a second defective virus genome engineered to express a target gene of interest, wherein the edAd or the host cells comprise nucleic acid sequences sufficient for expressing adenovirus (Ad) helper genes necessary for replication of the defective virus DNA; and wherein the edAd or the host cells comprise nucleic acid sequences sufficient from expressing helper functions necessary for producing infectious, replication defective virus particles corresponding to the second virus.

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TITLE

COMPOSITIONS AND METHODS FOR PREPARING VIRAL VECTORS

RELATED APPLICATIONS

[0001] The present application claims priority of U.S. Provisional Application No. 62/743,362, filed on October 9, 2018, which is incorporated by reference herein in its entirety.

FIELD

[0002] The present application generally relates to methods and compositions for producing recombinant viral vectors using encapsidation defective helper viruses.

BACKGROUND

[0003] Current recombinant virus production methods often employ adenovirus helper functions for preparing recombinant viral vectors for efficient gene delivery. Conventional methods often employ transfection methodologies resulting in less than optimal virus titers and difficult to scale up. When a helper adenovirus is used, the recombinant vector preparations are contaminated with helper viruses.

[0004] Therefore, there is a need to provide more efficient methods of producing high titer virus preparations that are not contaminated with encapsidated helper viruses or other undesirable effects.

SUMMARY

[0005] One aspect of the present application relates to an encapsidation defective adenovirus (edAd) for the production of recombinant virus vectors. The edAd comprises one or more mutations in its genome that results in (1) significantly reduced production or non-production of one or more encapsidation essential proteins, and/or (2) production of one or more defective encapsidation essential proteins.

[0006] Another aspect of the present application relates to a packaging cell line for production of an edAd of the present application. The packaging cell is capable of producing one or more gene products that allow encapsidation of edAd within the packaging cell.

[0007] Another aspect of the present application relates to a method for producing a recombinant virus (RV). The method comprises the steps of: (a) infecting a producer cell with one or more edAds to produce an infected producer cell, (b) incubating the infected producer cell under conditions that allow production of a RV having a RV genome; and (c) harvesting the recombinant virus, wherein either the edAd or the producer cell comprises the RV genome.

[0008] Another aspect of the present application relates to a producer cell for the production of a recombinant virus. The producer cell comprises (a) a genome of the recombination virus, or (b) genes encoding products required for the production of the recombination virus, or both (a) and (b).

BRIEF DESCRIPTION OF THE DRAWINGS

[0009] FIGs. 1A and 1B depict adenoviral particles and their structural components. FIG. 1C shows adenoviral transcriptional units, including polypeptides coded therefrom. The late unit encodes proteins that constitute mutation targets for creating an encapsidation defective Ad (edAd).

[0010] FIG. 2 is a schematic diagram depicting the functional outcomes associated with infection of host cells with a packaging competent Ad (pcAd) or an edAd. Specifically, a pcAd will produce infectious, packaged adenovirus particles; an edAd will not.

[0011] FIG. 3 is a schematic diagram depicting wt (panel A) and mutant Ad (edAd, panels B-E) genomes. The mutant Ads in panels B-E include pIIIa deletions, which render them encapsidation-defective. The edAd-dp3 (panel B) has a wild type E1 region which allows for DNA replication in host cells without E1a and E1b expression. The edAd-1g and edAd-2g (panels C and D) include deletions in E1 and E3 and can only replicate their DNA in cell lines, such as 293 cells, which express E1a and E1b proteins. The edAd-2g additionally has e4 deletion to further increase the packaging capacity. The edAd-2g virus can grow in 911E4 cell line with pIIIa expression. The edAd-2g-E1 (panel E) has additional deletions in E3, E4 and pIIIa but with a wild type E1 region.

[0012] FIG. 4 is a schematic diagram depicting several different edAds in panels A-E, each carrying a pIIIa deletion rendering them encapsidation-defective. In addition, each of the edAds has an integrated AAV vector for producing replication defective AAV particles expressing a desired target gene. The AAV vector sequences are shown as replacing Ad E3 sequences (panels A, B, D) or Ad E1 sequences (panels C, E). The edAd1g-AAV-i1-cre in panel C has an insertion at Ad E3 region with a CMV-Cre expression unit for providing Cre-mediated activation of helper gene functions necessary for rAAV production. The expression of the helper can be activated through cre mediated deletion or inversion a stop sequence.

[0013] FIG. 5 is a schematic diagram depicting production of edAds. Here, the structural elements missing in the edAd (such as pIIIa) are supplied by a host cell stably transformed to express the missing products in trans.

[0014] FIG. 6 is a schematic diagram depicting a conventional triple plasmid transfection method for producing rAAV particles in host cells expressing E1a and E1b helper functions (such as 293 cells).

[0015] FIG. 7 is a schematic diagram depicting production of both rAAVs vectors and pcAd or wt Ads when co-transfecting a host cell with a plasmid containing a defective AAV genome with AAV ITRs that is engineered to express a desired target gene and a plasmid co-expressing Rep and Cap (to provide helper functions for rAAV production) and also infecting the same host cells with wt Ad or pcAd, which provides helper functions for production of rAAVs. By products of wtAd and pcAd are also produced.

[0016] FIG. 8 is a schematic diagram depicting production of rAAVs vectors by infecting host cells with an edAd. In this case, the host cells (HeLa) are stably transformed with an AAV rep and cap expression plasmid and a defective AAV genome with AAV ITRs that is engineered to express a desired target gene. Although an infectious edAd-dp3 with E1 region is applied to the host cells, the resulting rAAVs are free from adenovirus contamination.

[0017] FIG. 9 is a schematic diagram depicting production of rAAVs by infecting 293 cells stably transformed to express E1a, E1b and inducible Rep-CapCap with an edAd, such as edAd1g-AAV-i1-Cre in FIG. 4, panel C. In this case, expression of Cre from the edAd excises a loxed fragment in an intron/polyA built into the Rep expression unit to activate Rep expression. The edAd also contains a defective AAV genome with AAV ITRs that is engineered to express a desired target gene. The resulting rAAVs are free from Ad contamination. Cre can be substituted with other expression induction system.

[0018] FIG. 10 is a schematic diagram depicting production of rAAVs by infecting an edAd (edAd1g-AAV-E1) which has a wt E1a/E1b region in Hela or A549 host cells. The edAd also contains a defective AAV genome with AAV ITRs that is engineered to express a desired target gene. The Hela and A549 host cells are stably transformed for expression of AAV Rep and Cap. The resulting rAAVs are free from Ad contamination.

[0019] FIG. 11 is a schematic diagram depicting production of rAAVs with an edAd with E1a/E1b, E2, E3, and E4 deletions in 293 cells providing E1a/E1b, E2 and E4 helper functions. The edAd2g-AAV-cre has a cre gene is designed to remove a loxed fragment in the intron built in the rep to activate rep expression. The edAd2g-AAV/(Cre) also contains a defective AAV genome with AAV ITRs that is engineered to express a desired target gene. The resulting rAAVs are free from Ad contamination.

[0020] FIG. 12 is a schematic diagram depicting components necessary for producing first (panel A), second (panel B) and third (panel C) generation lentivirus vector particles.

[0021] FIG. 13 is a schematic diagram depicting production of first generation lentivirus particles by infecting host cells stably transformed to express E1a and E1b with the three edAds depicted. The resulting lentivirus particles are free from Ad contamination. One of multiple adenovirus encoded elements can also be integrated into the host cell line. This example can be modified by only used one edAd carrying a combination of necessary elements for vector production while the rest elements are integrated into the host cell line.

[0022] FIG. 14 is a schematic diagram depicting production of second generation lentivirus particles by infecting host cells stably transformed to express E1a and E1b with the three edAds depicted. The resulting lentivirus particles are free from Ad contamination. One of multiple adenovirus encoded elements can also be integrated into the host cell line. This example can be modified by only used one edAd carrying a combination of necessary elements for vector production while the rest elements are integrated into the host cell line.

[0023] FIG. 15 is a schematic diagram depicting production of second generation lentivirus particles by infecting host cells stably transformed to express E1a/E1b with edAd1g-gen2pl-env particles engineered to express the lentiviral Env-Gag-Pol-Tat-Rev genes. In this case, a defective lentivirus genome engineered to express a desired transgene is stably transformed in the host cells. The resulting lentivirus particles are free from Ad contamination. This example can be modified by only used one edAd carrying a combination of necessary elements for vector production while the rest elements are integrated into the host cell line.

[0024] FIG. 16 is a schematic diagram depicting production of third generation lentiviral particles with a Tat-independent system comprising four edAds. In this case, host cells stably transformed to express E1a/E1b are infected with the four edAds as indicated. The edAd1g-transgene particles include a defective lentiviral self-inactivating (SIN) transfer vector containing a central copy of the polypurine tract *cis*-active sequence (cPPT) present in all lentiviral genomes for efficient nuclear import, an MSCV LTR promoter (MU3) as an internal promoter for driving the expression of the transgene, as well as a WPRE (W) element for high-level transgene expression. The other three edAds are engineered to provide helper functions from lentiviral gag-pol and rev proteins, and from the vesicular stomatitis virus (VSV)-G envelope glycoprotein. This example can be modified by only used one edAd carrying a combination necessary elements for vector production while the rest elements are integrated into the host cell line.

[0025] FIG. 17 is a schematic diagram depicting production of third generation lentiviral particles with a Tat-independent system comprising four edAds as previously described in FIG. 16, with the exception that one or more of the edAds including E1a/E1b. In this case, the host cells (such as Hela or A549) do not express any helper functions; instead, the helper functions are solely provided by the combination of edAds depicted (which do not depict inclusion of the E1a/E1b sequences). Only one adenovirus with wild type E1 region is necessary. This example can be modified by only used one edAd carrying a combination necessary elements for vector production while the rest elements are integrated into the host host cell line.

[0026] FIG. 18 is a schematic diagram depicting production of first generation lentivirus particles by infecting host cells stably transformed to express E1a and E1b with the two edAds depicted. The lentivirus genome is now integrated into host chromosome. The resulting lentivirus particles are free from Ad contamination. This example can be modified by only used one edAd carrying a combination necessary elements for vector production while the rest elements are integrated into the host host cell line.

[0027] FIG. 19 is a schematic diagram depicting production of third generation lentivirus particles by infecting host cells stably transformed to express E1a, E1b, E4 with one edAds depicted. The lentivirus genome is now integrated into host chromosome. The resulting lentivirus particles are free from Ad contamination.

While the present disclosure will now be described in detail, and it is done so in connection with the illustrative embodiments, it is not limited by the particular embodiments illustrated in the figures and the appended claims.

DETAILED DESCRIPTION

[0028] Reference will be made in detail to certain aspects and exemplary embodiments of the application, illustrating examples in the accompanying structures and figures. The aspects of the application will be described in conjunction with the exemplary embodiments, including methods, materials and examples, such description is non-limiting and the scope of the application is intended to encompass all equivalents, alternatives, and modifications, either generally known, or incorporated here. Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this application belongs. One of skill in the art will recognize many techniques and materials similar or equivalent to those described here, which could be used in the practice of the aspects and embodiments of the present application. The described aspects and embodiments of the application are not limited to the methods and materials described.

[0029] As used in this specification and the appended claims, the singular forms "a," "an" and "the" include plural referents unless the content clearly dictates otherwise.

[0030] Ranges may be expressed herein as from "about" one particular value, and/or to "about" another particular value. When such a range is expressed, another embodiment includes from the one particular value and/or to the other particular value. Similarly, when values are expressed as approximations, by use of the antecedent "about," it will be understood that the particular value forms another embodiment. It will be further understood that the endpoints of each of the ranges are significant both in relation to the other endpoint, and independently of the other endpoint. It is also understood that there are a number of values disclosed herein, and that each value is also herein disclosed as "about" that particular value in addition to the value itself. For example, if the value "10" is disclosed, then "about 10" is also disclosed. It is also understood that when a value is disclosed that "less than or equal to "the value," greater than or equal to the value" and possible ranges between values are also disclosed, as appropriately understood by the skilled artisan. For example, if the value "10" is disclosed the "less than or equal to 10" as well as "greater than or equal to 10" is also disclosed.

[0031] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this application belongs. Generally, the nomenclature used herein and the laboratory procedures in cell culture, molecular genetics, and nucleic acid chemistry and hybridization described below are those well known and commonly employed in the art. Standard techniques are used for recombinant nucleic acid methods, polynucleotide synthesis, and microbial culture and transformation (*e.g.*, electroporation, lipofection). Generally, enzymatic reactions and purification steps are performed according to the manufacturer's specifications. The techniques and procedures are generally performed according to conventional methods in the art and various general references (see generally, Sambrook et al. Molecular Cloning: A Laboratory Manual, 2d ed. (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., which is incorporated herein by reference) which are provided throughout this document. Units, prefixes, and symbols may be denoted in their SI accepted form. Unless otherwise indicated, nucleic acids are written left to right in 5' to 3' orientation; amino acid sequences are written left to right in amino to carboxyl orientation, respectively. Numeric ranges are inclusive of the numbers defining the range and include each integer within the defined range. Amino acids may be referred to herein by either their commonly known three letter symbols or by the one-letter symbols recommended by the IUPAC-IUB Biochemical nomenclature Commission. Nucleotides, likewise, may be referred to by their commonly accepted single-letter codes.

Definitions

[0032] The term "nucleic acid" as used herein, encompasses both RNA and DNA, including cDNA, genomic DNA, and synthetic (*e.g.*, chemically synthesized) DNA. A nucleic acid can be double-stranded or single-stranded. A single-stranded nucleic acid can be the sense strand or the antisense strand. In addition, a nucleic acid can be circular or linear.

[0033] An "isolated nucleic acid" refers to a nucleic acid that is separated from other nucleic acid molecules that are present in a viral genome, including nucleic acids that normally flank one or both sides of the nucleic acid in a viral genome. The term "isolated" as used herein with respect to nucleic acids also includes any non-naturally-occurring nucleic acid sequence, since such non-naturally-occurring sequences are not found in nature and do not have immediately contiguous sequences in a naturally-occurring genome.

[0034] An isolated nucleic acid can be, for example, a DNA molecule, provided one of the nucleic acid sequences normally found immediately flanking that DNA molecule in a naturally-occurring genome is removed or absent. Thus, an isolated nucleic acid includes, without limitation, a DNA molecule that exists as a separate molecule (*e.g.*, a chemically synthesized nucleic acid, or a cDNA or genomic DNA fragment produced by PCR or restriction endonuclease treatment) independent of other sequences as well as DNA that is incorporated into a vector, an autonomously replicating plasmid, a virus (*e.g.*, any paramyxovirus, retrovirus, lentivirus, adenovirus, or herpes virus), or into the genomic DNA of a prokaryote or eukaryote. In addition, an isolated nucleic acid can include an engineered nucleic acid such as a DNA molecule that is part of a hybrid or fusion nucleic acid. A nucleic acid existing among hundreds to millions of other nucleic acids within, for example, cDNA libraries or genomic libraries, or gel slices containing a genomic DNA restriction digest, is not considered an isolated nucleic acid.

[0035] In some embodiments, a nucleic acid molecule can encode the genome of an adenovirus with the exception that the genome lacks all or a portion of at least of one adenovirus polypeptide-encoding sequence. Any appropriate molecular cloning technique (*e.g.*, recombination or site-directed mutagenesis) can be used to generate an adenovirus nucleic acid molecule that lacks all or a portion of a fiber protein-encoding sequence, a V protein-encoding sequence, hexon protein-encoding sequence, penton base protein-encoding sequence, VA RNA-encoding sequence, or pIII protein-encoding sequence. Likewise, any appropriate molecular cloning technique (*e.g.*, PCR, recombination, or restriction site cloning) can be used to introduce a nucleic acid sequence into a nucleic acid molecule of an adenovirus. The nucleic acid molecules provided herein can be incorporated into viruses by standard techniques. For example, recombinant techniques can be used to insert a nucleic acid molecule provided herein into an infective viral genome or sub-genome within a plasmid or other vector. In some cases, a plasmid or other vector can additionally express luciferase or another reporter gene. The viral genome can then be transfected into mammalian cells to rescue the modified adenovirus. Alternately, modified subgenome sequences can be co-transfected into cells with other subgenome sequence such that the mammalian cells recombine the subgenomes into an intact genome making new virus.

[0036] "Gene transfer" or "gene delivery" refers to methods or systems for inserting foreign DNA into host cells. Gene transfer can result in transient expression of non-integrated transferred DNA, extrachromosomal replication and expression of transferred replicons (*e.g.*, episomes), or integration of transferred genetic material into the genomic DNA of host cells.

[0037] By "vector" is meant any genetic element, such as a plasmid, phage, transposon, cosmid, chromosome, artificial chromosome, virus, virion, etc., which is capable of replication when associated with the proper control elements and which can transfer gene sequences between cells. Thus, the term includes cloning and expression vehicles, as well as viral vectors.

[0038] By "adeno-associated virus inverted terminal repeats" or "AAV ITRs" is meant the art-recognized regions found at each end of the AAV genome which function together in *cis* as origins of DNA replication and as packaging signals for the viral genome. AAV ITRs, together with the AAV rep coding region, provide for the efficient excision and rescue from, and integration of a nucleotide sequence interposed between two flanking ITRs into a mammalian cell genome.

[0039] The nucleotide sequences of AAV ITR regions are known. *See*, *e.g.*, Kotin, R. M. (1994) Human Gene Therapy 5, 793–801; Bems, K. I. "Parvoviridae and their Replication" in Fundamental Virology, 2d ed., (B. N. Fields and D. M. Knipe, eds.) for the AAV-2 sequence. As used herein, an "AAV ITR" need not have the wild-type nucleotide sequence depicted in the previously cited references, but may be altered, *e.g.*, by the insertion, deletion or substitution of nucleotides. Additionally, the AAV ITR may be derived from any of several AAV serotypes, including without limitation, AAV-1, AAV-2, AAV-3, AAV-4, AAV-5, AAV-6, AAV-7, AAV-9, AAV-10, AAV-11, AAV-12, and AAV-13. Furthermore, 5′ and 3′ ITRs which flank a selected nucleotide sequence in an AAV vector need not necessarily be identical or derived from the same AAV serotype or isolate, so long as they function as intended, i.e., to allow for excision and rescue of the sequence of interest from a host cell genome or vector, and to allow integration of the heterologous sequence into the recipient cell genome when AAV Rep gene products are present in the cell.

[0040] By "AAV rep coding region" is meant the art-recognized region of the AAV genome which encodes the replication proteins of the virus which are required to replicate the viral genome and to insert the viral genome into a host genome during latent infection. The term also includes functional homologues thereof such as the human herpesvirus 6 (HHV-6) rep gene which is also known to mediate AAV-2 DNA replication (Thomson et al. (1994) Virology 204, 304–311). For a further description of the AAV rep coding region, see, *e.g.*, Muzyczka, N. (1992) Current Topics in Microbiol. and Immunol. 158, 97–129; Kotin, R. M. (1994) Human Gene Therapy 5, 793–801. The rep coding region, as used herein, can be derived from any viral serotype, such as the AAV serotypes described above. The region need not include all of the wild-type genes but may be altered, *e.g.*, by the insertion, deletion or substitution of nucleotides, so long as the rep genes present provide for sufficient integration functions when expressed in a suitable recipient cell.

[0041] The term "long forms of Rep" refers to the Rep 78 and Rep 68 gene products of the AAV rep coding region, including functional homologues thereof. The long forms of Rep are normally expressed under the direction of the AAV p5 promoter.

[0042] The term "short forms of Rep" refers to the Rep 52 and Rep 40 gene products of the AAV rep coding region, including functional homologues thereof. The short forms of Rep are expressed under the direction of the AAV p19 promoter.

[0043] By "AAV cap coding region" is meant the art-recognized region of the AAV genome which encodes the coat proteins of the virus which are required for packaging the viral genome. For a further description of the cap coding region, see, *e.g.*, Muzyczka, N. (1992) Current Topics in Microbiol. and Immunol. 158, 97–129; Kotin, R. M. (1994) Human Gene Therapy 5, 793–801. The AAV cap coding region, as used herein, can be derived from any AAV serotype, as described above. The region need not include all of the wild-type cap genes but may be altered, *e.g.*, by the insertion, deletion or substitution of nucleotides, so long as the genes provide for sufficient packaging functions when present in a host cell along with an AAV vector.

[0044] The term "AAV coding region" refers to a nucleic acid molecule that includes the two major AAV open reading frames corresponding to the AAV rep and cap coding regions; *e.g.*, a nucleic acid molecule comprising a nucleotide sequence substantially homologous to base pairs 310 through 4,440 of the wild-type AAV genome. Thus, for purposes of the present application, an AAV coding region does not include those sequences corresponding to the AAV p5 promoter region, and does not include the AAV ITRs.

[0045] By an "AAV vector" is meant a vector derived from an adeno-associated virus serotype, including without limitation, AAV-1, AAV-2, AAV-3, AAV-4, AAV-5, AAV-6, AAV-7, AAV-9, AAV-10, AAV-11, AAV-12, and AAV-13. AAV vectors can have one or more of the AAV wild-type genes deleted in whole or part, preferably the rep and/or cap genes, but retain functional flanking ITR sequences. Functional ITR sequences are necessary for the rescue, replication and packaging of the AAV virion. Thus, an AAV vector is defined herein to include at least those sequences required *in cis* for replication and packaging (*e.g.*, functional ITRs) of the virus. The ITRs need not be the wild-type nucleotide sequences, and may be altered, *e.g.*, by the insertion, deletion or substitution of nucleotides, so long as the sequences provide for functional rescue, replication and packaging.

[0046] "AAV helper functions" refer to AAV-derived coding sequences that can be expressed to provide AAV gene products that, in turn, function *in trans* for productive AAV replication. Thus, AAV helper functions include the rep and cap regions. The rep expression products have been shown to possess many functions, including, among others: recognition, binding and nicking of the AAV origin of DNA replication; DNA helicase activity; and modulation of transcription from AAV (or other heterologous) promoters. The cap expression products supply necessary packaging functions. AAV helper functions are used herein to complement AAV functions *in trans* that are missing from AAV vectors.

[0047] The term "AAV helper construct" refers generally to a nucleic acid molecule that includes nucleotide sequences providing AAV functions deleted from an AAV vector which is to be used to produce a transducing vector for delivery of a nucleotide sequence of interest. AAV helper constructs are commonly used to provide transient expression of AAV rep and/or cap genes to complement missing AAV functions that are necessary for lytic AAV replication; however, helper constructs lack AAV ITRs and can neither replicate nor package themselves. AAV helper constructs can be in the form of a plasmid, phage, transposon, cosmid, virus, or virion. A number of AAV helper constructs have been described, such as the commonly used plasmids pAAV/Ad and pIM29+45 which encode both Rep and Cap expression products. See, *e.g.*, Samulski et al. (1989) J. Virology 63, 3822–3828; McCarty et al. (1991) J. Virology 65, 2936–2945. A number of other vectors have been described which encode Rep and/or Cap expression products. See, *e.g.*, U.S. Pat. No. 5,139,941.

[0048] The term "accessory functions" refers to non-AAV derived viral and/or cellular functions upon which AAV is dependent for its replication. Thus, the term captures DNAs, RNAs and protein that are required for AAV replication, including those moieties involved in activation of AAV gene transcription, stage specific AAV mRNA splicing, AAV DNA replication, synthesis of Cap expression products and AAV capsid assembly. Viral-based accessory functions can be derived from any of the known helper viruses such as adenovirus, herpesvirus (other than herpes simplex virus type-1) and vaccinia virus.

[0049] For example, adenovirus-derived accessory functions have been widely studied, and a number of adenovirus genes involved in accessory functions have been identified and partially characterized. Specifically, early adenoviral E1A, E1B 55K, E2A, E4, and VA RNA gene regions are thought to participate in the accessory process. Herpesvirus-derived accessory functions have been described. See, *e.g.*, Young et al. (1979) Prog. Med. Virol. 25, 113. Vaccinia virus-derived accessory functions have also been described. See, *e.g.*, Carter, B. J. (1990), supra., Schlehofer et al. (1986) Virology 152, 110–117.

[0050] The term "accessory function vector" refers generally to a nucleic acid molecule that includes nucleotide sequences providing accessory functions. An accessory function vector can be transfected into a suitable host cell, wherein the vector is then capable of supporting AAV virion production in the host cell. Expressly excluded from the term are infectious viral particles as they exist in nature, such as adenovirus, herpesvirus or vaccinia virus particles. Thus, accessory function vectors can be in the form of a plasmid, phage, transposon, cosmid or virus that has been modified from its naturally occurring form.

[0051] By "recombinant virus" is meant a virus that has been genetically altered, *e.g.*, by the addition or insertion of a heterologous nucleic acid construct into the particle.

[0052] By "AAV virion" is meant a complete virus particle, such as a wild-type (wt) AAV virus particle (comprising a linear, single-stranded AAV nucleic acid genome associated with an AAV capsid protein coat). In this regard, single-stranded AAV nucleic acid molecules of either complementary sense, i.e., "sense" or "antisense" strands, can be packaged into any one AAV virion and both strands are equally infectious.

[0053] A "recombinant AAV virion," or "rAAV virion" is defined herein as an infectious, replication-defective virus composed of an AAV protein shell encapsulating a heterologous nucleotide sequence of interest that is flanked on both sides by AAV ITRs. A rAAV virion is produced in a suitable host cell comprising an AAV vector, AAV helper functions, and accessory functions. In this manner, the host cell is rendered capable of encoding AAV polypeptides that are required for packaging the AAV vector (containing a recombinant nucleotide sequence of interest) into infectious recombinant virion particles for subsequent gene delivery.

[0054] The term “transfection” is used to refer to the uptake of foreign DNA by a cell. A cell has been “transfected” when exogenous DNA has been introduced inside the cell membrane. A number of transfection techniques are generally known in the art. See, *e.g.*, Graham et al. (1973) Virology, 52, 456; Sambrook et al. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratories, New York; Davis et al. (1986) Basic Methods in Molecular Biology, Elsevier; Chu et al. (1981) Gene 13, 197. Such techniques can be used to introduce one or more exogenous DNA moieties, such as a nucleotide integration vector and other nucleic acid molecules, into suitable host cells. The term captures chemical, electrical, and viral-mediated transfection procedures.

[0055] The term “host cell” denotes, for example, microorganisms, yeast cells, insect cells, and mammalian cells, that can be, or have been, used as recipients of an AAV helper construct, an AAV vector plasmid, an accessory function vector, or other transfer DNA. The term includes the progeny of the original cell which has been transfected. Thus, a “host cell” as used herein generally refers to a cell which has been transfected with an exogenous DNA sequence. It is understood that the progeny of a single parental cell may not necessarily be completely identical in morphology or in genomic or total DNA complement to the original parent, due to natural, accidental, or deliberate mutation.

[0056] As used herein, the term “cell line” refers to a population of cells capable of continuous or prolonged growth and division in vitro. Often, cell lines are clonal populations derived from a single progenitor cell. It is further known in the art that spontaneous or induced changes can occur in karyotype during storage or transfer of such clonal populations. Therefore, cells derived from the cell line referred to may not be precisely identical to the ancestral cells or cultures, and the cell line referred to includes such variants.

[0057] The term “heterologous” as it relates to nucleic acid sequences such as coding sequences and control sequences, denotes sequences that are not normally joined together, and/or are not normally associated with a particular cell. Thus, a “heterologous” region of a nucleic acid construct or a vector is a segment of nucleic acid within or attached to another nucleic acid molecule that is not found in association with the other molecule in nature. For example, a heterologous region of a nucleic acid construct could include a coding sequence flanked by sequences not found in association with the coding sequence in nature. Another example of a heterologous coding sequence is a construct where the coding sequence itself is not found in nature (*e.g.*, synthetic sequences having codons different from the native gene). Similarly, a cell transformed with a construct which is not normally present in the cell would be considered heterologous for purposes of this application. Allelic variation or naturally occurring mutational events do not give rise to heterologous DNA, as used herein.

[0058] A "coding sequence" or a sequence which "encodes" a particular protein, is a nucleic acid sequence which is transcribed (in the case of DNA) and translated (in the case of mRNA) into a polypeptide in vitro or in vivo when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a start codon at the 5′ (amino) terminus and a translation stop codon at the 3′ (carboxy) terminus. A coding sequence can include, but is not limited to, cDNA from prokaryotic or eukaryotic mRNA, genomic DNA sequences from prokaryotic or eukaryotic DNA, and even synthetic DNA sequences. A transcription termination sequence will usually be located 3′ to the coding sequence.

[0059] A "nucleic acid" sequence refers to a DNA or RNA sequence. The term captures sequences that include any of the known base analogues of DNA and RNA such as, but not limited to, 4-acetylcytosine, 8-hydroxy-N6-methyladenosine, aziridinylcytosine, pseudoisocytosine, 5-(carboxyhydroxylmethyl) uracil, 5-fluorouracil, 5-bromouracil, 5-carboxymethylaminomethyl-2-thiouracil, 5-carboxymethylaminomethyluracil, dihydrouracil, inosine, N6-isopentenyladenine, 1-methyladenine, 1-methylpseudo-uracil, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-methyladenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxy-aminomethyl-2-thiouracil, beta-D-mannosylqueosine, 5′-methoxycarbonylmethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid, oxybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, N-uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid, pseudouracil, 2-thiocytosine, and 2,6-diaminopurine.

[0060] The term DNA "control sequences" refers collectively to promoter sequences, polyadenylation signals, transcription termination sequences, upstream regulatory domains, origins of replication, internal ribosome entry sites ("IRES"), enhancers, and the like, which collectively provide for the replication, transcription and translation of a coding sequence in a recipient cell. Not all of these control sequences need always be present so long as the selected coding sequence is capable of being replicated, transcribed and translated in an appropriate host cell.

[0061] The term "promoter region" is used herein in its ordinary sense to refer to a DNA regulatory sequence to which RNA polymerase binds, initiating transcription of a downstream (3′ direction) coding sequence.

[0062] The phrase "operably linked" refers to an arrangement of elements wherein the components so described are configured so as to perform their usual function. Thus, control sequences operably linked to a coding sequence are capable of effecting the expression of the coding sequence. The control sequences need not be contiguous with the coding sequence, so long as they function to direct the expression thereof. Thus, for example, intervening untranslated yet transcribed sequences can be present between a promoter sequence and the coding sequence and the promoter sequence can still be considered "operably linked" to the coding sequence.

[0063] By "isolated," when referring to a nucleotide sequence, is meant that the indicated molecule is present in the substantial absence of other biological macromolecules of the same type. Thus, an "isolated nucleic acid molecule which encodes a particular polypeptide" refers to a nucleic acid molecule which is substantially free of other nucleic acid molecules that do not encode the subject polypeptide; however, the molecule may include some additional bases or moieties which do not deleteriously affect the basic characteristics of the composition.

[0064] For the purpose of describing the relative position of nucleotide sequences in a particular nucleic acid molecule throughout the instant application, such as when a particular nucleotide sequence is described as being situated "upstream," "downstream," "3′," or "5′" relative to another sequence, it is to be understood that it is the position of the sequences in the "sense" or "coding" strand of a DNA molecule that is being referred to, as is conventional in the art.

[0065] "Homology" refers to the percent of identity between two polynucleotide or two polypeptide moieties. The correspondence between the sequences from one moiety to another can be determined by techniques known in the art. For example, homology can be determined by a direct comparison of the sequence information between two polypeptide molecules by aligning the sequence information and using readily available computer programs. Alternatively, homology can be determined by hybridization of polynucleotides under conditions which allow for the formation of stable duplexes between homologous regions, followed by digestion with single stranded-specific nuclease(s), and size determination of the digested fragments. Two DNA, or two polypeptide sequences are "substantially homologous" to each other when at least about 80%, preferably at least about

90%, and most preferably at least about 95% of the nucleotides or amino acids match over a defined length of the molecules, as determined using the methods above.

[0066] A "functional homologue" or a "functional equivalent" of a given polypeptide includes molecules derived from the native polypeptide sequence, as well as recombinantly produced or chemically synthesized polypeptides which function in a manner similar to the reference molecule to achieve a desired result. Thus, a functional homologue of AAV Rep68 or Rep78 encompasses derivatives and analogues of those polypeptides, including derivatives and analogues with any single or multiple amino acid additions, substitutions and/or deletions occurring internally or at the amino or carboxy termini thereof, so long as integration activity remains.

[0067] A "functional homologue" or a "functional equivalent" of a given adenoviral nucleotide region includes similar regions derived from a heterologous adenovirus serotype, nucleotide regions derived from another virus or from a cellular source, and recombinantly produced or chemically synthesized polynucleotides which function in a manner similar to the reference nucleotide region to achieve a desired result. Thus, a functional homologue of an adenoviral VA RNA gene region or an adenoviral E2A gene region encompasses derivatives and analogues of such gene regions—including derivatives and analogues with any single or multiple nucleotide base additions, substitutions and/or deletions occurring within the regions, so long as the homologue retains the ability to provide its inherent accessory function to support AAV virion production at levels detectable above background.

[0068] The term "wild-type AAV" as used herein refers to both wild-type and pseudo-wild-type AAV. "Pseudo-wild-type AAV" are replication-competent AAV virions produced by either homologous or non-homologous recombination between an AAV vector carrying ITRs and an AAV helper vector carrying rep and cap genes. Pseudo-wild-type AAV have nucleic acid sequences that differ from wild-type AAV sequences.

[0069] The term "encapsidation essential gene product" refers to an adenoviral gene product essential for encapsidating an adenoviral genome to produce infectious adenovirus particles. Encapsidation typically include "late phase" adenoviral gene products. Exemplary encapsidation essential adenoviral gene products include capsid protein IX, encapsidation protein IVa2, protein 13.6, encapsidation protein 52K, capsid protein precursor pIIIa, penton base (capsid protein III), core protein precursor pVII, core protein V, core protein precursor pX, capsid protein precursor pVI, hexon (capsid protein II), protease, hexon assembly protein 100K, protein 33K, encapsidation protein 22K, capsid protein precursor pVIII, protein UXP, and fiber (capsid protein IV).

[0070] The term "encapsidation non-essential gene product" refers to an adenoviral gene product that is dispensable for encapsidating an adenoviral genome to produce infectious adenovirus particles. Encapsidation non-essential adenoviral gene products are typically translated from adenovirus E1, E2, E3 and E4 transcriptional units. Thus, encapsidation non-essential adenoviral gene products may include gene products expressed from the adenoviral E1 transcriptional unit (*e.g.*, E1A, E1B-19K, E1B-55K); gene products expressed from the adenoviral E2/E2A transcriptional unit (*e.g.*, Iva2, pol, pTP, DBP); gene products expressed from the adenoviral E3 transcriptional unit (*e.g.*, E3 CR1 alpha0, E3 gp19, E3 14.7 K, E3 RID-beta); gene products expressed from the E4 transcriptional unit (*e.g.*, E4 34K, E4 ORF1, E4 ORFB, E4 ORF3, E4 ORF4, E4 ORF6/7); or a combination thereof.

[0071] The term "edAd" refers to an adenovirus mutant having one or more mutations in one or more encapsidation essential adenoviral gene products.

[0072] As used herein, the term "heterologous" nucleic acid sequence refers to a sequence that originates from a foreign species, or, if from the same species, it may be substantially modified from the original form. Alternatively, an unchanged nucleic acid sequence that is not expressed normally in a cell is a heterologous nucleic acid sequence. Preferably, the heterologous sequence is operatively linked to a promoter, resulting in a chimeric gene. The heterologous nucleic acid sequence is preferably under control of either the viral LTR promoter-enhancer signals or of an internal promoter, and retained signals within the retroviral LTR can still bring about efficient expression of the transgene.

[0073] A promoter sequence may be homologous or heterologous to the desired gene sequence. A wide range of promoters may be utilized, including a viral or a mammalian promoter. Cell or tissue specific promoters can be utilized to target expression of gene sequences in specific cell populations. Suitable mammalian and viral promoters for the present application are available in the art. A suitable promoter is one which is inducible or conditional.

[0074] Optionally during the cloning stage, the nucleic acid construct referred to as the transfer vector, having the packaging signal and the heterologous cloning site, also contains a selectable marker gene. Marker genes are utilized to assay for the presence of the vector, and thus, to confirm infection and integration. The presence of a marker gene ensures the selection and growth of only those host cells which express the inserts. Typical selection genes encode proteins that confer resistance to antibiotics and other toxic substances *e.g.*, histidinol, puromycin, hygromycin, neomycin, methotrexate etc. and cell surface markers.

[0075] The recombinant virus of the application is capable of transferring a nucleic acid sequence into a mammalian cell. The term, "nucleic acid sequence", refers to any nucleic acid molecule, preferably DNA, as discussed in detail herein. The nucleic acid molecule may be derived from a variety of sources, including DNA, cDNA, synthetic DNA, RNA or combinations thereof. Such nucleic acid sequences may comprise genomic DNA which may or may not include naturally occurring introns. Moreover, such genomic DNA may be obtained in association with promoter regions, poly A sequences or other associated sequences. Genomic DNA may be extracted and purified from suitable cells by means well known in the art. Alternatively, messenger RNA (mRNA) can be isolated from cells and used to produce cDNA by reverse transcription or other means.

Encapsidation Defective Adenoviruses (edAds)

[0076] One aspect of this application relates to an encapsidation defective adenovirus (edAd) for the production of recombinant virus vectors. In some embodiments, the edAd comprises one or more mutations in its genome that results in (1) significantly reduced production or non-production of one or more encapsidation essential proteins, and/or (2) production of one or more defective encapsidation essential proteins. Such mutations may include substitution, insertion or deletion of nucleotides in the relevant genes. Such mutations may also include substitution, insertion or deletion of nucleotides in the regulatory region of relevant genes which result in the downregulate or eliminant the transcription of these genes.

[0077] The edAd also include the type of adenovirus with a temperature sensitive mutation which can package its genome at the permissive temperature but not at the non-permissive temperature. The production of this category of edAd are therefore carried out at the permissive temperature. The application of these edADs for producing recombinant vectors are therefore performed at the non-permissive temperature that does not support edAD packaging but still facilitate the recombinant viral vector production.

[0078] The adenovirus described here can be any adenovirus subtypes or from any species. Here we main use human adenovirus type 5 for the purpose of clarity and convenience.

[0079] As further described below, the edAd of the present application can be used for the production of other recombinant viruses.

[0080] FIGs. 1A and 1B depict adenoviral particles and their structural components. FIG. 1C shows adenoviral transcriptional units, including polypeptides coded therefrom. The late transcriptional unit encodes proteins that are mutation targets for creating an encapsidation defective Ad (edAd). As shown in FIG. 2, infection of a host cell with an edAd can allow for adenovirus DNA replication, but does not result in packaged adenovirus viral particles.

[0081] Table 1 lists a number of adenoviral gene products necessary for producing an encapsidated viral genome, along with their corresponding protein accession numbers. Nucleic acid accession numbers corresponding to these proteins include, but are not limited to those set forth in GenBank gi numbers 209842, 58478, or 2935210, and/or annotated in GenBank accession numbers M73260, X17016, or AF030154.

Table 1

Protein name	Protein identifier	Transcription unit
capsid protein IX	YP_068021.1	IX
encapsidation protein IVa2	YP_068022.1	IVa2
protein 13.6K	YP_001661328.1	L1
encapsidation protein 52K	YP_068025.1	L1
capsid protein precursor pIIIa	YP_068026.1	L1
penton base (capsid protein III)	YP_068027.1	L2
core protein precursor pVII	YP_068028.1	L2
core protein V	YP_068029.1	L2
core protein precursor pX	YP_068030.1	L2
capsid protein precursor pVI	YP_068031.1	L3
hexon (capsid protein II)	YP_068032.1	L3
protease	YP_068033.1	L3
hexon assembly protein 100K	YP_068035.1	L4
protein 33K	YP_068036.1	L4
encapsidation protein 22K	YP_068037.1	L4
capsid protein precursor pVIII	YP_068038.1	L4
protein UXP	YP_068047.1	U
fiber (capsid protein IV)	YP_068048.1	L5

[0082] The list of gene products necessary for encapsidation includes capsid protein IX, encapsidation protein IVa2, protein 13.6, encapsidation protein 52K, capsid protein precursor pIIIa, penton base (capsid protein III), core protein precursor pVII, core protein V, core protein precursor pX, capsid protein precursor pVI, hexon (capsid protein II), protease, hexon assembly protein 100K, protein 33K, encapsidation protein 22K, capsid protein precursor pVIII, protein UXP, and fiber (capsid protein IV). As used herein, these gene products constitute gene products essential for encapsidation (or “encapsidation essential gene products”) and are to be distinguished from other adenovirus gene products that are not directly associated with encapsidation, as further described below. In addition, the edAds of the present application are distinguished from adenoviral genomes containing mutations in *cis*-acting sequences necessary for encapsidation. In addition to loss of functions for proteins involved in the adenovirus encapsidation, temperature sensitive mutations of these proteins are also useful for the production of recombinant viral vectors. The encapsidation defective adenoviruses have three major features for recombinant viral vector production: 1. Avoid transfection by infection of edAd. 2. Replicate edAd genomes and increase the copies of genes that can help recombinant vector production yield. 3. Reduce or eliminate the generation of packaged adenovirus in the production host cells. The replication property can be maintained by keeping the early genes necessary such as (E1, E2, E4) in the edAd or complete the edAd without them with a host line with such genes integrated; for example edAd-1ag can replicate in 293 cells; edad-2g can replicate in 911E4 cell line while edAd-dp3 can replicate in any cell line that adAd-dp3 can infect without producing packaged adenovirus.

[0083] In one embodiment, the host cells for production of recombinant viral vectors lack the receptors for adenovirus. The adenovirus receptors such as integrins, CAR, CD46, Cd80, Cd86 and other adenovirus receptors can be expressed in the host cells to make them compatible for adenovirus infection.

[0084] In some embodiments, the edAd of the present application contains a virus genome having a mutation in one or more encapsidation essential genes and the mutation results in the non-production of an encapsidation essential gene product or the production of a defective encapsidation essential gene product. Exemplary mutation(s) include deletion(s), insertion(s), sequence replacement(s), and substitution(s) in the nucleic acid sequence(s) encoding any of the encapsidation essential adenovirus product(s) and/or encapsidation non-essential adenoviral gene products. As used herein, substitution mutations include temperature-sensitive mutations.

[0085] FIG. 3, panels B-D show various edAds, each differing in the extent to which adenovirus genes are deleted and/or replaced with other sequences, including for example, transgenes or defective viral genomes. Thus, the edAd genome may additionally comprise one or more mutations preventing expression of one or more adenovirus gene product(s) that are not directly associated with encapsidation, i.e. “encapsidation non-essential” adenoviral gene products. Ad encapsidation non-essential products include, for example, proteins expressed from the adenoviral E1 transcriptional unit (*e.g.,* E1A, E1B-19K, E1B-55K); proteins expressed from the adenoviral E2/E2A transcriptional unit (*e.g.*, Iva2, pol, pTP, DBP); proteins expressed from the adenoviral E3 transcriptional unit (*e.g.*, E3 CR1 alpha 0, E3 gp19, E3 14.7 K, E3 RID-beta); and proteins expressed from the E4 transcriptional unit (*e.g.*, E4 34K, E4 ORF1, E4 ORFB, E4 ORF3, E4 ORF4, E4 ORF6/7).

[0086] In one embodiment, the edAd comprises a mutation preventing expression of Ad pIIIa. In other embodiments, the edAd comprises mutations preventing expression of other encapsidation essential proteins.

[0087] In some embodiments, the edAd further includes one or more mutations preventing expression of one or more Ad encapsidation non-essential protein(s), including but not limited to deletions or sequence replacements in E1 alone (FIG. 4, panels B, C, D, E); deletions or sequence replacements in E3 alone (FIG. 4, panel A); deletions or sequence replacements in both E1 and E3 (FIG. 3, panels C, D and FIG. 4, panels B, C, D, E); and deletions in E1, E2, and E3 (FIG. 3, panel D and FIG. 4, panels D and E).

[0088] In yet another embodiment, the edAd comprises a mutation preventing expression of Ad hexon assembly protein 100K. In other embodiments, the edAd with 100k deletion comprises mutations preventing pIIIa. In further embodiment, edAd comprises mutations preventing expression of other encapsidation essential proteins. The edAd further includes one or more mutations preventing expression of one or more Ad encapsidation non-essential protein(s), including but not limited to deletions or sequence replacements in E1 alone; deletions or sequence replacements in E3 alone; deletions or sequence replacements in both E1 and E3; and deletions in E1, E3, and E4.

[0089] In some embodiments, certain adenoviral gene sequences are replaced with defective viral genomes engineered to express a desired target gene as shown in FIG. 4, panels A-E. In certain preferred embodiments, the defective viral genome is from AAV or a lentivirus, such as HIV-1, HIV-2 or SIV.

[0090] Alternatively, or in addition, an adenoviral gene sequence may be replaced with an expression cassette for expressing *e.g.*, a recombinase enzyme, such as Cre (*see e.g.*, FIG. 4, panel C) or Flp to provide activation of one or more helper gene products as further described below. In some embodiments, the Ad genome in the edAd is engineered to include cis-acting DNA signals for recombination (*e.g.*, loxP, FRT etc.) so as to regulate expression of one or more helper functions. For example, the Ad genome in an edAd can be modified to include a "loxed fragment" containing loxP sites flanking a fragment in an intron within a Rep-Cap expression unit stably integrated into host cells such that loxed fragment serves as substrate for cre-mediated excision resulting in activation of AAV Rep/Cap expression. To facilitate this recombination (and accompanying deletion), packaging cells may be transfected with edAd genomic DNA or producer cells infected with edAd virus particles may further include stably or transiently transfected recombinase genes, such as Cre recombinase (with loxP) or Flp recombinase (with FRT).

[0091] In another embodiment, the edAd comprises a mutation preventing expression of Ad hexon. In yet another embodiment, the edAd comprises a mutation preventing expression of Ad penton. In further embodiment, the edAd comprises a mutation preventing expression of Ad fiber protein. In other embodiments, the edAd comprises mutations preventing expression of other encapsidation essential proteins as shown in Table 1.

[0092] In one embodiment, the edAd has defective encapsidation essential proteins which make intact empty capsids and defective in packaging adenovirus genome. In another embodiment, the edAd has defective encapsidation essential proteins which make defective empty capids and defective in packaging adenovirus genome. In a further embodiment, the edAd has defective encapsidation essential proteins which does not empty capsids.

Methods for Producing edAds

[0093] Another aspect of the present application relates to a method for producing the edAd of the present application. In some embodiments, the method comprises the steps of (a) infecting a packaging cell with an edAd to generate an infected packaging cell, (b) incubating the infected packaging cell under conditions that allow reproduction of the edAd, and (c) harvesting reproduced edAd, wherein the packaging cell is capable of producing one or more gene products that allow encapsidation of edAd within the packaging cell.

[0094] In some embodiments, the method comprises: (a) transfecting an edAd genome into a packaging cell to produce a transfected packaging cell, the edAd genome comprising one or more mutations that prevent the expression of one or more adenoviral encapsidation essential gene product(s) or result in the expression of one or more defective adenoviral encapsidation essential gene product(s); (b) culturing the transfected packaging cell under conditions that allow for production of infectious edAd particles; and (c) harvesting the infectious edAd particles, wherein the packaging cell is capable of producing the one or more functional adenoviral encapsidation essential gene products that allow encapsidation of edAd within the packaging cell.

[0095] The edAd genome can be generated with technologies well known in the art. Any appropriate molecular biology and biochemical method (e.g., nucleic acid sequencing) can be used to identify the presence and location of a deletion introduced into an adenovirus nucleic acid. For example, nucleic acids can be separated by size using gel electrophoresis to confirm that portions have been removed relative to the length of the original nucleic acid. In some cases, antibodies that recognize various epitopes on the encoded polypeptide (e.g., a fiber polypeptide) can be used to detect the presence or absence polypeptides targeted for deletion.

[0096] In one embodiment, edAd is generated from selected adenovirus plasmid such as pFG140, pTG3602, pAdEasy-1, pAdEasy-2 (from addgene) using standard molecular biology technology such homologous recombination, restriction enzyme digestion (include cas9/gRNA), cre-mediated recombinant to create the desired mutation and insertion in adenovirus genomes. For example, pIIIa gene is replaced by homologous recombination as previously published by M Barry's group (Crosby CM, Barry MA. IIIa deleted adenovirus as a single-cycle genome replicating vector. Virology. 2014; 462-463:158–165. doi:10.1016/j.virol.2014.05.030). Alternatively, hexon gene is partially deleted by using designed gRNA and cas9 digestions. The AAV genomes and other genes are introduced into adenovirus genome by ligation or using pshuttle vector and cre recombinase in appropriate bacteria. The resulting adenovirus plasmids are rescued in the host cells with transfected plasmid or integrated gene which supply the missing elements that are essential for adenovirus replication and packaging. The recued edAd is then be expanded using a corresponding packaging cell line.

Packaging Cell Lines for edAd

[0097] In another aspect, the present invention provides a packaging cell line for producing infectious edAds. In some embodiments, the packaging cell is capable of supplying one or more gene products that allow replication and encapsidation of an edAd within the host packaging cell.

[0098] The packaging cell can be generated by transient or stable transfection or infection for the production of one or more gene products that allow encapsidation of the edAd within the packaging cell. The packaging cell can be derived from any suitable cell line. Exemplary producer cell lines for use with the methods described herein include, but are not limited to 293, 911E4, A549, PER.C6, Hela, BHK, COS and CHO. In some embodiments, the packaging cell is derived from a mammalian cell. In some embodiments, the packaging cell is derived from a 293 cell. In another embodiments, the packaging cell is derived from a Hela cell. In another embodiments, the packaging cell is derived from a CHO cell.

[0099] In some embodiments, the packaging cell is capable of expressing or expresses an essential encapsidation gene product that is lacking or defective in the edAd genome. In some embodiments, the packaging cell is capable of expression or expresses an essential encapsidation gene product and a non-essential encapsidation gene product that are lacking or defective in the edAd genome.

[0100] In yet another embodiment, edAd with temperature sensitive mutation can be grown at a permissive temperature. Examples of temperature sensitive mutations include, but are not limited to, a deletion in the E3 region as described in Isolation and phenotypic characterization of human adenovirus type 2 temperature-sensitive mutants. Martin GR, Warocquier R, Cousin C, D'Halluin JC, Boulanger PA. J Gen Virol. 1978 Nov;41(2):303-14. Temperature-sensitive (ts) mutants may fail to grow at 39"5 °C but develop normally at 33 °C. Complementation tests in doubly infected cell cultures at restrictive temperature can be used to identify them. They are characterized phenotypically according to their soluble capsid antigen production. They may be defective in soluble hexon production, total penton (penton base+fibre), and other gene that prevent them from packaging virus. This property can complement edAd for AAV production.

[0101] The packaging cell may be produced by transfection or viral infection. In some embodiments, the packaging cell line is stably transformed with one or more adenoviral gene products complementing the one or more defects in the edAd genome (i.e., mutations affecting encapsidation essential and encapsidation non-essential products).

Method for Producing a Recombinant Virus with edAd

[0102] Another aspect of the present application relates to a method for producing a recombinant virus (RV). The method comprises the steps of: (a) infecting a host cell with one or more edAds to produce an infected host cell, (b) incubating the infected host cell under conditions that allow production of a RV having a RV genome; and (c) harvesting the recombinant virus, wherein either the edAd or the host cell comprises the RV genome.

[0103] The RV can be a DNA virus, RNA virus, poliovirus, poxvirus, retrovirus, Sindbis virus, an alphavirus, astrovirus, coronavirus, orthomyxovirus, papovavirus, paramyxovirus, parvovirus, picornavirus, poxvirus, togavirus or any other virus, including serotypes thereof, and pseudotypes thereof.

[0104] In some embodiments, the RV is a recombinant autonomous parvovirus. The recombinant parvovirus can be any member of autonomous parvovirus. Exemplary parvovirus include minute virus of mice (MVM), H1, LuIII, B19, CPV, Boca virus, FPV and others. An edAd carrying at least one of the elements from selected NS, VP genes, inducing gene (for example cre, tet-on, tet-off etc) or vector genomes, can be used to infect the host cells which will supply the elements that are missing from edAd from the parvovirus replication and packaging. The host cells will supply the missing adenovirus early genes for the replication of adenovirus genome as well.

Production of recombinant AAV vectors

[0105] In some embodiments, the RV is a recombinant AAV. The recombinant AAV can be any serotype. Exemplary AAV serotypes include AAV-1, AAV-2, AAV-3, AAV-4, AAV-5, AAV-6, AAV-7, AAV-9, AAV-10, AAV-11, AAV-12, and AAV-13. An edAd carrying at least one of the elements from selected aav-rep-cap, adenovirus E1, E2, or E4; or inducing elements (such as cre, cas9/gRNA, tet-on, tet-off) or vector genomes, can be used to infect the host cells which will supply the elements that are missing from edAd from the AAV replication and packaging. The host cells will supply the missing adenovirus early genes for the replication of adenovirus genome as well.

[0106] A conventional way for producing replication defective viral particles for gene delivery is to perform a double or triple transfection of plasmids. By way of example, **FIG. 6** shows a method for producing infectious, replication defective rAAVs in cells (such as 293 cells) stably transformed to express the helper functions specified by E1a and E1b. One plasmid contains a defective viral genome containing essential *cis*-acting sequences for replication, such as ITRs or LTRs, wherein a desired transgene or target gene of interest is inserted between flanking ITRs or flanking LTRs for expression thereof. The other plasmids are designed to express other helper functions necessary for producing the rAAV, such as AAV Rep, AAV Cap, Ad E2a, VARNA and E4. **FIG. 7** shows that when Ad helper function are provided by infection of host cells with a wtAd (or packaging competent Ad, pcAd), the recovered virus particles include both rAAVs and wtAd.

[0107] In some embodiments, recombinant AAV vectors are produced using the edAd of the present application. Nucleic acid molecules encoding edAd include all the naturally-occurring sequences of an adenovirus (e.g., an Ad5 virus) with the exception that it lacks all or a portion of at least of one of the following adenovirus sequences: fiber protein-encoding sequence, V protein-encoding sequence, hexon-encoding sequence, penton base-encoding sequence, pIII protein-encoding sequence, or other early or late gene product-encoding sequences. Examples of adenoviral nucleic acid sequences that encode polypeptides include, without limitation, those set forth in GenBank gi numbers 209842, 58478, or 2935210, and/or annotated in GenBank accession numbers M73260, X17016, or AF030154.

[0108] In some embodiments, a mutation or deletion of all or a portion of the nucleic acid encoding one or more of the following polypeptides are engineered into a nucleic acid encoding an adenovirus: fiber protein-encoding sequence, V protein-encoding sequence, hexon-encoding sequence, penton base-encoding sequence, VA RNA-encoding sequence, pIII protein-encoding sequence, or other early or late gene product-encoding sequences. Such deletions can be any length that results in the deletion of one or more encoded amino acids. For example, portions of a nucleic acid sequence of an adenovirus can be removed such that an encoded polypeptide lacks 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, or more amino acid residues). The portion or portions to be deleted can be removed from any location along the length of the sequence. For example, a portion of an adenovirus nucleic acid sequence can be removed at the 5′ end, the 3′ end, or an internal region of an adenovirus nucleic acid such as a fiber protein-encoding sequence, V protein-encoding sequence, hexon-encoding sequence, penton base-encoding sequence, VA RNA-encoding sequence, pIII protein-encoding sequence, or other early or late gene product-encoding sequences.

[0109] Any appropriate molecular biology and biochemical method (e.g., nucleic acid sequencing) can be used to identify the presence and location of a deletion introduced into an adenovirus nucleic acid. For example, nucleic acids can be separated by size using gel electrophoresis to confirm that portions have been removed relative to the length of the original nucleic acid. In some cases, antibodies that recognize various epitopes on the encoded polypeptide (e.g., a fiber polypeptide) can be used to detect the presence or absence polypeptides targeted for deletion.

[0110] In some embodiments, AAV vectors are produced using an adenovirus as a helper. Currently there are two ways of using adenovirus helper for AAV production. One is to use adenovirus DNA, which relies on a transfection method and it is not scalable. The other is to wild type (wt) adenovirus or adenoviruses with deletions in the E1a/E1b region. This results in contamination of adenovirus with encapsidated DNA, which is very difficult to be removed from the AAV preparations.

[0111] The edAd provides one or more necessary helper functions for AAV production which include E1a, E1b, VA RNA, E2, E4 and other adenovirus genes. The edAd complements AAV replication and packaging while edAd itself will not produce adenovirus with encapsidated genome in the AAV production host cells.

[0112] In some embodiments, the edAd are further modified to have more features that can complement AAV production in difference conditions. E1/E1b or E3 can be deleted to make edAd1g, which allows AAV vector genomes to be carried in edAd1g and other factors essential for the production system. For example, cre, FLP and crispr elements can also be carried by the said adenovirus. Further deletions in adenovirus genome can be made to accommodate other essential elements. Those elements essential for AAV production can be integrated into the host cells.

[0113] In some embodiments, the AAV genomes are constructed into the edAd. This is desirable in some situations and the AAV genome copies will be increased dramatically when edAD undergoes extensive replications. Other genes that may be important for inducing rep and cap expression may be constructed into edAd. In some embodiments, AAV genomes are cloned into edAd. In other embodiments, genes that used to control integrated host genes are built into edAd as well. Such genes include Cas9/gRNA or other variant of crispr enzymes, cre recombinase, FLP recombinanse and other proteins that can regulate gene expression.

[0114] In some embodiments, edAd is used for AAV vector production. The AAV production host cells are infected with an edAd. Elements which are essential for AAV production but not carried by the edAd are supplied in *trans* by transfection or be integrated into the production host cells. The AAV production host cells do not have the factor/elements to restore edAd packaging so that no adenovirus genome are packaged to adenovirus capsids.

[0115] The edAd virus may have different configurations. For example, in 293 cells based host cells, edAd1g can be used since E1a and E1 can be removed as it is supplied in the host cells. In contrast, for production in host cell based on CHO, E1a and E1b region will have to be included in the edAD viruses.

[0116] Also provided herein are host cells for producing rAAV virions. In certain embodiments, a host cell of the present invention comprises a nucleic acid encoding AAV helper functions. Upon introduction of an AAV vector and expression of accessory functions in the host cell, rAAV virions are produced. In certain preferred embodiments, a host cell of the present invention also includes one or more accessory functions.

[0117] The present invention further provides methods of using accessory function vectors to produce rAAV and the rAAV virions produced by such methods. In certain embodiments, a method of the present invention includes the steps of (1) introducing an AAV vector genome into a suitable host cell; (2) introducing an AAV helper virus of the present invention into the host cell; (3) expressing accessory functions in the host cell; and (4) culturing the host cell to produce rAAV virions. The AAV vector and AAV helper function vector can be transfected into the host cell, either sequentially or simultaneously, using well-known techniques. Accessory functions may be expressed in any of several ways, including infecting the host cell with a suitable helper virus (such as adenovirus, herpesvirus, or vaccinia virus), or by transfecting one or more accessory function vectors into the host cell. It is also well known in the art that certain cell lines, e.g., 293 cells, inherently express one or more accessory functions.

[0118] The rAAV virions produced using the present invention may be used to introduce genetic material into animals, including humans, or isolated animal cells for a variety of research and therapeutic uses. For example, rAAV virions produced using the methods of the present invention may be used to express a protein in animals to gather preclinical data or to screen for potential drug candidates. Alternatively, the rAAV virions may be used to transfer genetic material into a human to cure a genetic defect or to effect a desired treatment.

[0119] The general method for using the edAd to produce parvovirus vectors are following this procedure: in cells that have been transfected with AAV rep&cap or AAV vector plasmids, edAd is used to deliver the required helper functions for AAV production. The vectors can then harvested in the media or in the cells at various time. The rep&cap function can be integrated into the host cells or delivered by viral or non-viral vectors. The AAV vector plasmids can also be integrated into the host cell lines or delivered by a viral vectors. In one particular embodiment, the AAV vector sequence is in the genome of edAd. Multiple edAds to carry additional factors can be for rAAV reproduction if necessary.

[0120] In some embodiments, the infectious edAd virus particles are co-expressed with one or more helper functions for replication of AAV (*e.g.,* AAV Rep and/or AAV Cap) in the producer cell line. To facilitate replication of the AAV, the producer cells are transfected with or otherwise include an AAV vector construct comprising essential *cis*-active sequences *e.g*., AAV ITRs for production of replication-defective AAV particles. The AAV Rep and Cap genes can be stably integrated into the producer cell line or they may be delivered by viral or non-viral vectors. The AAV vector plasmids can also be integrated into the producer cell line or delivered by viral or non-viral vectors. In a particular embodiment, the AAV vector sequences are cloned into edAd DNA construct for producing the adAd virus particles. Multiple edADs can be used for producing a given rAAV or a plurality of different rAAVs.

[0121] In a particular embodiment, a method for producing a rAAV comprises infecting a host cell with an edAd, wherein the host cell is stably transformed to express AAV Rep and Cap, and is stably transformed with a defective AAV genome engineered to express a desired target gene as shown in **FIG. 8**.

[0122] In another embodiment, a method for producing a rAAV comprises infecting a host cell with an edAd, where the edAd genome comprises a defective AAV genome engineered to express a desired target gene, and where the host cell (such as Hela or A549) is stably transformed to express the Ad E1a, Ad E1b, AAV Rep, and AAV Cap proteins as shown in **FIG. 9**. In some embodiments, the edAd is edAd-100k.

[0123] In another embodiment, a method for producing a rAAV comprises infecting a host cell with an edAd, where the edAd genome comprises a defective AAV genome engineered to express Cre recombinase and a desired target gene of interest, and where the host cell is stably transformed with the Ad E1, E2 and E4 genes, and is stably transformed to express AAV Rep, and AAV Cap proteins as shown in **FIG. 11**. In this case, expression of Cre recombinase from the edAd excises a "loxed fragment" in an intron built into a Rep-Cap expression unit to activate expression of Rep-Cap proteins. The loxed fragment contains loxP sites flanking a fragment in the intron that serves as substrate for cre-mediated excision resulting in activation of AAV Rep/Cap expression.

[0124] The AAV viruses provided herein can be used in a wide range of MOI from 0.01 to 10^6. It can be used to infect the cells before for after induction of other elements for viral vector production.

Other Recombinant Viruses

[0125] In some embodiments, the RV is a recombinant lentivirus. In some embodiments, the recombinant lentivirus is HIV-1, HIV-2 or SIV.

[0126] In one embodiments, the RV is a recombinant retrovirus. In some embodiments, the recombinant lentivirus is Moloney murine leukemia virus.

[0127] In some embodiments, the RV is a recombinant vaccinia virus.

[0128] In some embodiments, the RV is a recombinant herpes virus. Exemplary herpesviruses include, but are not limited to, herpes simplex virus (HSV)-1, HSV-2, human herpesvirus (HHV)-1, HHV-2, HHV-3, HHV-4, HHV-7, HHV-8, and varicella zoster, human cytomegalovirus (HCMV).

[0129] To produce a stock infectious replication defective recombinant virus particles (also referred to as virus vectors), such as recombinant AAV (rAAV), recombinant lentiviruses, and others, the edAds are used in combination with structural elements from the recombinant virus (*e.g.*, AAV, lentiviruses etc.) to produce infectious viral particles corresponding to the recombinant virus. Unlike other methodologies involving double or triple transfection of plasmids, infection of host cells with one or more edAds significantly increases the transduction efficiencies resulting in higher titers and little or no contamination.

[0130] In some embodiments, the producer cells are stably transformed with nucleic acids engineered to express one or more Ad helper functions, one or more AAV helper functions, or a combination thereof. In a particular embodiment, the host cells are 293 cells. In other embodiments, the host cells are Hela cells or A549 cells.

[0131] In some embodiments, the producer cells are stably transformed with nucleic acids engineered to express Ad E1a and Ad E1b. In other embodiments, the producer cells are stably transformed with nucleic acids engineered to express AAV Rep and/or AAV Cap. In other embodiments, the producer cells are stably transformed with nucleic acids engineered to express one or more helper functions essential for producing infectious lentivirus particles. In other embodiments, the producer cells are stably transformed with a defective virus genome engineered to express a target gene of interest.

[0132] In some embodiments, the producer cells are infected with a plurality of different edAds. In one embodiment, each of edAd in the plurality expresses one or more helper functions.

[0133] In one embodiment, producer cells are co-infected with a first edAd engineered to express a Cre recombinase and a second edAd comprising a Rep-Cap expression unit comprising a "loxed fragment" in an intron that serves as substrate for Cre-mediated excision resulting in activation of AAV Rep/Cap expression.

[0134] In another embodiment, one or more helper gene(s) may be activated for expression using the Clustered Regularly Interspaced Short Palindromic Repeat/Cas (CRISPR/Cas9) system. By this system, Cas9 nucleases can be directed by short RNAs to induce precise cleavage at specific sites in DNA, and can edit multiple sites in the genome by allowing for coding of several sequences in a single CRISPR array. A single Cas enzyme can be programmed by a short RNA molecule (referred to as the "guide" RNA) to recognize a target DNA. In other words, the Cas enzyme can be recruited to a specific target DNA using short RNA molecules as guide RNAs to provide for specificity of the CRISPR-mediated nucleic acid cleavage. Any other crispr systems such as cpf1,cas13 et., are also included in this invention

[0135] There are three CRISPR types, the most commonly used type for gene correction or disruption to date is type II. For example, the CRISPR RNA targeting sequences are transcribed from DNA sequences clustered within the CRISPR array. In order to operate, the CRISPR targeting RNA, or precursor crRNA (pre-crRNA), is transcribed and the RNA is processed to separate the individual RNAs (crRNAs) dependent on the presence of a trans-activating CRISPR RNA (tracrRNA) that has sequence complementarity to the CRISPR repeat. When the trans RNA hybridizes to the CRISPR repeat, it initiates processing by the double-stranded RNA specific ribonuclease, RNAse III, forming tandem tracrRNA: crRNA duplexes, which can be synthetically made as single guide RNAs (sgRNAs) for genome engineering purposes. The Cas9 nuclease, which is activated and specifically responds to the DNA sequence complementary to the crRNA by cleaving it. A target sequence must contain a specific sequence on its 3' end, called the protospacer adjacent motif (PAM) in the DNA to be cleaved which is not present in the CRISPR RNA that recognizes the target sequence.

[0136] In addition to the naturally occurring guide RNAs, synthetic guide RNAs can be fused to a CRISPR vector. The design of guide RNAs with target-recognition sequences and other essential elements (e.g., hairpin and scaffold sequence) using bioinformatics methods is described (see, e.g., Mali et al., Science 339: 823-826 (2013)).

[0137] In one embodiment, producer cells are co-infected with a first edAd encoding a functional Type II CRISPR-Cas9 protein and a second edAd encoding a guide RNA sequence targeting one or more helper gene(s) for CRISPR-mediated activation of the one or more helper gene(s) analogous to the Cre/lox system above.

[0138] In a variation of the above-described method, the CRISPR-Cas9 system may be used with the edAds of the present application to provide a means for deletion of host sequences, replacement of mutated host sequences with wild-type host sequences, or targeted activation of a host gene. In this variation, host cells are co-infected with a first edAd comprising a defective virus genome engineered to express a functional Type II CRISPR-Cas9 protein, and a second edAd comprising a guide RNA sequence targeting a host gene for CRISPR-mediated deletion of host sequences, replacement of mutated host sequences with wild-type host sequences, or targeted activation of a host gene.

[0139] In some embodiments, the present application provides a method for producing a recombinant lentivirus capable of infecting a non-dividing cell, which comprises infecting a suitable producer cell with one or more edAd carrying the packaging functions encoded by gag, pol and env, as well as rev and tat. For example, a first edAd virus can provide a nucleic acid encoding a viral gag and a viral pol and the same edAd or another edAd can provide a nucleic acid encoding a viral env to produce a producer cell. Introducing a defective lentivirus vector engineered to express a heterologous gene, herein identified as a transfer vector, into that producer cell yields a producer cell which releases infectious viral particles carrying the foreign gene of interest.

[0140] In one embodiment, the edAd can be used to produce recombinant herpes vector. The essential gene for herpes vector production can be carried using an edAd and infect the herpes vector production cell to facilitation herpes vector production.

[0141] *Herpesvirus (HSV) complementation system.* Current HSV-based designs generally comprise two replication-deficient HSV strains engineered to individually harbor Rep/Cap and AAV vector sequences. One of ordinary skill can appreciate that the encapsidation defective herpes can be used for producing rAAV or lentiviral vectors or other recombinant vector similarly. The encapsidation defective and packaging defective herpes virus can be made by creating mutations or deletions in herpes structural genes. The encapsidation defective and packaging defective herpes can be used to produce RV-utilizing methods disclosed herein may be adapted to HSV-based systems.

[0142] In view of the foregoing, the instant application provides compositions and methods for producing high titer recombinant virus. The virus particle preparations can be used to infect target cells using techniques known in the art. Thus the instant application will find use in both *in vivo* gene therapy applications, as well as *ex vivo* gene therapy applications, where target cells are removed from a host, and transformed in culture, and then returned back to the host.

Producer Cell Lines

[0143] Another aspect of the present application relates to a producer cell for the production of a recombinant virus. The producer cell comprises (a) a genome of the recombination virus, or (b) genes encoding products required for the production of the recombination virus, or both (a) and (b).

[0144] The producer cell can be generated by transient or stable transfection or infection. In some embodiments, the producer cell line is stably transformed with (a) a genome of the recombination virus, or (b) genes encoding products required for the production of the recombination virus, or both (a) and (b).

[0145] The producer cell can be derived from any suitable cell line. Exemplary producer cell lines for use with the methods described herein include, but are not limited to 293, 911E4, Hela, COS, A549, CHO, PER.C6 and BHK. In some embodiments, the producer cell is derived from a mammalian cell. In some embodiments, the producer cell is derived from a 293 cell. In another embodiments, the producer cell is derived from a Hela cell. In another embodiments, the producer cell is derived from a CHO cell.

[0146] The producer cell line is used in conjunction with its corresponding edAd for viral vector production. Typically, the missing essential early genes from edAd is complemented with integrated copy in the producer cell line so edAd can replicate adenovirus genomes but still cannot package adenovirus genomes because of the defects in encapsidation genes.

[0147] one or more viral gene products corresponding to a second virus that are necessary for producing infectious, replication-defective virus particles corresponding to the second virus. More specifically, infection of the producer cell line with the edAds results in the production of infectious, replication-defective virus particles corresponding to the second virus. Given that the producer cell line is infected with edAd virus particles, the producer cell line must be permissive for or genetically modified to support entry and/or replication of adenovirus particles. Methods for producing packaging cell lines are well known in the art and require no further discussion.

[0148] The present application is further illustrated by the following examples that should not be construed as limiting. The contents of all references, patents, and published patent applications cited throughout this application, as well as the Figures and Tables, are incorporated herein by reference.

Examples

Example 1: Exemplary edAd genomes

[0149] FIG. 3, panel B-D and FIG. 4, panel A-E show several examples of edAd genomes. The edAd genomes can be generated using standard recombinant DNA technologies well known in the art. The starting adenovirus 5 plasmids based edAd are pTG3602, pFG140, pAdeasy plasmids. The desired genes to be deleted such as pIIIa, hexon, penton, fiber etc. are digested with designed cas9/gRNA and then resulting fragment are ligated by self-ligation. The obtaining plasmids are rescued in the corresponding host cells with missing adenovirus encapsidation genes. Further addition of other components for viral vectors is carried out by homologouse recombinantion or cre-mediated recombination using a shuttle vector in the bacteria or in the corresponding cell lines compatible for rescuing edAd.

Example 2: Production of edAd

[0150] FIG. 5 shows two exemplary methods for the production of edAd. For each edAd, a special production cell line is made so that gene(s) missing or defective in the edAd are expressed in the production cell line.

Generation of edAd-d100k

[0151] edAd-d100k was produced as following: pAdEasy-1 is digested with BamHI, and the subfragment containing Ad5 sequences 21696 to 35995 was isolated and subcloned into pUC19 at BamHI, yielding pUC19-Ad-BamHI. The pUC19-Ad-BamHI/Δ100K was made by removing the NheI fragment of the 100K gene (Ad5 sequences 24999 to 25686) from pUC19-Ad-BamHI. The adenovirus fragment containing the deletion in 100k gene was then released from the pUC19-Ad-BamHI/Δ100K plasmid with BamHI and ligated to back to the large BamHI subfragment of pAdEasy-1 to obtain pAd-easy-d100. Adenovirus edAd-d100k was then rescued in a cell line 293-100k.

[0152] Generation of 293-100k cells

[0153] The cell line 293-100k was generated by cloning chemically synthesized 100k gene in pcDNA3 (pcDNA3-100k) under the control of CMV promoter. Two micrograms of the pcDNA3-100K plasmid was linearized with ClaI restriction enzyme digestion and transfected into 293 cells by the lipofectamine. Transfected cells were selected in DMEM medium with G-418 at 600 μg/ml. The obtained clones were confirmed by PCR and western blot. The 293-100k clone was confirmed for its ability to support the growth of the temperature-sensitive (ts) Ad5 100K mutant, H5ts116 (initially made by H. Ginsberg) at the nonpermissive temperature of 39°C.

[0154] Generation of edAd-E1-d100k

[0155] edAd-d100k was used to infect Hela cell expressing 100k, which is transfected with the left arm fragment of Ad5 (cla I fragment). The plaque is selected to confirm the presence E1 and 100k deletion.

[0156] Generation of edAd-d100k-cre

[0157] A SalI fragment encompassing the cre gene was ligated into the SalI site of pShuttleCMV, generating pShuttleCMVcre. The cre-carrying shuttle plasmid was linearized with PmeI and coelectroporated with pAd-easy-d100 into Escherichia coli BJ5183. In this manner, targeted recombination between the two plasmids generated the full-length edAd-d100-cre vector genome which is $E1^-$, $E3^-$ and $100k^-$, within a bacterial plasmid. Similarly, the pShuttle-AAV-CMVlacZ plasmid was coelectroporated with pAdEasy-1 to generate the [$E1^-$, $E3^-$]Ad-AAV-CMV-lacZ-containing plasmid. These adenoviruses were then rescued from 293-100k cells.

Example 3: Production of recombinant AAV vectors with edAd

[0158] Generation of IIIa encapsidation deficient adenovirus for rAAV production

[0159] The starting material is pTG3602 which contains full length Ad5 genome (JOURNAL OF VIROLOGY, July 1996, p. 4805–4810 CHARTIER et al). The E3 or E1 and E3 regions were removed by using typical molecular biology techniques to obtain pAd-d3 or pAd-d13. The IIIa region was removed from the plasmid by using CRISPR nuclease digestion and self ligation of the plasmids pAd-d3 or pAd-d13 to obtain pdIIIa-d3 (**SEQ ID NO:1**) and pdIIIa-d13 (**SEQ ID NO:2**) so the IIIa functions were removed from adenovirus. The resulting constructs were confirmed by sequencing before they were used for rescuing infectious adenovirus in 293-IIIa.

[0160] To provide IIIa in trans, the Ad5 IIIa cDNA was used to generate a 293 stable cell line (293-IIIa). Ad5 with IIIa deletion was rescued by transfection and propagation in 293-IIIa cells.

[0161] The IIIa deletion in pdIIIa-d3 and pdIIIa-d13 were similar to Ad6-IIIa deletion as described by Crosby CM and Barry MA in Virology. 2014 Aug; 462-463:158-65. doi: 10.1016/j.virol.2014.05.030. Epub 2014 Jul 2.

[0162] Function tests were using similar method described by Crosby CM and Barry MA (supra).

[0163] To confirm that IIIa deleted adenoviruses were competent for AAV production, AAV expression cassette with GFP gene flanked by AAV ITR was cloned into pIIIa-d3 or pAd-d13 in the E1 region (may use E3 or other regions) and rescued the infectious IIIa-d13-AAV-GFP **(SEQ ID NO:3)** and IIIa-d3-AAV-GFP in 293-IIIa cell line. AAV vectors were produced in the following conditions. IIIa-d13-AAV-GFP virus DNA was amplified 1E+5 fold during production without producing infectious adenovirus.

TEST ID	Transfection Components (plasmids)	Infection Components (adenovirus)	Host Cells	rAAV yield (vg/cell	Infectious adenovirus produced	misc
1	pAd pRepCap pAAV-GFP	none	293	~1E(+3)-1E(+6)	none	
2	pRepCap pAAV-GFP	Wt Ad	293	~1E(+2)-1E(+4)	+++++	
3	pRepCap pAAV-GFP	IIIa-d13	293	~1E(+3)-1E(+6)	none	
4	pRepCap pAAV-GFP	IIIa-d3	Hela	~1E(+3)-1E(+5)	none	
5		IIIa-d3-AAV-GFP	B50 (Hela w/Rep&Cap)	~1E(+4)-1E(+6)	none	
6		IIIa-d13-AAV-GFP	293-RepCap	~1E(+4)-1E(+6)	none	

[0164] One limitation of rAAVs is that their genome-packaging capacity is only ~5 kb. For certain diseases the packaging limit of AAV does not allow the delivery of a full-length therapeutic protein by a single AAV vector. In view of the limitations imposed by the packaging capacity of AAV, dual-vector approaches may be employed, whereby a transgene is split across two separate rAAV vectors. Co-infection of a cell with these two rAAVs can then result in the transcription of an assembled mRNA that could not be encoded by a single AAV vector, because of the DNA packaging limits of AAV. One of ordinary skill will understand that the presently disclosed methods can be adapted to produce such dual vectors for expressing transgenes that exceed the packaging capacity of adeno-associated virus capsids. B50 cell line is transfected with pAAV-CB-EGFP and the cell clone with resueable AAV-CB-EGFP is named as B50-AAV-CB-EGFP, which has both AAV rep & cap sequences and AAV genomes. edAd-E1-d100k is then used to infect B50-AAV-CB-EGFP at a moi 1, 5 and 10.

[0165] Generation of edAd-d100k-dp3

[0166] To make 100k and pIIIa double deletions in adenovirus, pAd-easy-d100 was digested with cas9/gRNA which releases the pIIIa gene fragment. The resulting large fragment was self-ligated to obtain pAd-easy-d100dp3. The edAd-d100k-dp3 was rescued and amplified in 293-100k-pIIIa cell line.

[0167] Generation of edAd-d100k-dp3-cre

[0168] Plasmid pshuttle-CMV-Cre was made and used for recombination with pAd-easy-d100dp3 using an ad-easy kit. The resulting adenovirus was rescued and amplified in 293-100k-pIIIa cell.

[0169] AAV production using edAd-d100k-dp3-cre

[0170] Cell line with integrated AAV genome and cre-activable rep and cap, 293-dsGFP-12 (provided by Xiao Xiao), is infected edAd-d100k-dp3-cre.

<u>Example 4: Production of recombinant lentivirus vectors with edAd</u>

[0171] The present application also provides method for producing recombinant lentivirus capable of infecting non-dividing cells as well as methods and means for making same. The virus is useful for the in vivo and ex vivo transfer and expression of nucleic acid sequences.

[0172] The lentiviral genome and the proviral DNA have the three genes found in retroviruses; gag, pol and env, which are flanked by two LTR sequences. The gag gene encodes the internal structural (matrix, capsid and nucleocapsid) proteins; the pot gene encodes the RNA-directed DNA polymerase (reverse transcriptase), a protease and an integrase; and the env gene encodes viral envelope glycoproteins. The 5' and 3' LTR's serve to promote transcription and polyadenylation of the virion RNA's. The LTR contains all other cis-acting sequences necessary for viral replication. Lentiviruses have additional genes including vif, vpr, tat, rev, vpu, nef and vpx (in HIV-1, HIV-2 and/or SIV).

[0173] Adjacent to the 5' LTR are sequences necessary for reverse transcription of the genome (the tRNA primer binding site) and for efficient encapsidation of viral RNA into particles (the Psi site). If the sequences necessary for encapsidation (or packaging of retroviral RNA into infectious virions) are missing from the viral genome, the cis defect prevents encapsidation of genomic RNA. However, the resulting mutant remains capable of directing the synthesis of all virion proteins.

[0174] The present application provides a method of producing a recombinant lentivirus capable of infecting a non-dividing cell comprising infecting a suitable host cell with one or more edAd carrying the packaging functions, namely gag, pol and env, as well as rev and tat. For example, a first edAd virus can provide a nucleic acid encoding a viral gag and a viral pol and the same edAd or another edAd can provide a nucleic acid encoding a viral env to produce a packaging cell. Introducing a vector providing a heterologous gene, herein identified as a transfer vector, into that packaging cell yields a producer cell which releases infectious viral particles carrying the foreign gene of interest.

[0175] A lentiviral vector described herein may be packaged by three non-overlapping expression constructs, two expressing HIV proteins and the other the envelope of a different virus. Moreover, all HIV sequences known to be required for encapsidation and reverse transcription are absent from the constructs, with the exception of the portion of the gag gene that contributes to the stem-loop structure of the HIV-1 packaging motif.

[0176] A second strategy to improve vector biosafety takes advantage of the complexity of the lentiviral genome. The minimal set of HIV-1 genes required to generate an efficient vector was identified and all the other HIV reading frames were eliminated from the system. As the products of the removed genes are important for the completion of the virus life cycle and for pathogenesis, no recombinant can acquire the pathogenetic features of the parental virus. All four accessory genes of HIV could be deleted from the packaging construct without compromising gene transduction. The tat gene is crucial for HIV replication. The tat gene product is one of the most powerful transcriptional activators known and plays a pivotal role in the exceedingly high replication rates that characterize HIV-induced disease.

[0177] Examples of retroviral-derived env genes include, but are not limited to Moloney murine leukemia virus (MoMuLV or MMLV), Harvey murine sarcoma virus (HaMuSV or HSV), murine mammary tumor virus (MuMTV or MMTV), gibbon ape leukemia virus (GaLV or GALV), human immunodeficiency virus (HIV) and Rous sarcoma virus (RSV). Other env genes that may be used include vesicular stomatitis virus (VSV) protein G (VSV-G), as well as those of hepatitis- and influenza viruses.

[0178] The vector providing the viral env nucleic acid sequence is associated operably with regulatory sequences, *e.g.*, a promoter or enhancer. The regulatory sequence can be any eukaryotic promoter or enhancer, including for example, the Moloney murine leukemia virus promoter-enhancer element, the human cytomegalovirus (HCMV) enhancer or the vaccinia P7.5 promoter. In some cases, such as the Moloney murine leukemia virus promoter-enhancer element, the promoter-enhancer elements are located within or adjacent to the LTR-sequences.

[0179] Preferably, the regulatory sequence is one which is not endogenous to the lentivirus from which the vector is being constructed. Thus, if the vector is being made from SIV, the SIV regulatory sequence found in the SIV LTR would be replaced by a regulatory element which does not originate from SIV.

[0180] While VSV C protein is a desirable env gene because VSV G confers broad host range on the recombinant virus, VSV G can be deleterious to the host cell. Thus, when a gene such as that for VSV G is used, it is preferred to employ an inducible promoter system so that VSV G expression can be regulated to minimize host toxicity when VSV C is expression is not required.

[0181] For example, the tetracycline-regulatable gene expression system of Gossen & Bujard (Proc. Natl. Acad. Sci. (1992) 89:5547-5551) can be employed to provide for inducible expression of VSV G when tetracycline is withdrawn from the transferred cell. Thus, the tet/VP16 transactivator is present on a first vector and the VSV C coding sequence is cloned downstream from a promoter controlled by tet operator sequences on another vector.

[0182] Such a hybrid promoter can be inserted in place of the 3' U3 region of the LTR of a transfer vector. As a result of transduction of target cells by the vector particles produced by the use of such a transfer vector, the hybrid promoter will be copied to the 5' U3 region on reverse transcription. In the target cells, such a conditional expression of a gene can be activated to express full-length packageable vector transcripts only in the presence of tTA--for example, after transduction of an appropriate packaging cell line expressing tTA.

[0183] Use of such vectors in producer cells allows one to “turn on” the production of the packageable vector mRNA messages at high levels only when needed. In contrast, on transduction of cells which do not express tTA, the hybrid promoter becomes transcriptionally silent. Such transcriptional silence was maintained even in the presence of HIV Tat protein, which is known to be capable of upregulating basal transcriptional activity of heterologous promoters. The promoter system significantly reduces the chance of mobilization of the vector genome even if transduced cells are infected by wild type HIV-1.

[0184] Another embodiment relates to a retroviral vector system based on lentivirus in which sequence homology (sequence overlap) between coding sequences of packaging and transfer vector constructs is eliminated. Importantly, vector particles produced by the use of such constructs retain high levels of transduction potential. Use of such constructs in a vector production system is expected to most significantly decrease the frequency of recombination events, which is a significant advance in biosafety associated with such a vector system.

[0185] It is known that throughout the gag-pol coding mRNA, several *cis*-acting repression sequences (CRS) are present. The sequences prevent transport of mRNA's to the cell cytoplasm and therefore prevent encoded protein expression. To suppress the action of CRS, HIV-1 mRNA's contain an anti-repression signal called RRE to which Rev protein may bind. HIV-1 mRNA-Rev complexes then are efficiently transported to the cell cytoplasm where the complex dissociates and mRNA becomes available for translation.

[0186] At least two approaches are available for choosing the minimal amounts of HIV sequences necessary in Gag and Gag-Pol expressing packaging vectors. First, only the gag-pol gene could be inserted. In that case, all, or at least most of the CRS will need to be identified and mutated without effecting the encoded amino acid sequence. If that is accomplished, the Rev gene can be eliminated from the vector system.

[0187] Second, the minimal RRE element can be introduced to the gag-pol expression cassette so that the sequence thereof will be part of the resulting mRNA. In that case, expression of Gag and Gag-Pol polyproteins will require presence of the anti-repressor, Rev. Rev protein itself, however, does not need to be part of the gag-pol expression vector but could be provided *in trans* from independent and, preferably, non-overlapping with the gag-pol expression cassette.

[0188] In the system where Rev protein is not required for efficient production of transfer vector mRNA, the rev gene and RRE element may be eliminated from the vector system as a further biosafety measure. In such a system, however, if the gag-pol gene in whole or in part is transferred into a vector recipient as the result of a homologous or a non-homologous recombination event the expression may occur.

[0189] In contrast, a vector system in which gag-pol gene expression is dependent on Rev may be a valuable safety alternative. Thus, if a Rev utilizing vector system is designed so all of the components do not have homologous sequences, in the unlikely event of recombination, which would result in transfer the of gag-pol sequences to the vector recipient, the expression thereof is much less likely to occur since the transferred recombinant must contain both the RRE element as well as Rev coding sequence capable of being expressed.

[0190] Given that the major interest in HIV-derived vectors concerns their ability to transduce nondividing and slowly dividing cells and tissues, nonoverlapping vectors were tested for transduction in cell cycle arrested cells. In contrast to MoMLV vectors, minimal HIV-derived vectors maintained transduction potential in both dividing and growth arrested cells.

[0191] Furthermore, an HIV-1 RNA element present in the packaging vector gag-pol mRNA was observed to lead to specific encapsidation of significant amounts of the message into released vector particles under certain conditions. The element serves as the HIV-1 major splice donor site (SD) and consists of at least nucleotides, GACUGGUGAG (SEQ ID NO: 1). In the absence of transfer vector expression, vector particles generated only by pMDLg/pRRE packaging construct have no detectable gag-pol RNA message. Analysis of total RNA extracted from the cells which produced the vector particles, showed that expression levels in all cases were similar. When 5' mRNA regions of the tested packaging vectors were compared, it became apparent that the specified above sequence is the determinant which provides specific encapsidation of the messages.

[0192] Preferably, the recombinant lentivirus produced by the method of the application is a derivative of human immunodeficiency virus (HIV). However, the env is preferably derived from a virus other than HIV.

[0193] The method of the present application provides, in some embodiments, at least one edAd and up to four edAd viruses which provide all of the functions required for packaging of recombinant lentivirus virions, such as, gag, pol, env, tat and rev, as discussed above. See **FIGs. 13-17**. As noted herein, that may be deleted functionally for unexpected benefits. There is no limitation on the number of vectors which are utilized so long as the vectors are used to transform and to produce the packaging cell line to yield recombinant lentivirus.

[0194] The edAd vectors are introduced infection into the producer cell line while additional elements are integrated into the producer cells to avoid transfection. The producer cell line produces viral particles that contain the vector genome. Methods for transfection or infection are well known by those of skill in the art. After infection or co-infection of the edAd viruses to the producer cell line, the recombinant virus is recovered from the culture media and titered by standard methods used by those of skill in the art.

[0195] Stable cell lines wherein the producer functions are configured to be expressed by a suitable producer cell are known. For example, see U.S. Pat. No. 5,686,279; and Ory et al., Proc. Natl. Acad. Sci. (1996) 93: 11400-11406, which describe packaging cells. Such stable cell line will be used along with at least one edAd of the present application.

[0196] Zufferey et al supra, describes a lentiviral producer in which sequences 3' of pol including the HIV-1 env gene are deleted. The construct contains tat and rev sequences and the 3' LTR is replaced with poly A sequences. The 5' LTR and psi sequences are replaced by another promoter, such as one which is inducible. For example, a CMV promoter or derivative thereof can be used.

[0197] The producer vectors of interest contain additional changes to the packaging functions to enhance lentiviral protein expression and to enhance safety. For example, all of the HIV sequences upstream of gag can be removed. Also, sequences downstream of env can be removed. Moreover, steps can be taken to modify the vector to enhance the splicing and translation of the RNA.

[0198] To provide an edAd with an even more remote possibility of generating replication competent lentivirus, an aspect of the present application provides for lentivirus packaging edAd wherein tat sequences, a regulating protein which promotes viral expression through a transcriptional mechanism, are deleted functionally. Thus, the tat gene can be deleted, in part or in whole, or various point mutations or other mutations can be made to the tat sequence to render the gene nonfunctional. An artisan can practice known techniques to render the tat gene non-functional.

[0199] Thus, according to the present application, a lentiviral packaging edAd is made to contain a promoter and other optional or requisite regulatory sequences as determined by the artisan, gag, pol, rev, env or a combination thereof, and with specific functional or actual excision of tat, and optionally other lentiviral accessory genes.

[0200] The 5' LTR of transfer vector constructs can be modified by substituting part or all of the transcriptional regulatory elements of the U3 region with heterologous enhancer/promoters. The changes can enhance the expression of transfer vector RNA in producer cells; allow vector production in the absence of the HIV tat gene; and serve to remove the upstream wild-type copy of the HIV LTR that can recombine with the 3'deleted version to “rescue” the above described SIN vectors. Thus, vectors containing the above-described alterations at the 5' LTR, 5' vectors, can find use as transfer vectors because of the sequences to enhance expression and in combination with packaging cells that do not express tat.

[0201] Such 5' vectors can also carry modifications at the 3' LTR as discussed hereinabove to yield improved transfer vectors which have not only enhanced expression and can be used in packaging cells that do not express tat but can be self-inactivating as well.

[0202] Transcription from the HIV LTR is highly dependent on the transactivator function of the tat protein. In the presence of tat, often expressed by the core packaging construct existing in producer cells, vector transcription from the HIV LTR is stimulated strongly. Given that full-length "viral" RNA has a full complement of packaging signals, the RNA is encapsidated efficiently into vector particles and transferred to target cells. The amount of vector RNA available for packaging in producer cells is a rate-limiting step in the production of infectious vector.

[0203] The enhancer or the enhancer and promoter regions of the 5' LTR may be substituted with the enhancer or the enhancer and promoter of the human cytomegalovirus (CMV) or Rous sarcoma virus (RSV), respectively, see **FIG. 12** for a schematic of constructs and code names of the hybrid vectors. The CCL and RRL vectors have complete substitution of the 5' U3 region.

[0204] The control lentivector HR2 and the panel of 5' hybrids were compared in producer cells transfected with the transfer vector, and with or without packaging constructs, which provide the tat transactivator. The transcriptional level of the four chimeric vectors is higher than that of a control lentivector both in the presence and in the absence of the packaging construct. All chimeric vectors efficiently transfer the transgene into target cells and the RRL vector performs as well as the control HR2 vector. Finally, integration of the vector in target cells was confirmed by examining transduced cells at an early and a later passage after transduction. No decrease was observed in the percentage of transgene-positive cells indicating that the vector had been integrated.

[0205] The high level of expression of the 5' LTR modified transfer vector RNA obtained in producer cells in the absence of a packaging construct indicates that the producing vector is functional in the absence of a functional tat gene. Functional deletion of the tat gene as indicated for the packaging plasmid disclosed hereinabove would confer a higher level of biosafety to the lentiviral vector system given the number of pathogenic activities associated with the tat protein. Thus, a lentiviral vector of significantly improved biosafety is a SIN transfer vector that does not contain a d-type copy of the HIV LTR either at the 5' or at the 3' end, which is used in conjunction with tat-less packaging vectors as described herein.

[0206] Viral supernatants can be harvested using standard techniques such as filtration of supernatants 48 hours post transfection. Viral titers can be determined by infection of, for example, 10^6 NIH 3T3 cells or 10^5 HeLa cells with an appropriate amount of viral supernatant, in the presence of 8 μg/ml polybrene (Sigma Chemical Co., St. Louis, Mo.). Forty-eight hours later, the transduction efficiency can be assayed.

[0207] For illustration purpose, the invention only illustrate the use of vsv0G envelop. Pseudotyped lentiviral vectors consist of vector particles bearing glycoproteins (GPs) derived from other enveloped viruses. There is an ever-growing list of alternative GPs for pseudotyping lentiviral vectors. These GOs have been reviewed in Curr Gene Ther. 2005 Aug; 5(4): 387–398 by Cronin. Additional GPs include but not limited to LCMV, RRV, SeV F, Ebola, Marburg, HN, JSRV, Rabies, Mokola, RD114, GALV etc.. Since GPs are generally toxic, edAd can carry it for high level expression and compliment lentiviral production.

[0208] An example for generating each generation of lentiviral vectors are illustrated in Figures 13-19.

[0209] It may be desirable to target the recombinant virus by linkage of the envelope protein with an antibody or a particular ligand for targeting to a receptor of a particular cell type. By inserting a sequence (including a regulatory region) of interest into the viral vector, along with another gene which encodes the ligand for a receptor on a specific target cell, for example, the vector is now target-specific. Retroviral vectors can be made target-specific by inserting, for example, a glycolipid or a protein. Targeting often is accomplished by using an antigen-binding portion of an antibody or a recombinant antibody-type molecule, such as a single chain antibody, to target the retroviral vector. Those of skill in the art will know of, or can readily ascertain without undue experimentation, specific methods to achieve delivery of a retroviral vector to a specific target.

Example 5. Adenovirus with deletions in protein V, hexon, IVa2, L1-52/55K, and L4-22K

[0210] Three viral core proteins (V, VII, and mu) interact with and condense the viral DNA and mediate interactions between the core and the capsid. A series of minor capsid components, IIIa, VI, VIII, and IX, contribute to capsid structure and stability. The IVa2 protein binds to the CG motif and the L4-22K protein binds the TTTG motif of the packaging A repeats in vitro and in vivo. The L1-52/55K protein has only been found to be associated with the packaging domain in vivo, and the L1-52/55K protein associates with IVa2 in vitro. The IVa2 and L1-52/55K proteins are required for packaging of Ad DNA into the capsid

[0211] A new 293 cell line that stably expresses the Ad5 L1-52/55K protein is isolated following selection with Geneticin as described for 293-L1 (J Virol. 2011 Aug; 85(15): 7849–7855. doi: 10.1128/JVI.00467-11).

[0212] Similar 293 cells for V, IVa2, hexon,and L4-22K expression are generated and named as 293-V, 293-Iva2, 294-L4, 293-hexon. The adenoviruses with deletions in these regions are made by first deleting these coding regions from pAd-d3 and pAd-d13 and then rescue the infectious viruses from the corresponding cell lines.

[0213] When tested in 293 cells, Ad-dV produced reporter gene product, but never plagued. Following infection as a helper for AAV in the compatible production cell line, viral genomes with V deleted can be replicated normally. However, progeny virions lack protein V and are unable to infect actively a second set of cells after the initial infection.

[0214] These advenoviruses support AAV production similarly as outlined in example I.

Example 6. Adenovirus with fiber deletion support rAAV production

[0215] Fiber protein binding to cellular receptors is the primary mode of transduction for Adenovirus. Therefore, fiberless Ad particle has severely reduced infectivity.

[0216] To develop an Ad5 Fiber Expressing Cell Line, the Ad5 fiber sequence is codon-optimized (co) and synthesized. Codon-optimization will improve protein expression levels and significantly reduce the possibility of homologous recombination in vitro. Expression fragment with the Ad5-Fiber-co is transfected into 293 cells using Polyfect (Qiagen, Valencia, CA, USA). Twenty-four hours after transfection. Geneticin was added (0.5 mg/mL). The cell line 293-Fibr is then confirmed by western blot.

[0217] To delete the fiber from pAd-d3 and pAd-d13, the specially designed crispr nucleases are used to release the fiber gene fragment and the resulting large fragments are recircularized by DNA ligase to obtain pdFibr-d3 and pdFibr-d13. pdFibr-d3 and pdFibr-d13 are then used to rescue viruses Ad-dFibr-d3 and Ad-dFibr-d13 in 293-Fibr cell line. The yield of Ad-dFibr-d3 and Ad-dFibr-d13 in 293-Fibr cell line is normal.

[0218] Ad-dFibr-d3 and Ad-dFibr-d13 with AAV-GFP are obtained by clone AAV-GFP into pdFibr-d3 and pdFibr-d13 before using them for rescue in 293-Fibr cell line.

[0219] Testing results were:

Test ID	Transfection components	Infection components	Host Cell	rAAV yield	Infectious adenovirus produced	misc
1	pAd pRepCap pAAV-GFP		293	+++++	none	
2	pRepCap pAAV-GFP	Wt Ad	293	++	+++++	
3	pRepCap pAAV-GFP	Ad-Fibr-d13	293	+++	None (adeno particles are not infectious)	
4	pRepCap pAAV-GFP	Ad-Fibr -d3	Hela	++	None (adeno particles are not infectious)	
5		Ad-Fibr -d3-AAV-GFP	B50 (Hela w/Rep&Cap)	+++++	None (adeno particles are not infectious)	
6		Ad-Fibr -d13-AAV-GFP	293-RepCap	+++++	None (adeno particles are not infectious)	

[0220] This results showed that fiber needs to combine with another factor, such as IIIa, to obtain adenovirus particles free AAV vectors.

[0221] Thus, the breadth and scope of the subject compositions and methods should not be limited by any of the above-described exemplary embodiments, but should be defined only in accordance with the following claims and their equivalents.

[0222] The above description is for the purpose of teaching the person of ordinary skill in the art how to practice the present application, and it is not intended to detail all those obvious modifications and variations of it which will become apparent to the skilled worker upon reading the description. While various embodiments have been described above, it should be understood that such disclosures have been presented by way of example only and are not limiting. Further, it is intended that all obvious modifications and variations be included within the scope of the present application, which is defined by the following claims. The claims are intended to cover the components and steps in any sequence, which is effective to meet the objectives there intended, unless the context specifically indicates the contrary. The breadth and scope of the subject compositions and methods should not be limited by any of the above-described exemplary embodiments, but should be defined only in accordance with the following claims and their equivalents.

What is claimed is

1. An encapsidation defective adenovirus (edAd) for the production of a recombinant virus, comprising:

an edAd genome with one or more mutations that result in (1) significantly reduced production or non-production of one or more encapsidation essential proteins, and/or (2) the production of one or more defective encapsidation essential proteins.

2. The edAd of Claim 1, wherein the one or more encapsidation essential proteins are selected from the group consisting of capsid protein IX, encapsidation protein IVa2, protein 13.6, encapsidation protein 52K, capsid protein precursor pIIIa, penton base (capsid protein III), core protein precursor pVII, core protein V, core protein precursor pX, capsid protein precursor pVI, hexon (capsid protein II), protease, hexon assembly protein 100K, protein 33K, encapsidation protein 22K, capsid protein precursor pVIII, protein UXP, and fiber (capsid protein IV).

3. The edAd of Claim 1, wherein the one or more mutations comprise a mutation that results in the non-expression of capsid protein precursor pIIIa.

4. The edAd of Claim 3, further comprising one or more deletions in protein V, hexon, Iva2, L1-52/55k and/or L4-22K.

5. The edAd of Claim 3, further comprising a deletion in the fiber protein.

6. The edAd of Claim 1, wherein the one or more mutations comprise the deletion of hexon assembly protein 100K.

7. The edAd of Claim 1, further comprising one or more deletions in adenovirus early genes.

8. The edAd of Claim 7, comprising a deletion in adenovirus early gene E1.

9. The edAd of Claim 7, comprising a deletion in adenovirus early genes E1 and E3.

10. The edAd of Claim 7, comprising deletions in adenovirus early genes E1, E3 and E4.

11. The edAd of Claim 1, further comprising a sequence that encodes the genome of a recombinant AAV.

12. The edAd of Claim 11, wherein the genome of the recombinant AAV comprises an expression cassette comprising a target gene operably linked to a control sequence, wherein the expression cassette is flanked on each end with an AAV ITR.

13. The edAd of Claim 1, further comprising the coding sequence of CRE operatively linked to a control sequence.

14. The edAd of Claim 1, further comprising the coding sequence of the gag and pol proteins of a lentivirus.

15. The edAd of Claim 1, further comprising the coding sequence of the VSV-G protein of a lentivirus.

16. A method for producing a recombinant virus (RV), comprising the steps of:

(a) infecting a producer cell with one or more edAds to produce an infected producer cell, wherein the one or more edAds are capable of DNA replication, but not virus particle formation, in the producer cell and wherein either the one or more edAds, or the producer cell, comprises a RV genome;

(b) incubating the infected producer cell under conditions that allow production of a RV having the RV genome; and

(c) harvesting the RV.

17. The method of Claim 16, wherein the RV is AAV.

18. The method of Claim 17, wherein the one or more edAds comprise an AAV genome.

19. A packaging cell for producing the edAd of Claim 1, wherein the packaging cell expresses one or more gene products that allow encapsidation of the edAd within the packaging cell.

20. The packaging cell of Claim 19, wherein the one or more gene products comprise adenovirus pIIIa gene product.

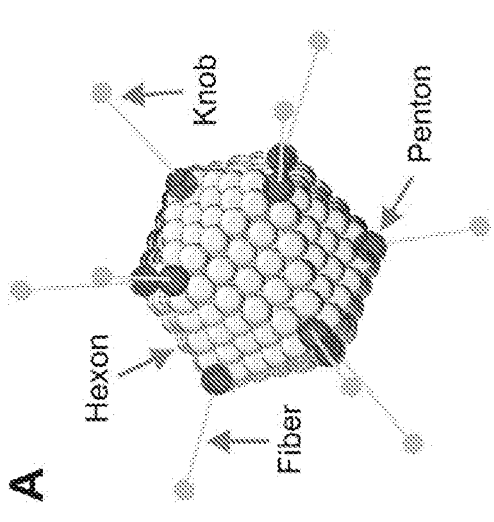


Figure 1A

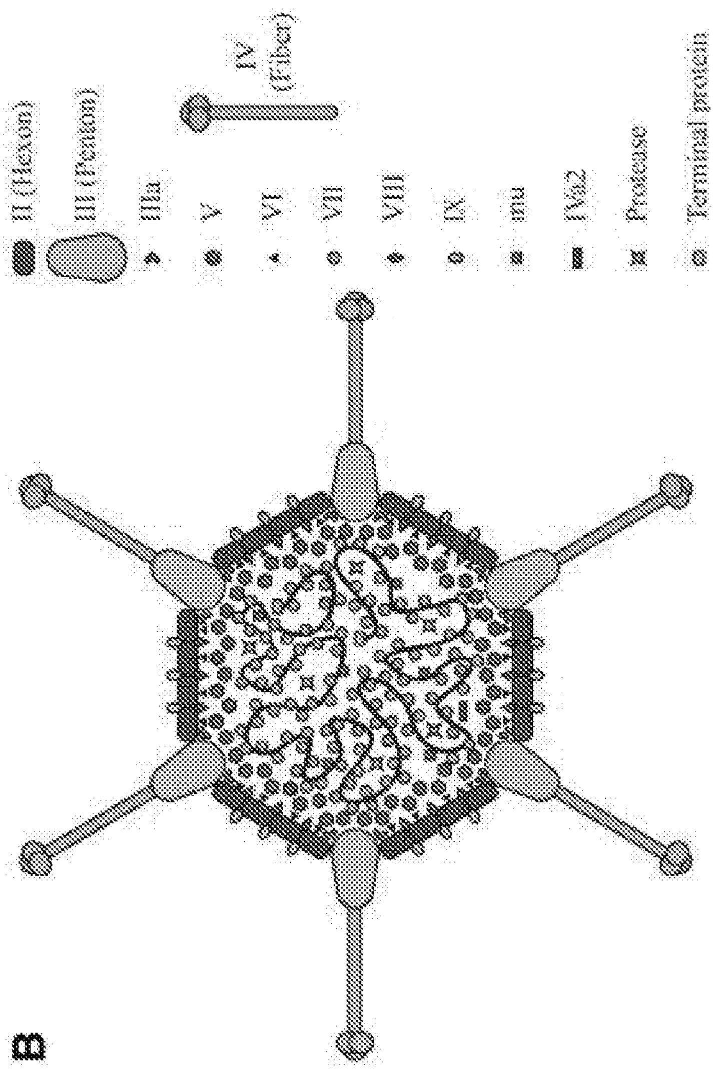


Figure 1B

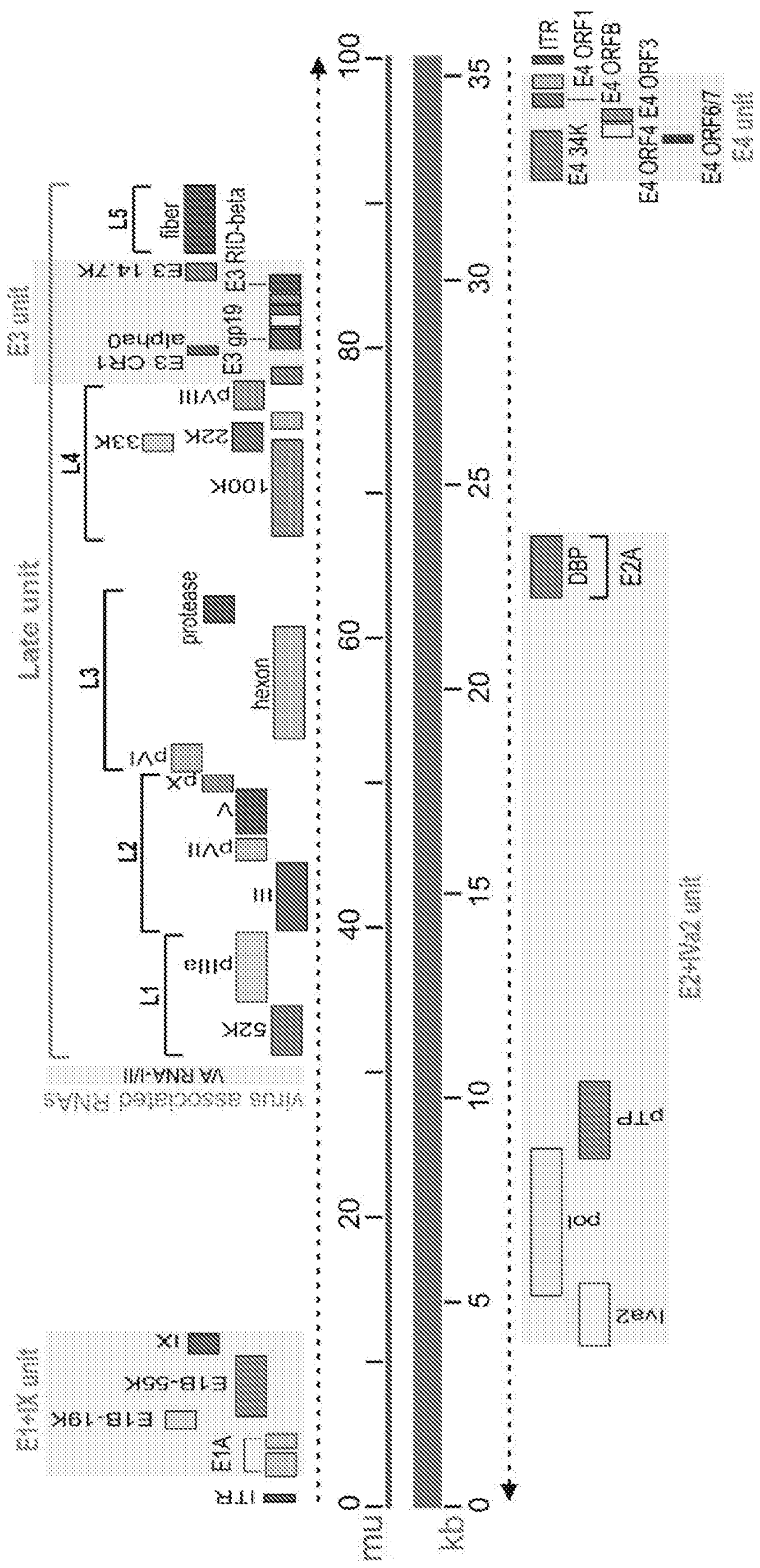

mu
kb
0
20
40
60
80
100
0
5
10
15
20
25
30
35
ITR
E1A
E1B-19K
E1B-55K
IX
E1+IX unit
virus associated RNAs
VA RNA-I/II
Late unit
L1
52K
pIIIa
L2
III
pVII
V
pX
L3
pVI
hexon
protease
L4
100K
33K
22K
pVIII
E3 unit
E3 CR1 alpha0
E3 gp19
E3 RID-beta
E3 14.7K
L5
fiber
Iva2
pol
pTP
E2+Iva2 unit
DBP
E2A
E4 34K
E4 ORF4
E4 ORF3
E4 ORF6/7
E4 ORFB
E4 ORF1
ITR
E4 unit

Figure 1C

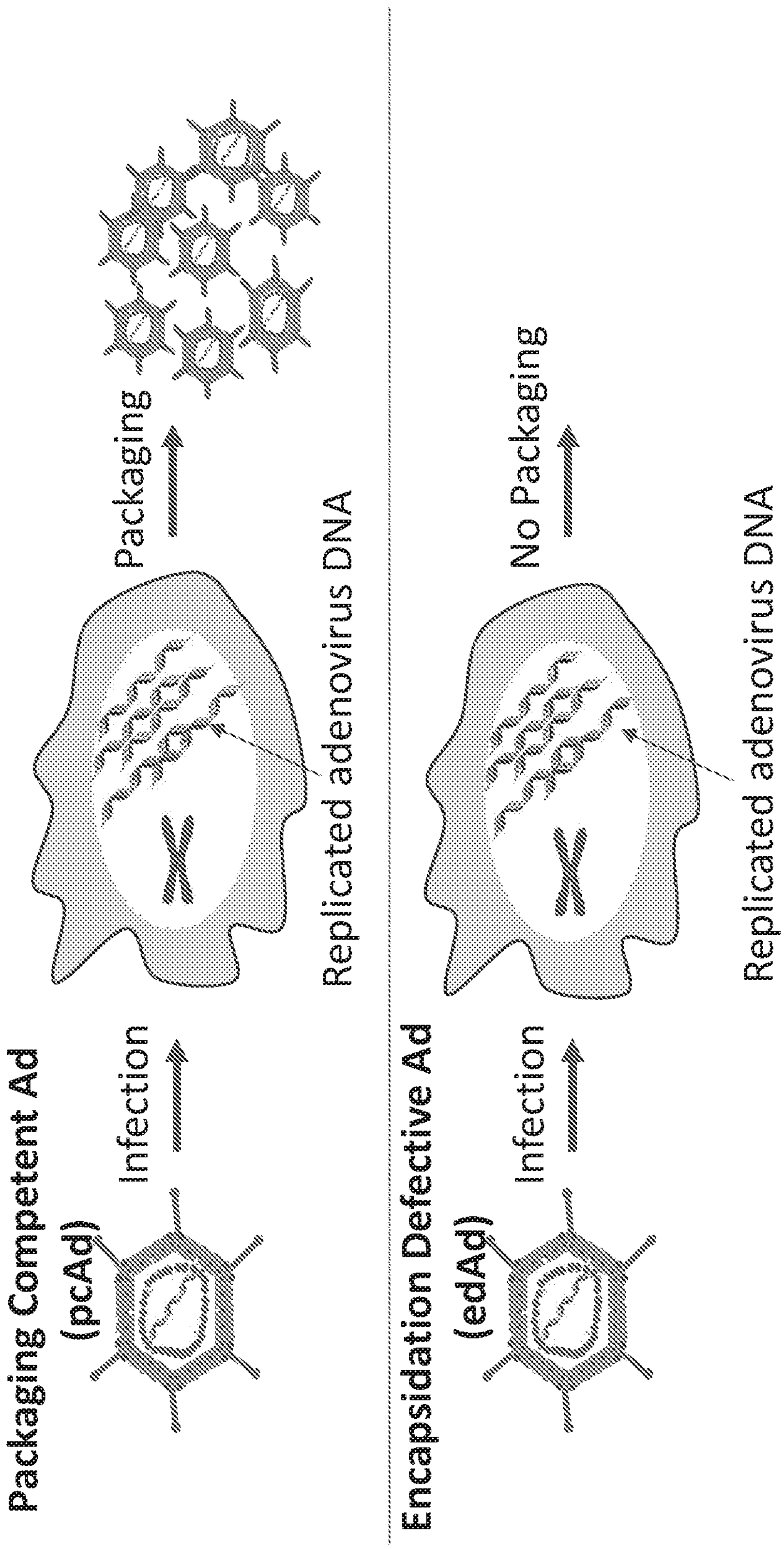

Packaging Competent Ad
(pcAd)
Infection
Replicated adenovirus DNA
Packaging
Encapsidation Defective Ad
(edAd)
Infection
Replicated adenovirus DNA
No Packaging

Figure 2

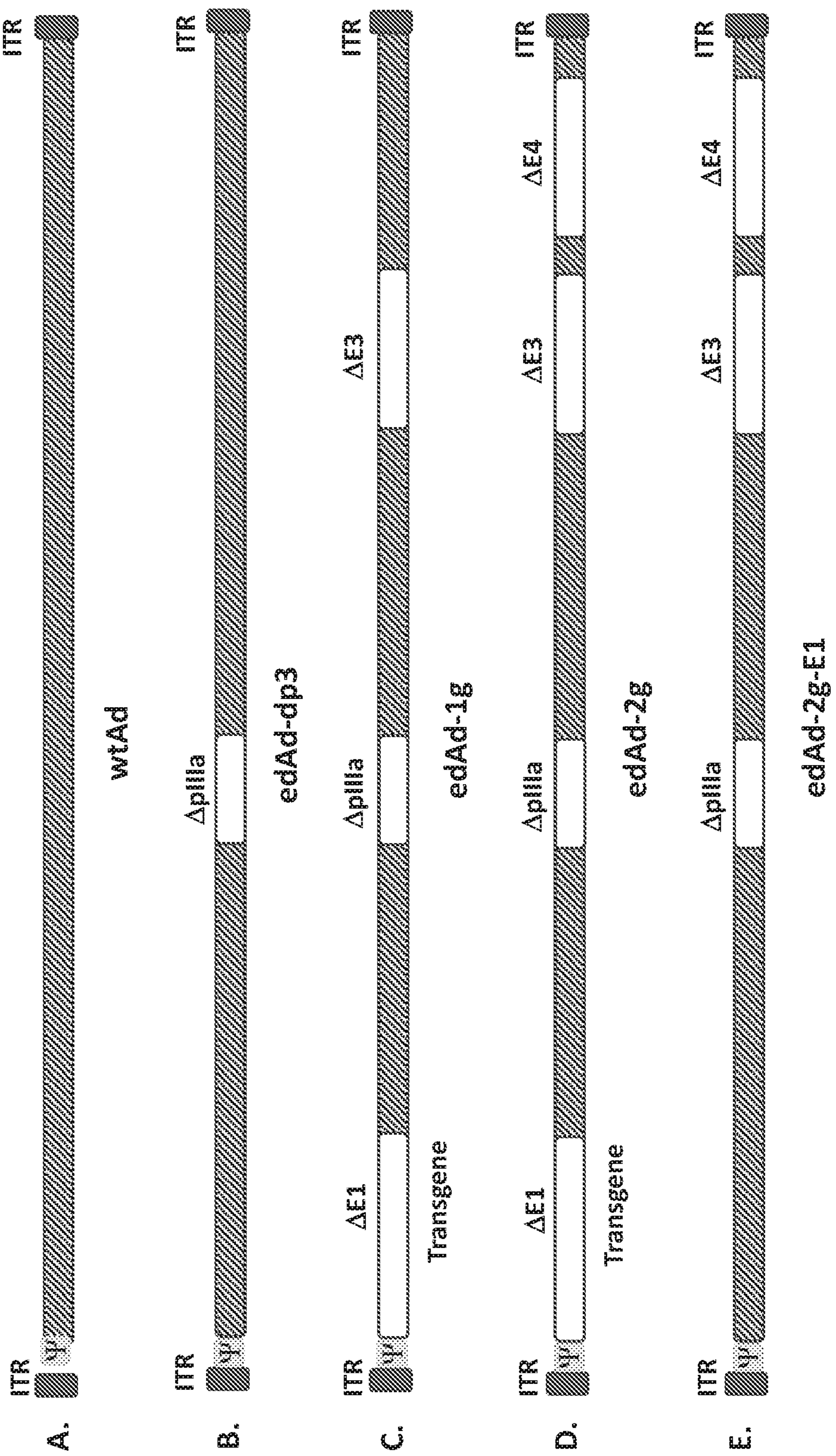

A.
ITR
Ψ
ITR
wtAd
B.
ITR
Ψ
ΔpIIIa
ITR
edAd-dp3
C.
ITR
Ψ
ΔE1
Transgene
ΔpIIIa
ΔE3
ITR
edAd-1g
D.
ITR
Ψ
ΔE1
Transgene
ΔpIIIa
ΔE3
ΔE4
ITR
edAd-2g
E.
ITR
Ψ
ΔpIIIa
ΔE3
ΔE4
ITR
edAd-2g-E1

Figure 3

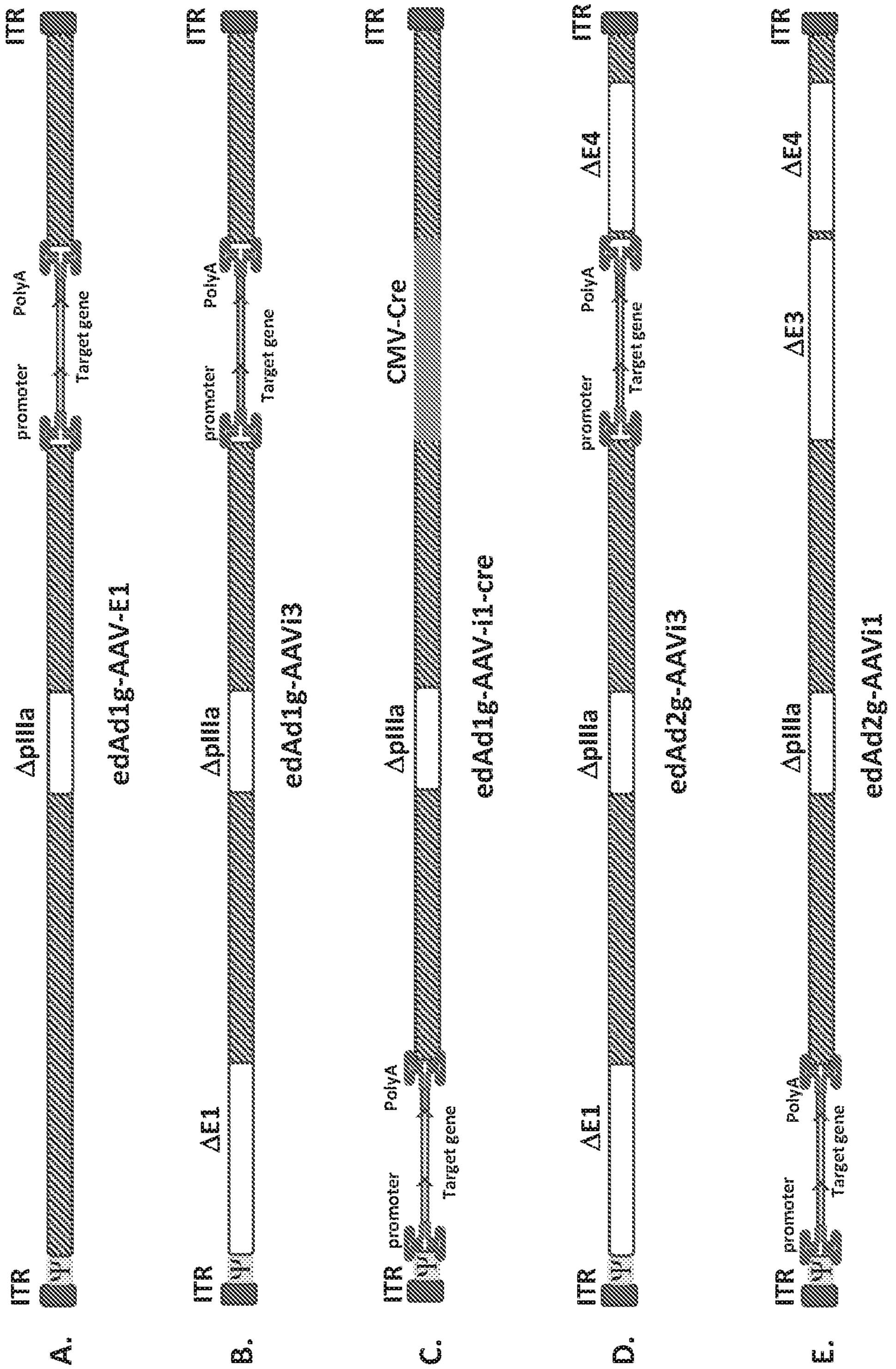

A.
ITR
Ψ
ΔpIIIa
promoter
Target gene
PolyA
ITR
edAd1g-AAV-E1
B.
ITR
Ψ
ΔE1
ΔpIIIa
promoter
Target gene
PolyA
ITR
edAd1g-AAVi3
C.
ITR
Ψ
promoter
Target gene
PolyA
ΔpIIIa
CMV-Cre
ITR
edAd1g-AAV-i1-cre
D.
ITR
Ψ
ΔE1
ΔpIIIa
promoter
Target gene
PolyA
ΔE4
ITR
edAd2g-AAVi3
E.
ITR
Ψ
promoter
Target gene
PolyA
ΔpIIIa
ΔE3
ΔE4
ITR
edAd2g-AAVi1

Figure 4

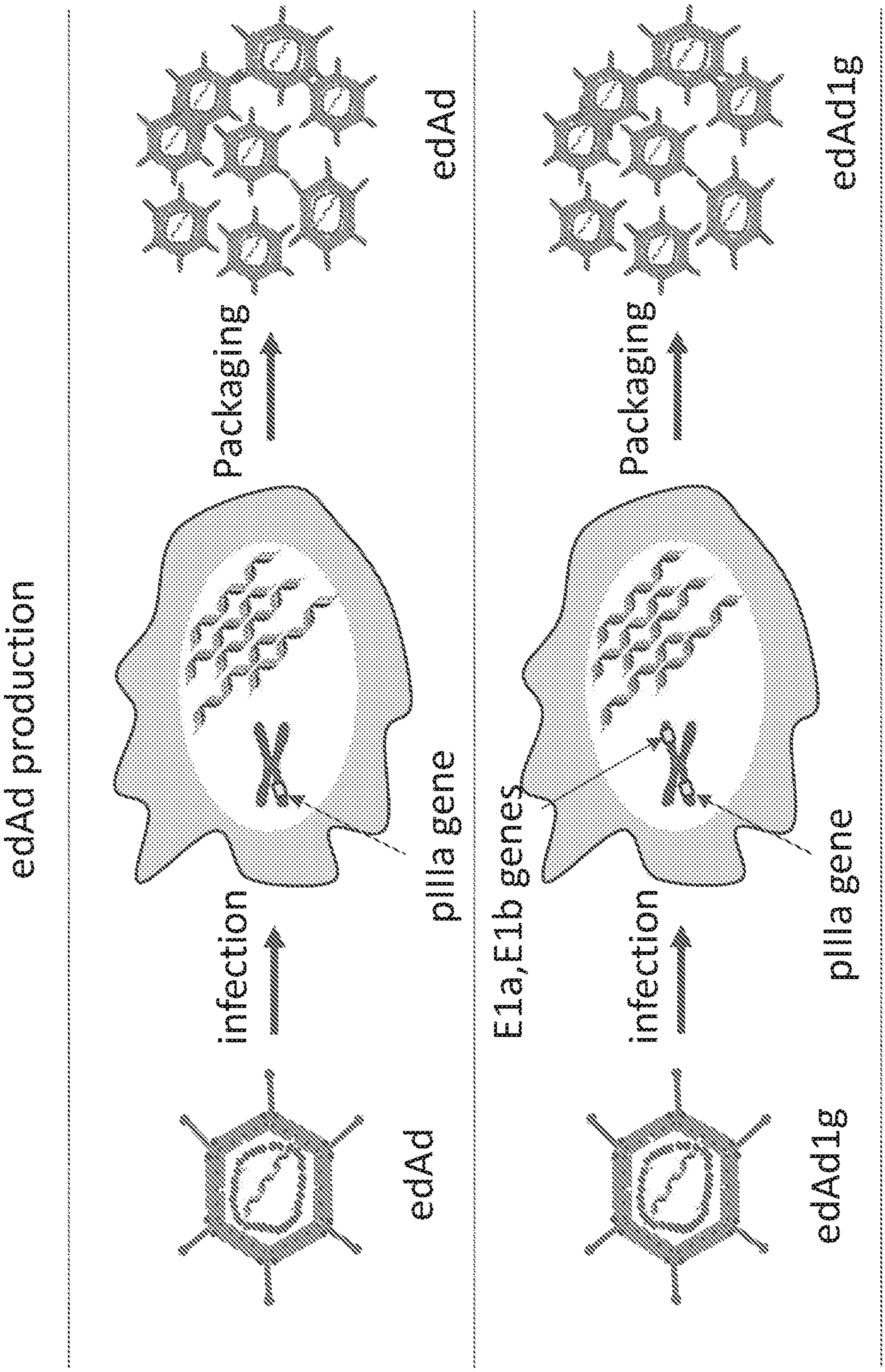

edAd production
edAd
infection
pIIIa gene
Packaging
edAd
E1a,E1b genes
edAd1g
infection
pIIIa gene
Packaging
edAd1g

Figure 5

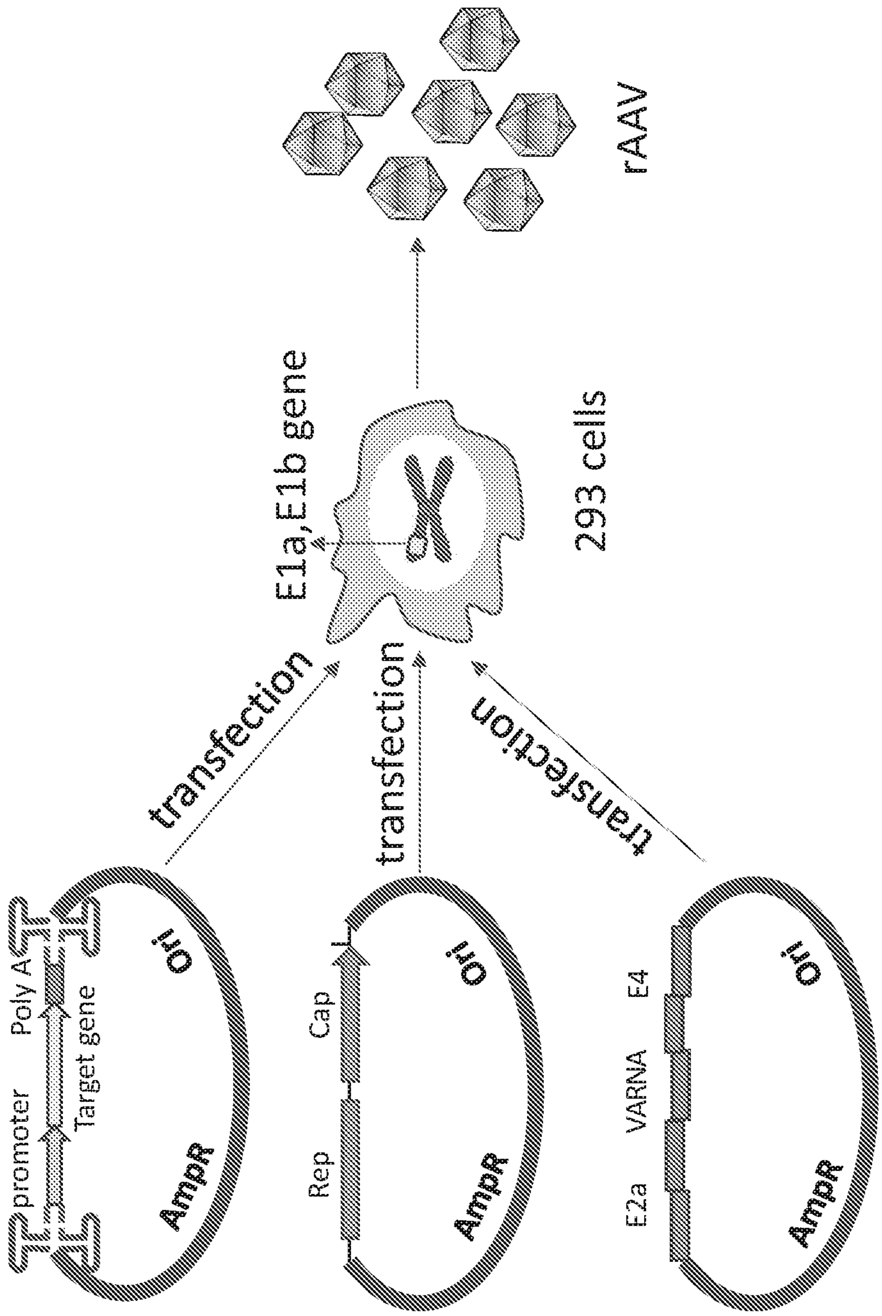

promoter
Target gene
Poly A
ori
AmpR
Rep
Cap
ori
AmpR
E2a
VARNA
E4
ori
AmpR
transfection
transfection
transfection
E1a,E1b gene
293 cells
rAAV

Figure 6

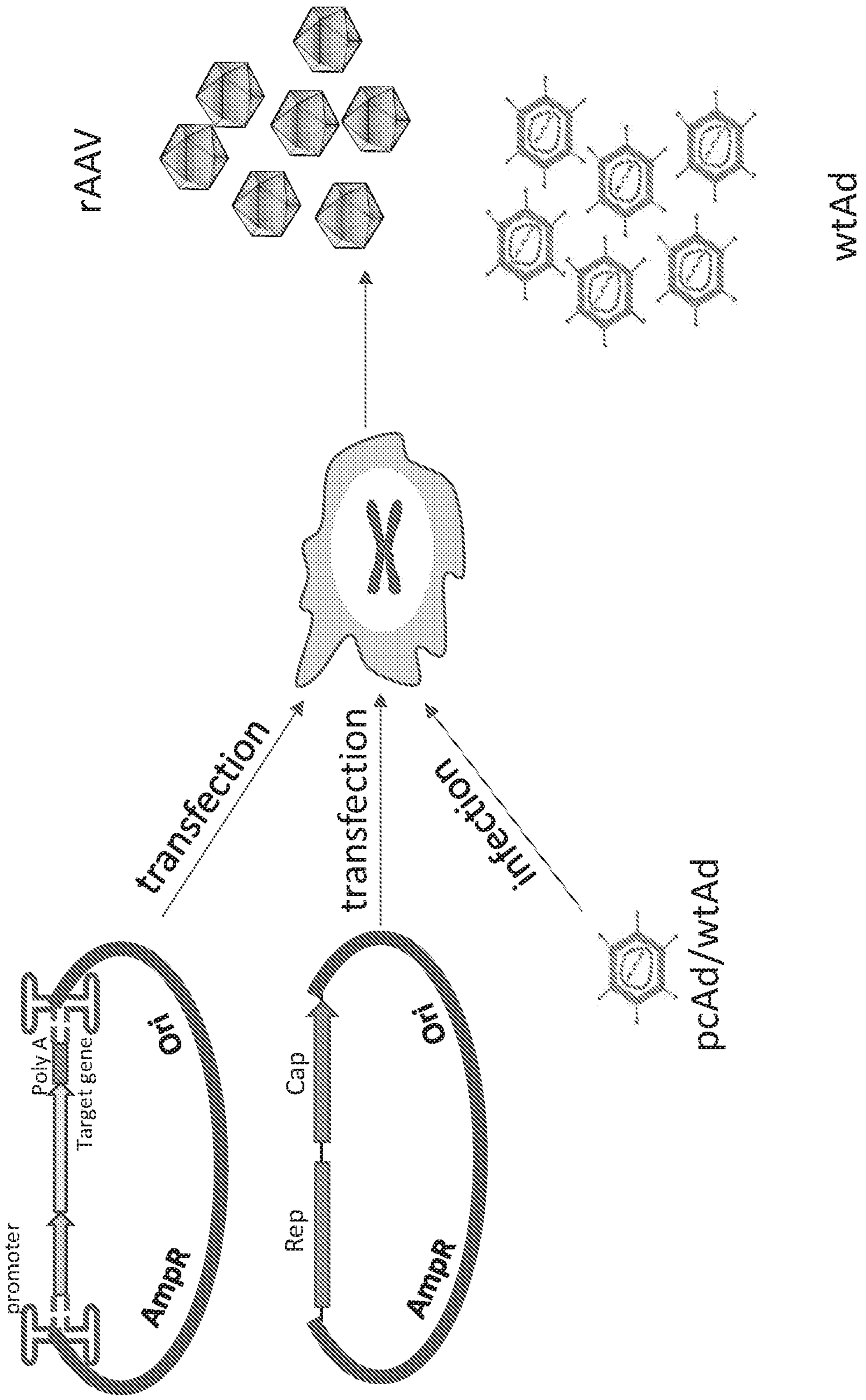

rAAV
wtAd
transfection
transfection
infection
pcAd/wtAd
promoter
Poly A
Target gene
AmpR
ori
Rep
Cap
AmpR
ori

Figure 7

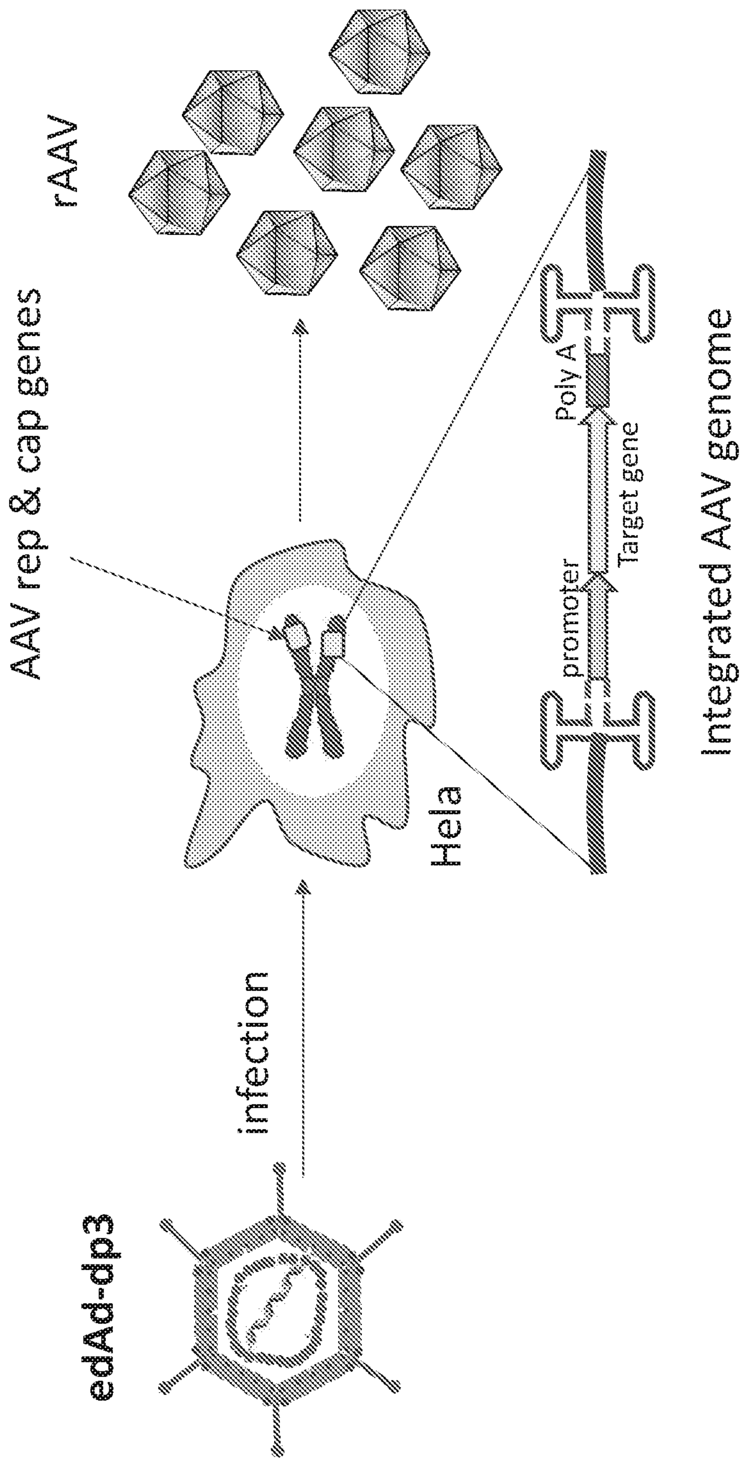

edAd-dp3
infection
Hela
AAV rep & cap genes
rAAV
promoter
Target gene
Poly A
Integrated AAV genome

Figure 8

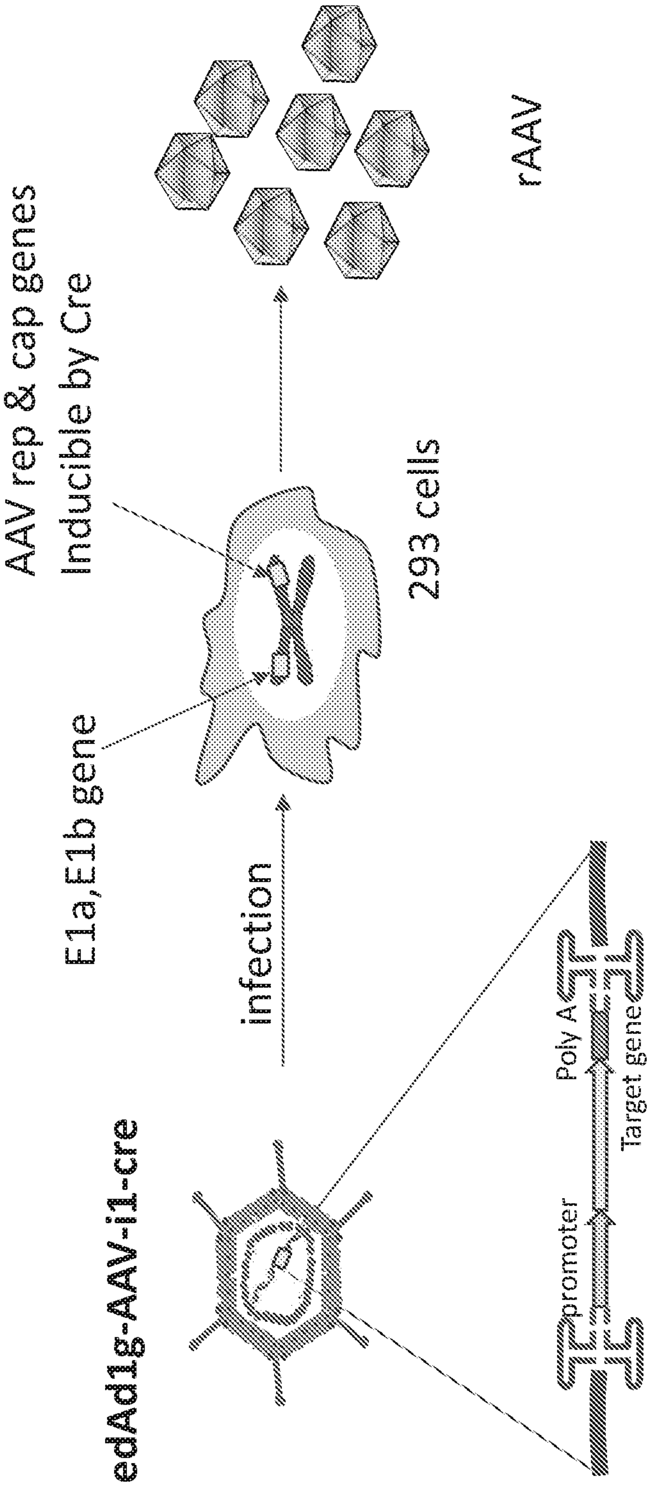

edAd1g-AAV-i1-cre
promoter
Target gene
Poly A
infection
E1a,E1b gene
AAV rep & cap genes
Inducible by Cre
293 cells
rAAV

Figure 9

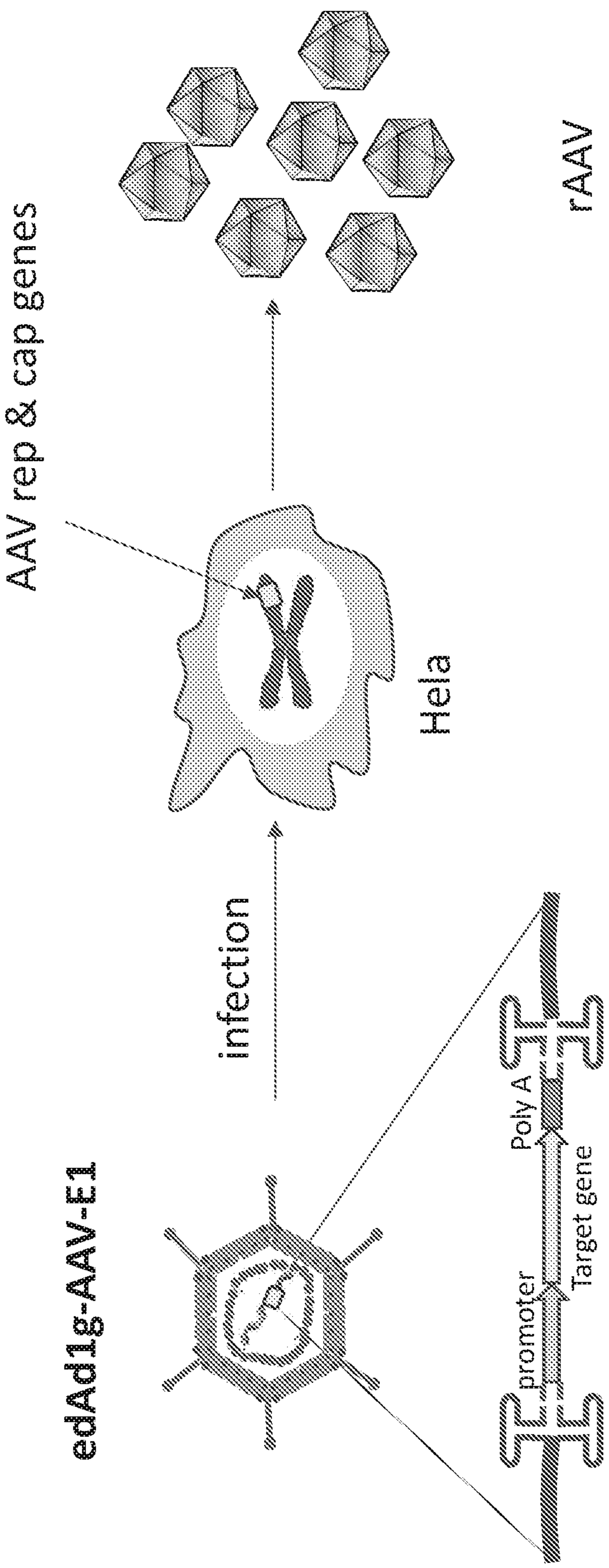

AAV rep & cap genes
rAAV
Hela
edAd1g-AAV-E1
infection
promoter
Target gene
Poly A

Figure 10

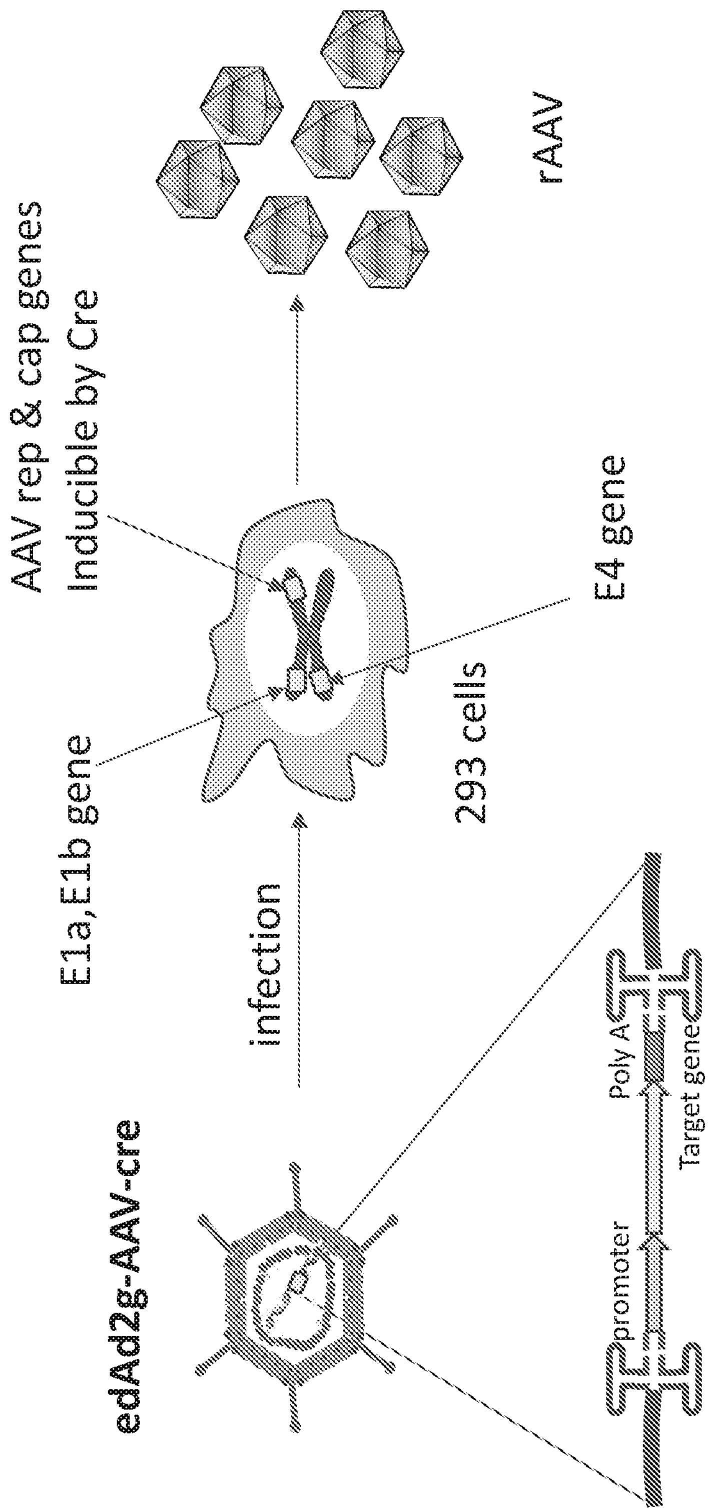

edAd2g-AAV-cre
infection
E1a,E1b gene
AAV rep & cap genes
Inducible by Cre
E4 gene
293 cells
rAAV
promoter
Target gene
Poly A

Figure 11

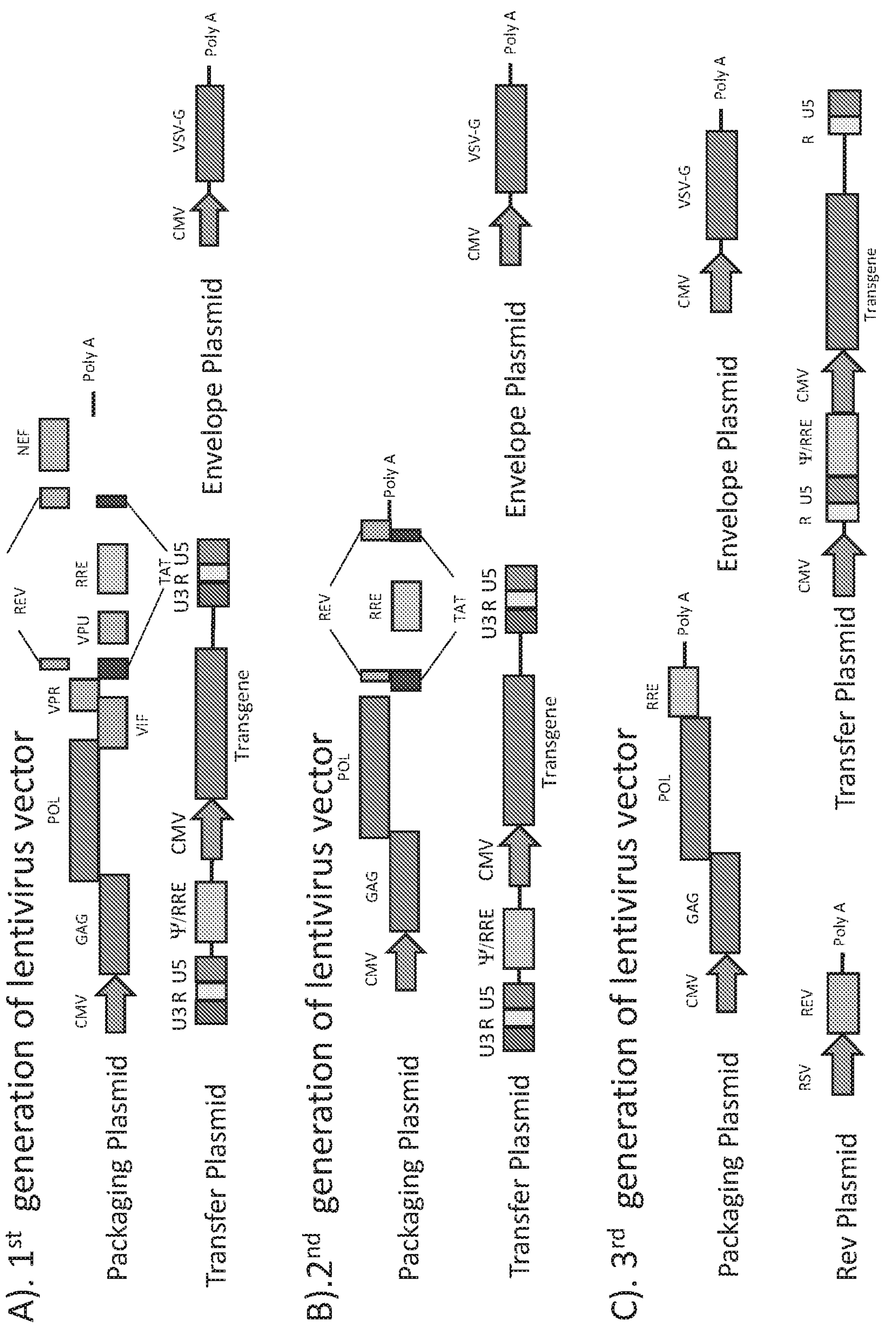

A). 1st generation of lentivirus vector
Packaging Plasmid
CMV
GAG
POL
VPR
ViF
REV
VPU
RRE
TAT
NEF
Poly A
Transfer Plasmid
U3 R U5
Ψ/RRE
CMV
Transgene
U3 R U5
Envelope Plasmid
CMV
VSV-G
Poly A
B).2nd generation of lentivirus vector
Packaging Plasmid
CMV
GAG
POL
REV
RRE
TAT
Poly A
Transfer Plasmid
U3 R U5
Ψ/RRE
CMV
Transgene
U3 R U5
Envelope Plasmid
CMV
VSV-G
Poly A
C). 3rd generation of lentivirus vector
Packaging Plasmid
CMV
GAG
POL
RRE
Poly A
Envelope Plasmid
CMV
VSV-G
Poly A
Rev Plasmid
RSV
REV
Poly A
Transfer Plasmid
CMV
R U5
Ψ/RRE
CMV
Transgene
R U5

Figure 12

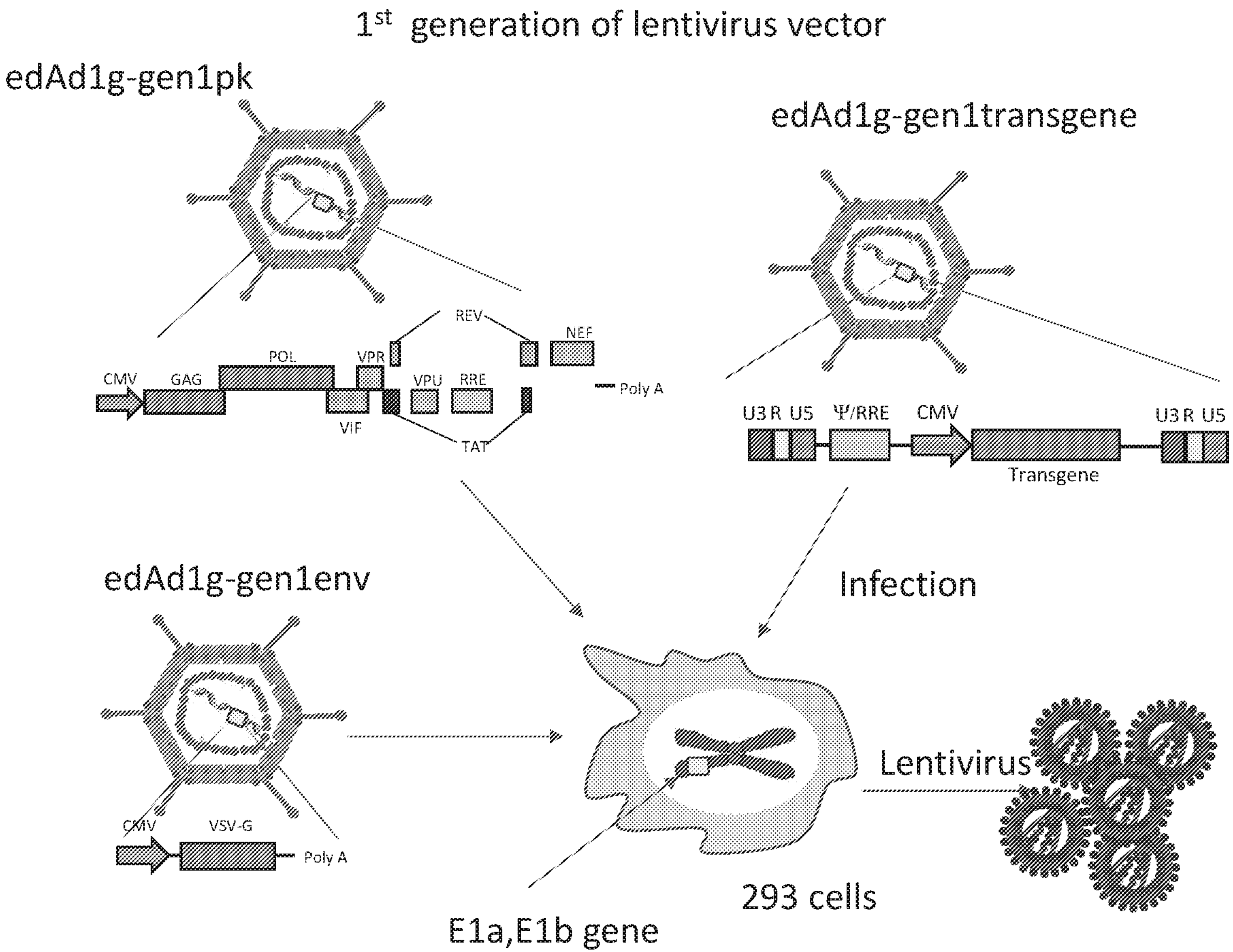
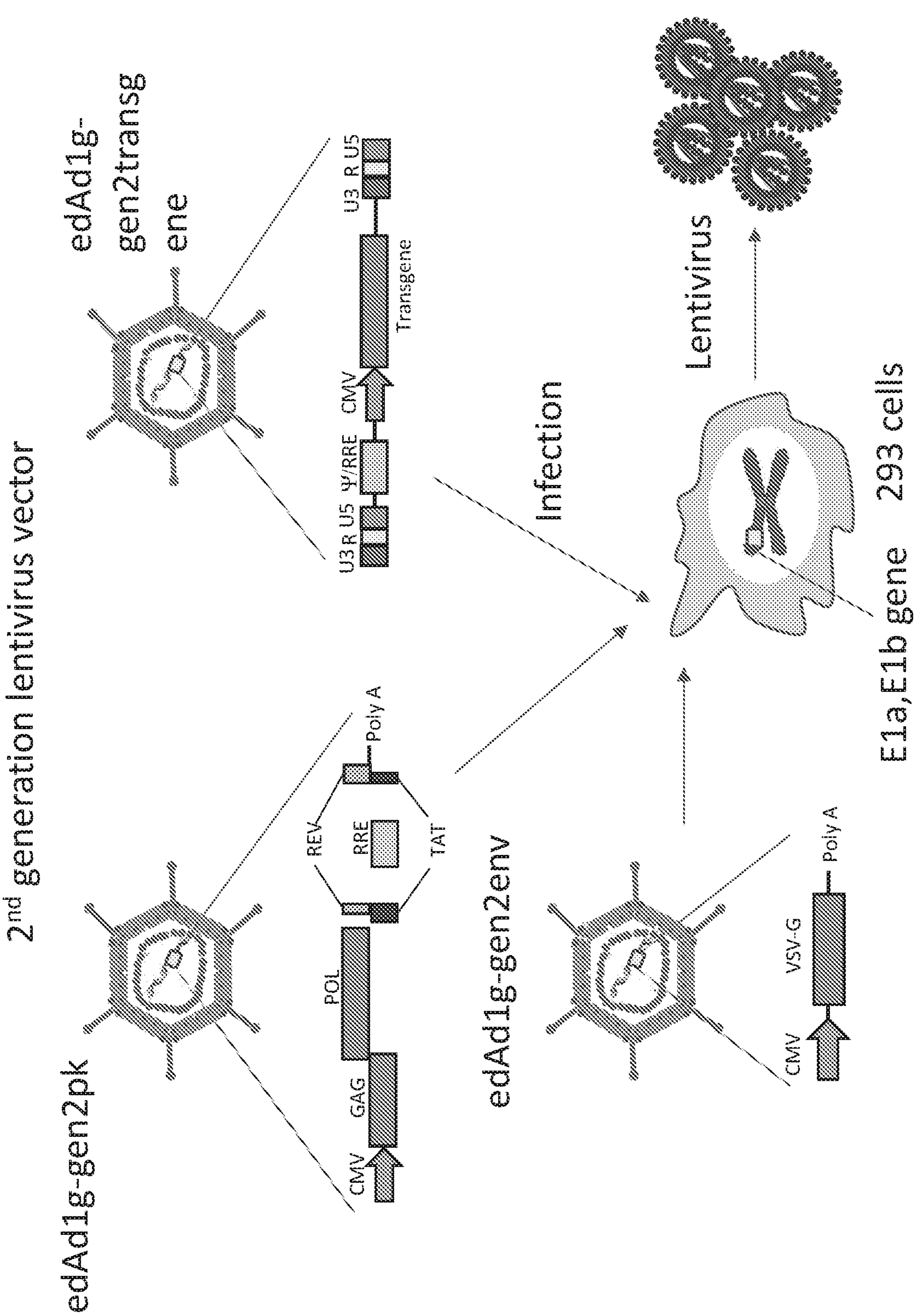

1st generation of lentivirus vector
edAd1g-gen1pk
CMV
GAG
POL
VPR
VIF
REV
VPU
RRE
TAT
NEF
Poly A
edAd1g-gen1transgene
U3 R U5
Ψ/RRE
CMV
Transgene
U3 R U5
edAd1g-gen1env
CMV
VSV-G
Poly A
Infection
Lentivirus
293 cells
E1a,E1b gene

Figure 13



2nd generation lentivirus vector
edAd1g-gen2pk
CMV
GAG
POL
REV
RRE
TAT
Poly A
edAd1g-gen2env
CMV
VSV-G
Poly A
edAd1g-gen2transgene
U3 R U5
Ψ/RRE
CMV
Transgene
U3 R U5
Infection
Lentivirus
293 cells
E1a,E1b gene

Figure 14

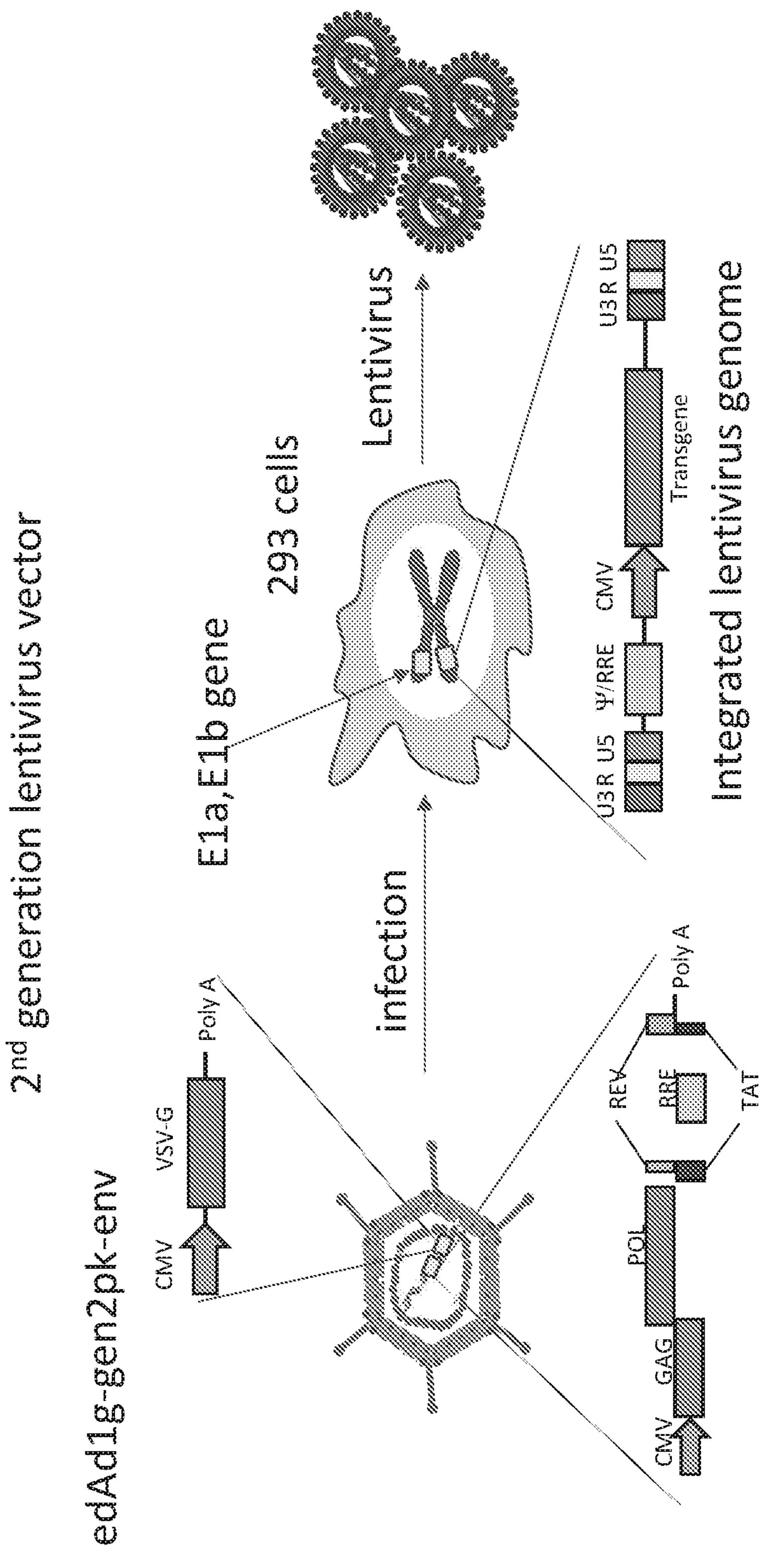

2nd generation lentivirus vector
edAd1g-gen2pk-env
CMV
VSV-G
Poly A
CMV
GAG
POL
REV
RRE
TAT
Poly A
infection
E1a,E1b gene
293 cells
Lentivirus
U3 R U5
Ψ/RRE
CMV
Transgene
U3 R U5
Integrated lentivirus genome

Figure 15

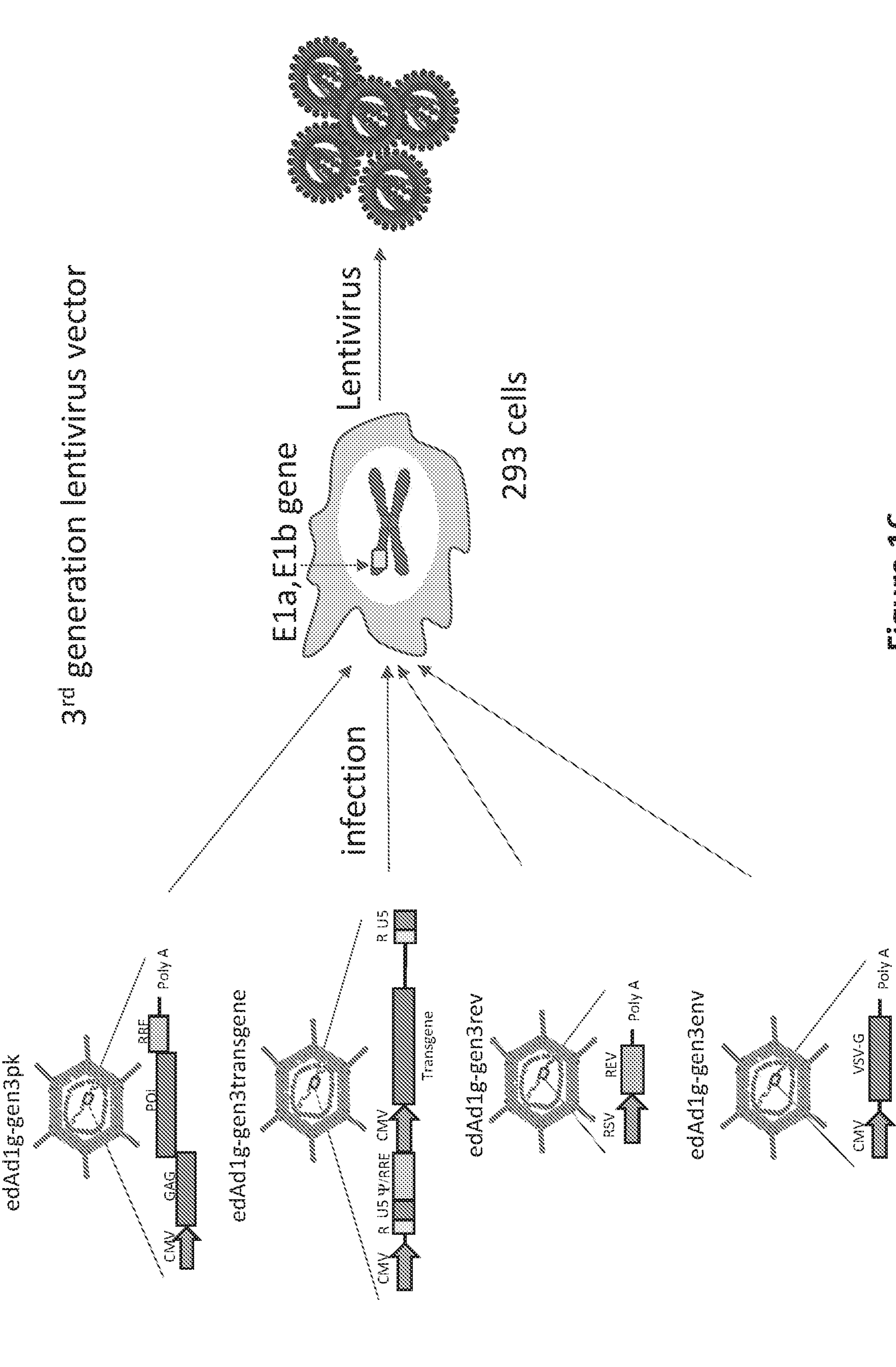

3rd generation lentivirus vector
E1a,E1b gene
Lentivirus
293 cells
infection
edAd1g-gen3pk
CMV
GAG
POI
RRE
Poly A
edAd1g-gen3transgene
CMV
R
U5
Ψ/RRE
CMV
Transgene
R U5
edAd1g-gen3rev
RSV
REV
Poly A
edAd1g-gen3env
CMV
VSV-G
Poly A

Figure 16

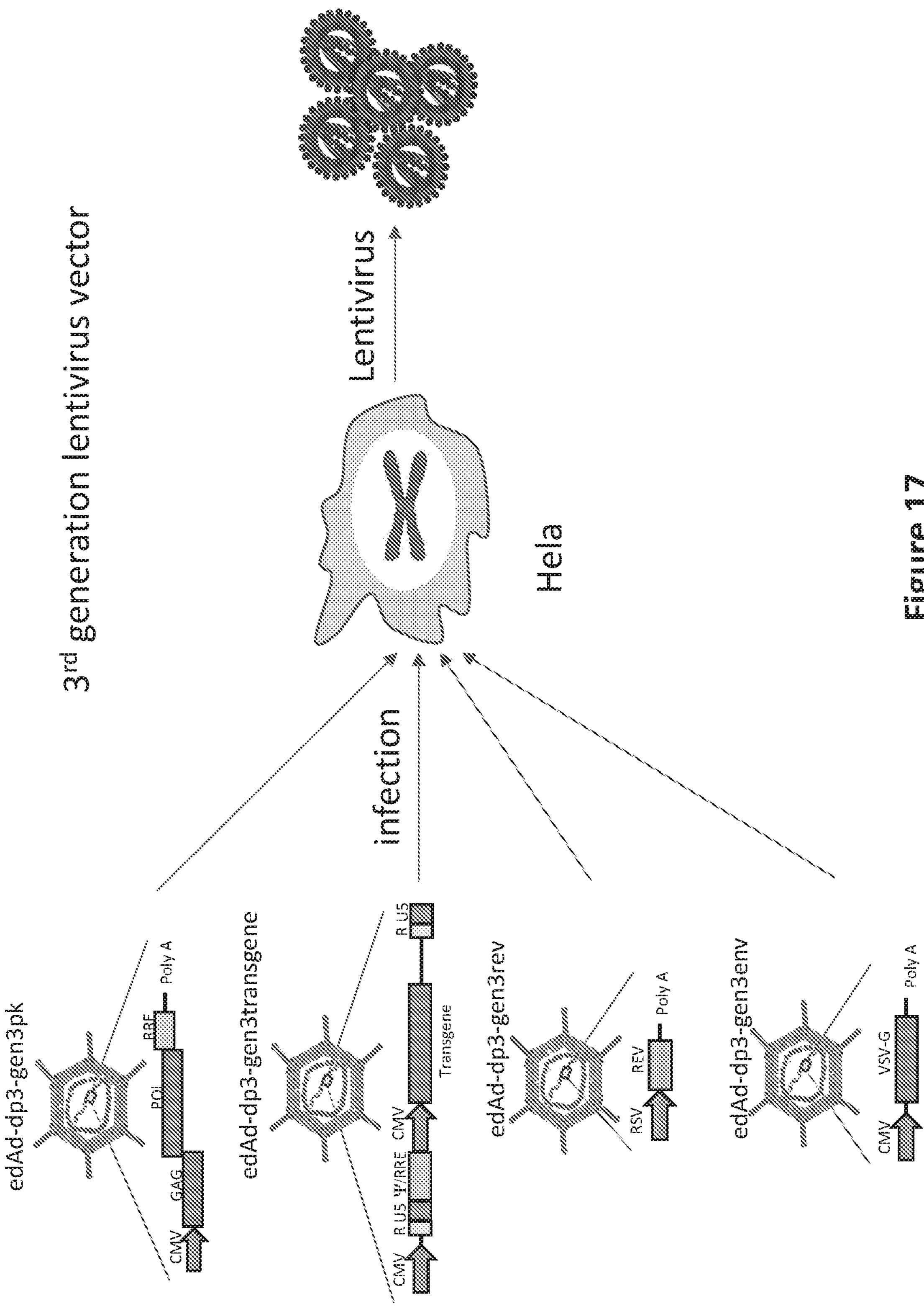
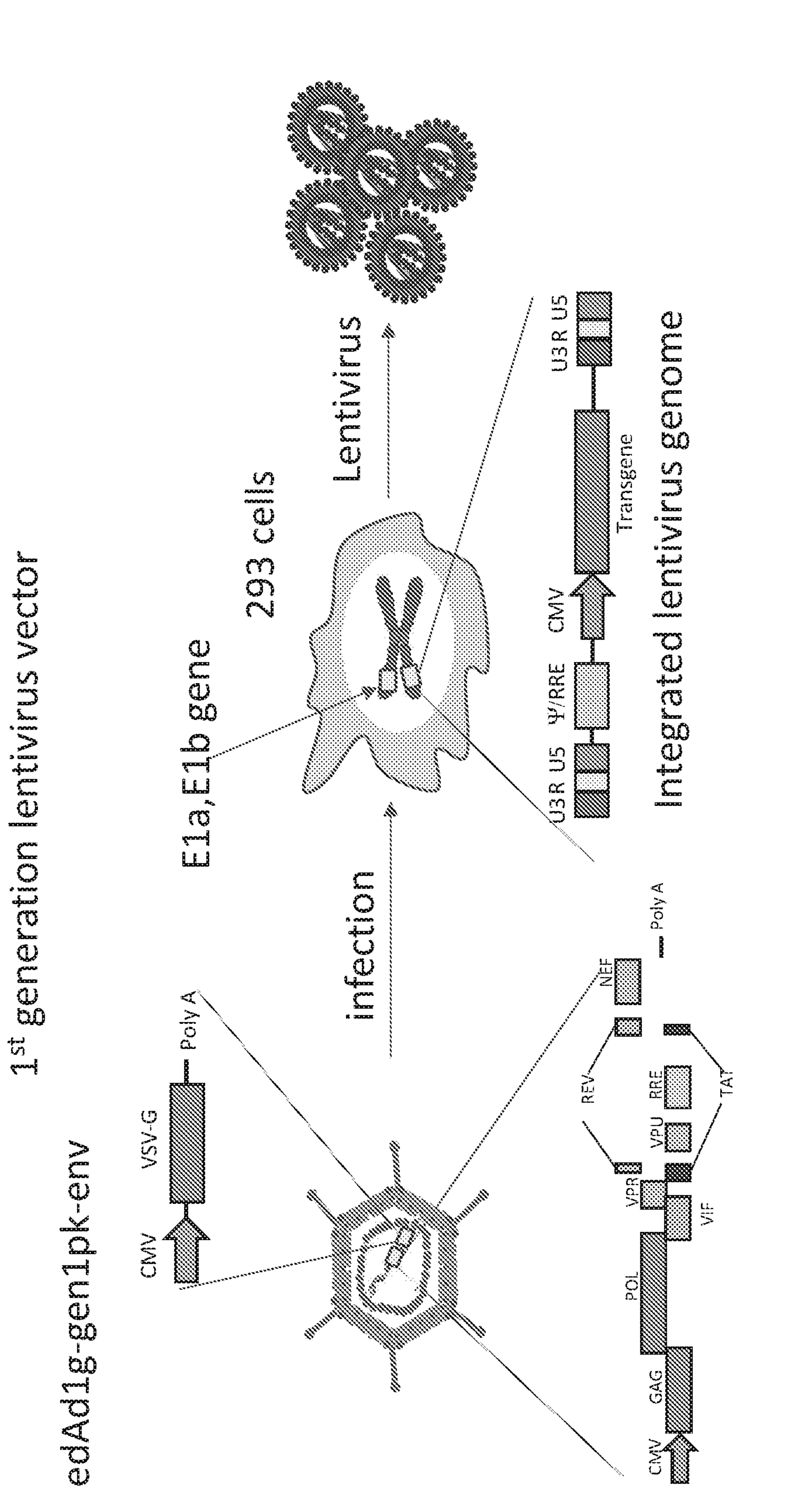

3rd generation lentivirus vector
edAd-dp3-gen3pk
CMV
GAG
POI
RRE
Poly A
edAd-dp3-gen3transgene
CMV
R U5 Ψ/RRE
CMV
Transgene
R U5
edAd-dp3-gen3rev
RSV
REV
Poly A
edAd-dp3-gen3env
CMV
VSV-G
Poly A
infection
Hela
Lentivirus

Figure 17



1st generation lentivirus vector
edAd1g-gen1pk-env
CMV
VSV-G
Poly A
E1a,E1b gene
293 cells
infection
Lentivirus
CMV
GAG
POL
VPR
VIF
VPU
REV
RRE
TAT
NEF
Poly A
U3 R U5
Ψ/RRE
CMV
Transgene
U3 R U5
Integrated lentivirus genome

Figure 18

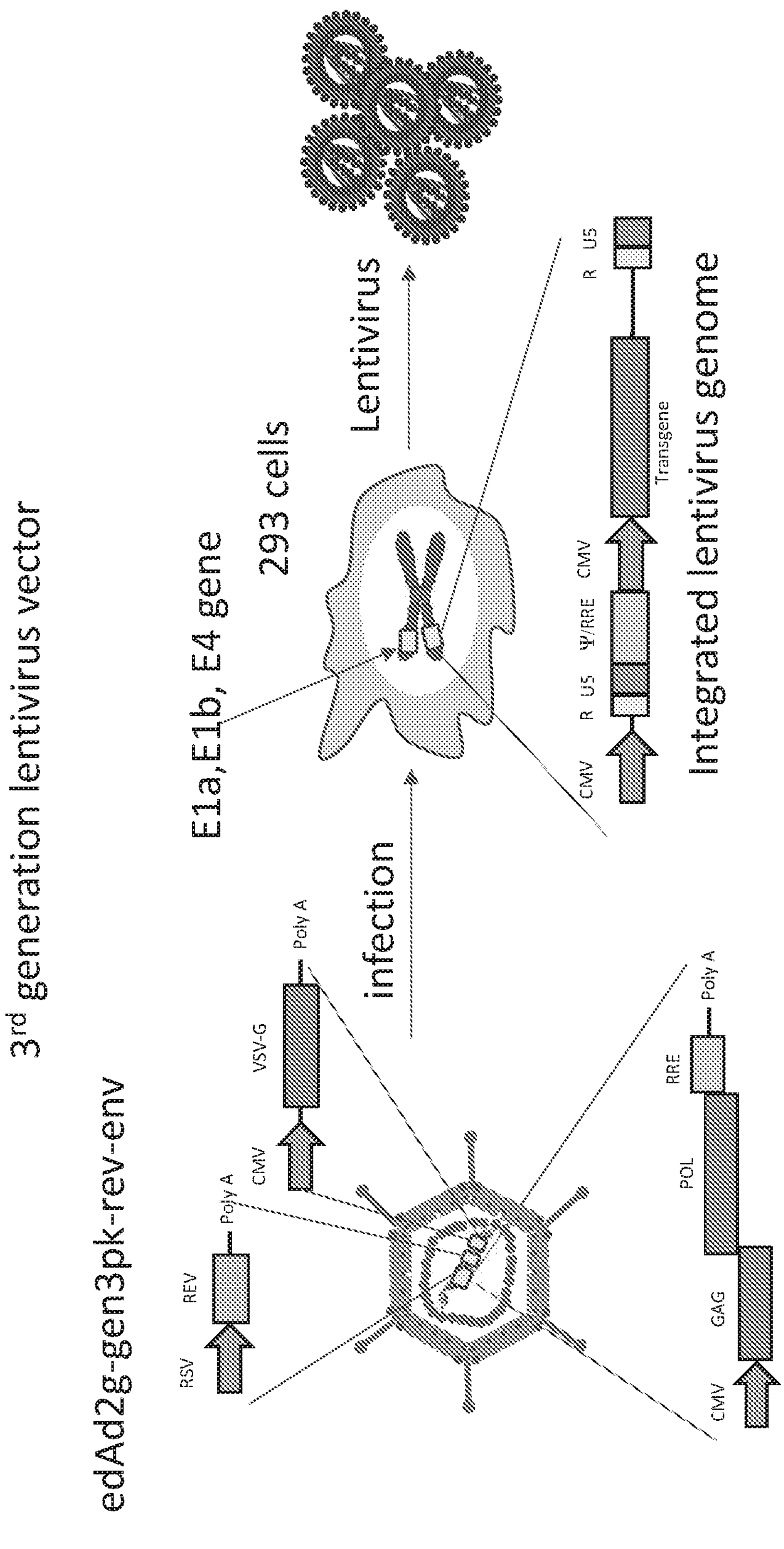

3rd generation lentivirus vector
edAd2g-gen3pk-rev-env
RSV
REV
Poly A
CMV
VSV-G
Poly A
CMV
GAG
POL
RRE
Poly A
infection
E1a,E1b, E4 gene
293 cells
Lentivirus
CMV
R
U5
Ψ/RRE
CMV
Transgene
R
U5
Integrated lentivirus genome

Figure 19