(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
02 November 2017 (02.11.2017)

WIPO | PCT

(10) International Publication Number
WO 2017/189901 A1

(51) International Patent Classification:
A61K 31/713 (2006.01) *C12N 15/861* (2006.01)
A61K 48/00 (2006.01) *C12N 15/63* (2006.01)
C07H 21/04 (2006.01) *G01N 33/50* (2006.01)
C12N 15/113 (2010.01) *G01N 33/53* (2006.01)

(21) International Application Number:
PCT/US2017/029933

(22) International Filing Date:
27 April 2017 (27.04.2017)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
62/328,139 27 April 2016 (27.04.2016) US

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report (Art. 21(3))*
— *with sequence listing part of description (Rule 5.2(a))*

WO 2017/189901 A1

(54) Title: SILENCING TRANSGENE EXPRESSION DURING VECTOR PRODUCTION

(57) Abstract: Embodiments of the disclosure concern particular vectors and their production in which transgenes are silenced through one or more untranslated sequences comprising a target sequence for a microRNA produced from the adenoviral associated RNA I (VA RNAI) (mivaRNAI) and/or a microRNA produced from the adenoviral VA RNAII (mivaRNAII). Examples of specific sequences are disclosed.

SILENCING TRANSGENE EXPRESSION DURING VECTOR PRODUCTION

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to U.S. Provisional Patent Application Serial No. 62/328,139, which is incorporated by reference herein in its entirety.

TECHNICAL FIELD

[0002] Embodiments of the disclosure concern at least the fields of cell biology, molecular biology, virology, and recombinant nucleic acid technology, for example.

BACKGROUND

[0003] Helper-dependent adenoviral vectors (HDAds) are devoid of all viral coding sequences and have proven to be excellent vectors for many gene and cell therapy applications because they can mediate high efficiency transduction of many different cells types from many different species in vivo and in vitro independent of the cell cycle, they have an enormous cloning capacity of 36 kb, do not integrate into the host genome and, provide long-term transgene expression with reduced toxicity[1]. Because HDAd do not contain any viral genes, they must be produced using a helper virus (HV)[2]. The HV is an E1-deleted adenovirus whose packaging signal is flanked by loxP sites. To produce HDAd, E1-complementing cells expressing Cre are co-infected with the HDAd and the HV wherein the packaging signal of the HV is excised by Cre-mediated site-specific recombination rendering it unpackagable but still able to undergo DNA replication and trans-complement HDAd production[2].

[0004] Occasionally, HDAds expressing certain transgenes cannot be produced because the transgene product is toxic to the producer cells, especially when present in large quantities, such as during vector production. For example, 10^5 to 10^6 progeny adenoviral genomes are produced post-infection, only about 20% of which are packaged into virions[3]. Because of such high transgene copy numbers during vector production, even transgene products that may not normally be toxic, may have toxic effects when present in such overwhelming quantities. And this problem is further exacerbated by the very strong promoters/enhancers that are normally employed in gene transfer vectors. Therefore, development of strategies to overcome this obstacle is important and necessary for the production of these recalcitrant HDAds.

[0005] Adenovirus, including the HV, expressed two RNA polymerase III-dependent non-coding RNAs, called virus-associated (VA) RNAI and VA RNAII[4,5]. VA RNAI functions to ensure high level adenoviral protein synthesis by binding to and inhibiting protein kinase R (PKR), part of the antiviral interferon response, while the role of VA RNAII is not well known. These short RNA transcripts (~160 nucleotides) have a stem-loop structure similar to pre-miRNA and are expressed throughout the virus life cycle, reaching very high concentrations during the late phase of infection (10^8 molecules of VA RNA I/cell and 10^7 molecules of VA RNAII/cell). Like pre-miRNA, VA RNAI and VA RNAII are exported to the cytosol by Exportin 5 where they are processed by Dicer into functional microRNAs, called mivaRNAs, which are incorporated into the RNA-induced silencing complex (RISC)[6,7,8]. Importantly, mivaRNAI derived from VA RNAI has been shown to target the mRNA from reporter transgenes engineered to contain the complementary target sequence in their 3' untranslated region (UTR), thereby inhibiting their expression[7,9,10,11]. As well, host cellular mRNAs targeted by VA RNAI-derived mivaRNAI resulting in downregulated expression have been identified[10,11].

[0006] The present disclosure satisfies a long-felt need in the art by providing simple strategies of exploiting expression of VA RNAI from the HV to downregulate transgene expression from the HDAd during its production. In this way, recalcitrant HDAds can be easily produced.

BRIEF SUMMARY

[0007] In one embodiment, there is a composition comprising a vector polynucleotide comprising an expression cassette, the expression cassette comprising: a) a coding sequence; and b) one or more untranslated sequences operably linked to the coding sequence, wherein the untranslated sequences comprise a target sequence for a microRNA produced from the adenoviral viral associated RNA I (VA RNAI) (mivaRNAI) and/or a microRNA produced from the adenoviral VA RNAII (mivaRNAII). The vector may be a viral vector, such as a helper-dependent adenoviral vector (HDAd), adeno-associated viral vector, lentiviral vector, or retroviral vector. In specific embodiments, the untranslated sequence is 5'-untranslated sequence, 3'-untranslated sequence, or both. The coding sequence may encode a therapeutic gene product and/or a diagnostic gene product. In certain aspects, the coding sequence encodes a gene product that is not a reporter gene product.

[0008] In specific embodiments, a mivaRNAI target sequence comprises a) the sequence 5'-AAAGGAGCACTCCCCCGTTGTCTG-3' (SEQ ID NO:1); b) a sequence that is at least 70, 75, 80, 85, 90, 95, 96, 97, 98, or 99% identical to 5'-AAAGGAGCACTCCCCCGTTGTCTG-3' (SEQ ID NO:2); or c) a sequence that is complementary to the sequence in a) or b). In specific cases, the 5'-terminal end of the sequence further comprises 1, 2, 3, 4, 5, or more nucleotides, and in specific examples the 1, 2, 3, 4, 5, or more nucleotides are A. In certain cases, the 5'-terminal end of the sequence lacks 1, 2, 3, 4, 5, or more nucleotides of the sequence. In some cases, the 3'-terminal end of the sequence further comprises 1, 2, 3, 4, 5, or more nucleotides. The 3'-terminal end of the sequence may lack 1, 2, 3, 4, 5, or more nucleotides of the sequence.

[0009] In particular embodiments, the mivaRNAI target sequence comprises: a) the sequence 5'-AAAAGGAGCACTCCCCCGTTGTCTG-3' (SEQ ID NO:3); b) a sequence that is at least 70, 75, 80, 85, 90, 95, 96, 97, 98, or 99% identical to 5'-AAAAGGAGCACTCCCCCGTTGTCTG-3' (SEQ ID NO:4); or c) a sequence that is complementary to the sequence in a) or b). In specific embodiments, the 5'-terminal end of the sequence further comprises 1, 2, 3, 4, 5, or more nucleotides, such as the 1, 2, 3, 4, 5, or more nucleotides being A. The 5'-terminal end of the sequence may lack 1, 2, 3, 4, 5, or more nucleotides of the sequence. In specific cases, the 3'-terminal end of the sequence further comprises 1, 2, 3, 4, 5, or more nucleotides. In some cases, the 3'-terminal end of the sequence lacks 1, 2, 3, 4, 5, or more nucleotides of the sequence.

[0010] In particular embodiments, the mivaRNAII target sequence comprises: a) the sequence 5'-AGGGGCTCGTCCCTGTTTCCG-3' (SEQ ID NO:5); b) a sequence that is at least 70, 75, 80, 85, 90, 95, 96, 97, 98, or 99% identical to 5'- AGGGGCTCGTCCCTGTTTCCG-3' (SEQ ID NO:6); or c) a sequence that is complementary to the sequence in a) or b). In specific embodiments, the 5'-terminal end of the sequence further comprises 1, 2, 3, 4, 5, or more nucleotides, such as the 1, 2, 3, 4, 5, or more nucleotides being A. In specific cases, the 5'-terminal end of the sequence lacks 1, 2, 3, 4, 5, or more nucleotides of the sequence. In some cases, the 3'-terminal end of the sequence further comprises 1, 2, 3, or more nucleotides. The 3'-terminal end of the sequence may lack 1, 2, 3, 4, 5, or more nucleotides of the sequence.

[0011] In specific embodiments, the vector is a helper-dependent adenoviral vector that derives from adenovirus serotype 1, adenovirus serotype 2, adenovirus serotype 5, adenovirus serotype 6, adenovirus serotype 35, or a chimera thereof.

[0012] In one embodiment, there are cells that comprise any composition of the disclosure. In specific embodiments, the cell comprises a helper virus that encodes VA RNAI, VA RNAII, or both. In particular cases, there is the following: 1) the helper virus (HV) is an E1-deleted adenovirus comprising a packaging signal flanked by loxP sites; 2) the HV comprises a packaging signal flanked by frt sites; 3) the HV comprises a pIX deletion so that only HDAd of a certain size can be packaged, 3) the HV comprises a mutated, inefficient packaging signal; or 4) the HV comprises an insertion of φC31 phage into it to delay its life cycle relative to the HDAd. The cell may or may not express Cre.

[0013] In one embodiment, there is a method of producing a composition of the disclosure, comprising the steps of: a) providing, obtaining, or generating an expression cassette comprising a coding sequence operably linked to a 3' untranslated region; and b) modifying the 3' untranslated sequence to comprise a target sequence for a microRNA produced from the adenoviral viral associated RNA I (VA RNAI) (mivaRNAI) and/or a microRNA produced from the adenoviral VA RNAII (mivaRNAII). The method may further comprise the step of incorporating the expression cassette into a vector, for example the expression cassette may be comprised in a helper-dependent adenoviral vector, an adenoviral associated vector, a lentiviral vector, or a retroviral vector. In some cases, the method comprises the step of transducing a cell with the vector, such as a helper-dependent adenoviral vector or adeno-associated viral vector, the cell comprises a helper virus. In some cases, the method further comprises the step of transducing the cell with a helper virus. The helper virus may encode VA RNAI, VA RNAII, or both. In specific cases, the helper virus is an E1-deleted adenovirus comprising a packaging signal flanked by loxP sites.

[0014] In one embodiment, there is a method of producing a vector in a cell, comprising the steps of: a) providing, obtaining, or generating in the cell a vector that includes an expression cassette that comprises a coding sequence operably linked to at least one untranslated region that comprises a target sequence for mivaRNAI and/or a target sequence for mivaRNAII; and b) exposing the vector to the mivaRNAI and/or mivaRNAII under suitable conditions such that expression of the coding sequence is inhibited upon binding of the mivaRNAI and/or mivaRNAII to their respective target sequences; and c) producing the vector. In specific embodiments, the vector is a helper-dependent adenoviral vector or adeno-associated viral vector. The mivaRNAI and/or mivaRNAII are expressed from a vector in the cell. The mivaRNAI and/or mivaRNAII may be expressed from a helper virus.

[0015] The foregoing has outlined rather broadly the features and technical advantages of the present invention in order that the detailed description of the invention that follows may be better understood. Additional features and advantages of the invention will be described hereinafter which form the subject of the claims of the invention. It should be appreciated by those skilled in the art that the conception and specific embodiment disclosed may be readily utilized as a basis for modifying or designing other structures for carrying out the same purposes of the present invention. It should also be realized by those skilled in the art that such equivalent constructions do not depart from the spirit and scope of the invention as set forth in the appended claims. The novel features which are believed to be characteristic of the invention, both as to its organization and method of operation, together with further objects and advantages will be better understood from the following description when considered in connection with the accompanying figures. It is to be expressly understood, however, that each of the figures is provided for the purpose of illustration and description only and is not intended as a definition of the limits of the present invention.

BRIEF DESCRIPTION OF THE DRAWINGS

[0016] For a more complete understanding of the present invention, reference is now made to the following descriptions taken in conjunction with the accompanying drawing, in which:

[0017] FIGS. 1A-1I. Amplification of (1A) HDAd-CAG-LacZ, (1B to 1E) HDAd-CAG-hyPB, and (1F to 1G) HDAd-CAG-hyPB-VAI. Total cellular DNA extracted from serial passages was digested with ApaLI and analyzed by agarose gel electrophesis to identify those passages containing peak vector titer which is indicated by the visible presence of vector-specific bands in the presence of the background cellular DNA smear. The lanes labeled pHV contain the HV plasmid digested with ApaLI and PacI to serve as a control for HV specific bands. The lanes labelled pHDAd contain the corresponding HDAd plasmid digested with ApaLI and PmeI to serve as a control for HDAd specific bands. Passage 0 (the initial transfection with pHDAd and infection with HV of the producer cells) for each amplification was not included in this analysis.

[0018] FIG. 2. Schematic of amplification and large-scale HDAd production. As described in the Results and shown in FIG. 1, the earliest passage that contains peak vector titer during vector amplification was passage 2 for HDAd-CAG-LacZ, passage 6 for HDAd-CAG-hyPB, and passage 3 for HDAd-CAG-hyPB-VAI. Thus, crude viral lysate from these passages

were used to coinfect a single 150 mm dish of producer cells along with HV. The resulting crude viral lysate from this single 150 mm dish was used to co-infect 2 L of producer cells along with HV and the HDAd was subsequently purified by CsCl ultracentrifugation.

[0019] FIGS 3A-3L. Appearance of virus band in CsCl gradient and vector genomic structure. (3A to 3I) The first continuous CsCl gradient for the indicated vector preparation is shown. (3J to 3L) The result of restriction analyses of virion DNA extracted from the indicated vector preparation is shown. The lanes labelled pHV contain HV plasmid digested with ApaLI and PacI to serve as a control for HV specific bands. The lanes labelled pHDAd-CAG-LacZ, pHDAd-CAG-hyPB, and pHDAd-CAG-hyPB-VAI contain the plasmid form of the indicated HDAd digested with ApaLI and PmeI to serve as a control for the HDAd-specific bands. Total viral particles (vp) obtained from each preparation is indicated.

[0020] FIG. 4. Strategy to downregulate transgene expression from HDAd during vector production. VA RNAI is expressed from the HV which is processed into mivaRNAI by Dicer and incorporated into the RISC and can cleave mRNA expressed from the HDAd which contains the mivaRNAI target sequence in the 3' UTR. The HV genome cannot be packaged into virions because of excision of the floxed packaging signal (Ψ) by Cre. Thus, following HDAd purification, transduction of target cells result in unimpeded transgene expression from the HDAd due to the absence of the HV.

[0021] FIG. 5. Modification of hyPBase expression cassette to permit downregulation by the HV. A sequence corresponding to nucleotide 119 to 159 from VA RNAI is inserted into the 3' UTR of the HDAd expression cassette (SEQ ID NO:7-8). The mivaRNAI target sequence is boxed. The 3' UTR is part of the multiple cloning site of the expression plasmid (SEQ ID NO:9).

[0022] FIGS. 6A and 6B. HDAd-CAG-hyPB-VAI expresses functional hyPBase. (6A) HDAd-PB-TR contains a 2.3 kb segment of DNA flanked by PB TRs. hyPB-mediated excision of this DNA segment converts a 4.1 kb PCR product to a 1.8 kb PCR product. (6B) PCR analyses of DNA extracted from human iPS cells infected with the indicated vectors. Lane 5 contains the PCR product obtained from uninfected human iPS cells to provide a control for non-specific amplification products. Lane 6 contains the PCR product from the plasmid used to make HDAd-PB-TR to provide a control for the 4.1 kb unexcised PCR product.

DETAILED DESCRIPTION

[0023] As used herein, the words "a" and "an" when used in the present specification in concert with the word comprising, including the claims, denote "one or more." Some embodiments of the invention may consist of or consist essentially of one or more elements, method steps, and/or methods of the invention. It is contemplated that any method or composition described herein can be implemented with respect to any other method or composition described herein.

[0024] Throughout this specification, unless the context requires otherwise, the words "comprise", "comprises" and "comprising" will be understood to imply the inclusion of a stated step or element or group of steps or elements but not the exclusion of any other step or element or group of steps or elements. By "consisting of" is meant including, and limited to, whatever follows the phrase "consisting of." Thus, the phrase "consisting of" indicates that the listed elements are required or mandatory, and that no other elements may be present. By "consisting essentially of" is meant including any elements listed after the phrase, and limited to other elements that do not interfere with or contribute to the activity or action specified in the disclosure for the listed elements. Thus, the phrase "consisting essentially of" indicates that the listed elements are required or mandatory, but that no other elements are optional and may or may not be present depending upon whether or not they affect the activity or action of the listed elements.

[0025] Reference throughout this specification to "one embodiment," "an embodiment," "a particular embodiment," "a related embodiment," "a certain embodiment," "an additional embodiment," or "a further embodiment" or combinations thereof means that a particular feature, structure or characteristic described in connection with the embodiment is included in at least one embodiment of the present invention. Thus, the appearances of the foregoing phrases in various places throughout this specification are not necessarily all referring to the same embodiment. Furthermore, the particular features, structures, or characteristics may be combined in any suitable manner in one or more embodiments.

[0026] In particular embodiments, the disclosure concerns compositions and methods related to efficient expression of a vector. In specific embodiments, a transgene capable of being expressed in a vector is in need of inhibition of its expression. Thus, the disclosure concerns vector or expression construct configurations that allow for silencing (in part or to undetectable

levels) of a transgene. In certain cases, one or more compositions and method(s) of using the composition(s) concern a vector polynucleotide comprising an expression cassette, the expression cassette comprising: a) a coding sequence; and b) one or more untranslated sequences operably linked to the coding sequence, wherein the untranslated sequences comprise a target sequence for a microRNA produced from the adenoviral viral associated RNA I (VA RNAI) (mivaRNAI) and/or a microRNA produced from the adenoviral VA RNAII (mivaRNAII). The vector may be of any kind, including adenoviral vectors, lentiviral vectors, helper-dependent adenoviral vectors (HDAd), adeno-associated viral vector, and so forth.

[0027] In specific but merely exemplary cases, helper-dependent adenoviral vectors (HDAd) that express certain transgene products are impossible to produce because the transgene product is toxic to the producer cells, especially when made in large amounts during vector production. Downregulating transgene expression from the HDAd during vector production is a way to solve this problem. In this disclosure the inventors show that this can be accomplished by inserting the target sequence for the adenoviral VA RNAI into the 3' untranslated region of the expression cassette in the HDAd. Thus during vector production, when the producer cells are co-infected with both the helper virus (HV) and the HDAd, the VA RNAI produced by the HV will target the transgene mRNA from the HDAd via the endogenous cellular RNAi pathway. Once the HDAd is produced and purified, transduction of the target cells results in unimpeded transgene expression because of the absence of HV. This simple and universal strategy permits for the robust production of otherwise recalcitrant HDAds.

[0028] Embodiments of the disclosure encompass methods and compositions related to downregulation of transgene expression including for production of a certain vector, such as helper virus-mediated downregulation of transgene expression that permits production of a particular vector, such as a recalcitrant helper-dependent adenoviral vector. Specific embodiments concern production of recalcitrant helper-dependent Ads.

EXAMPLES

[0029] The following examples are presented in order to more fully illustrate the preferred embodiments of the disclosure. They should in no way, however, be construed as limiting the broad scope of the disclosure.

EXAMPLE 1

PRODUCTION OF RECALCITRANT HELPER-DEPENDENT ADS

[0030] PiggyBac (PB) is a transposon isolated from cabbage looper moth *Tichoplusia ni* and encodes a transposase that catalyzes PB transposition[12]. Hyperactive PB transposase (hyPBase) bears 7 amino acid substitutions resulting in a 17-fold and 9-fold increase in excision and integration, respectively, compared to wildtype[13]. The inventors sought to produce a HDAd expressing hyPBase from the strong CAG promoter (HDAd-CAG-hyPB) using a well-established protocol[14,15] that entails serial coinfections (called passages) of the producer cells with the HDAd and the HV to increase the HDAd titer, followed by identification of the earliest passage containing peak HDAd titer which would then be used to initiate large-scale vector production. To determine the passages that comprise peak HDAd titers, total cellular DNA was extracted from the producer cells at each serial passage, and examined by agarose gel electrophoresis after ApaLI digestion. Serial passages with peak HDAd titers can be identified by the visible presence of HDAd-specific bands in the presence of the background cellular DNA smear, and is typically first attained at serial passage 2 or 3[14,15]. As expected, this was indeed the case for the control vector, HDAd-CAG-LacZ, amplified in parallel with HDAd-CAG-hyPB, in which maximum titer was first reached at serial passage 2 (**FIG. 1A**). In contrast, for the four independent amplifications of HDAd-CAG-hyPB, vector-specific bands were not visible until serial passage 5 or 6 (**FIGS. 1B to 1E**).

[0031] According to a protocol, large scale HDAd production is initiated using the earliest passage containing the highest intensity vector-specific band based on agarose gel analyses[14,15]. Thus, the earliest passage to contain maximum vector titer was passage 2 for the control HDAd-CAG-LacZ (**FIG. 1A**), and passage 6 for the four amplifications of HDAd-CAG-hyPB (**FIG. 1B to 1E**). Accordingly, crude viral lysate from passage 2 for HDAd-CAG-LacZ and crude viral lysate from passage 6 for HDAd-hyPB were used to co-infect a single 150 mm dish of producer cells along with HV to initiate large-scale vector production (**FIG. 2**). The resulting crude viral lysates from this 150 mm dish was used to co-infect 2 L of producer cells along with HV for large scale production, and the vectors were purified by CsCl centrifugation (**FIG. 2**). Following CsCl ultracentrifugation, a single band was observed in the gradient for HDAd-CAG-LacZ as expected (**FIG. 3A**). In contrast, multiple bands were observed in the CsCl gradient for the four preparations of HDAd-CAG-hyPB (**FIGS. 3B to 3E**) which is indicative of

genomic rearrangement of the HDAd and/or HV. To verify genomic structure, DNA was extracted from the virions obtained from the CsCl gradients, digested with ApaLI and analyzed by agarose gel electrophoresis. As expected, the DNA pattern of HDAd-CAG-LacZ was identical to the plasmid from which it was derived (except for the expected absence of the 2.5 kb fragment containing the bacterial plasmid DNA) indicating no DNA rearrangements (**FIG. 3J**). In contrast, the four preparations of HDAd-CAG-hyPB revealed different DNA patterns with some bands corresponding to the HDAd and some to the HV, as well as novel bands not expected of either, all of which is consistent with a mixed population of various rearranged HDAd and HV DNA (**FIG. 3K**). Because these preparations are useless for their intended purpose, their precise DNA rearrangements were not investigated further.

[0032] Given that HDAd-CAG-hyPB and HDAd-CAG-LacZ are identical except for the coding sequence in the expression cassette, it was reasonable to assume that expression of hyPBase was responsible for the delay in reaching peak titers during vector amplification and HDAd and HV genome rearrangements. Therefore, it was considered that downregulating hyPBase expression during vector amplification would result in successful vector production. The strategy to accomplish this is presented in **FIG 4**. Within producer cells co-infected with HV and HDAd, VA RNAI is expressed from the HV which is processed into mivaRNAs by Dicer and incorporated into the RISC and, importantly, this has been shown to downregulate expression cassettes bearing the complementary mivaRNAI sequence in the 3'UTR[7,9,10,11]. Thus, downregulating transgene expression from the HDAd should be achievable during vector production by simply inserting the mivaRNAI target sequence into the 3'UTR of the HDAd's expression cassette. Conversely, once the HDAd is purified, this modification would not inhibit transgene expression by the HDAd in the transduced target cell because of the absence of the HV.

[0033] To evaluate this strategy, HDAd-CAG-hyPB-VAI was created which bears, within the 3' UTR, nucleotides 119 to 159 from VA RNAI within which resides the mivaRNAI target sequence (**FIG. 5**). In four independent amplifications of HDAd-CAG-hyPB-VAI, peak vector titers were first reached by passage 2 to 3 (**FIG. 1F to 1I**). For all four amplifications, passage 3 was chosen to initiate large scale vector production (**FIG. 2**), and in all 4 cases a single virus band was obtained in the CsCl gradient (**FIGS. 3F to 3I**). Restriction analysis of the DNA extracted from these four vector preparations revealed the expected pattern, indistinguishable from the plasmid from which the vector was derived (except for the expected absence of the 2.5

kb fragment containing the bacterial plasmid DNA) indicating the absence of DNA rearrangements (**FIG. 3l**).

[0034] To confirm that purified HDAd-CAG-hyPB-VAI expresses functional hyPBase, human induced pluriopotent stem (iPS) cells[16] were coinfected with HDAd-CAG-hyPB-VAI and HDAd-PB-TR. HDAd-PB-TR contains a 2.3 kb segment of DNA flanked by PB terminal repeats (TRs) and is thus excisable in the presence of hyPBase (**FIG. 6A**). As controls, iPS cells were also infected with each vector alone, or mock infected. The next day, total DNA was extracted from the treated cells and subjected to PCR analyses. In the absence of hyPBase-mediated excision, a 4.1 kb PCR product is expected, and this is converted to a 1.8 kb PCR product following hyPBase-mediated excision (**FIG. 6A**). The plasmid pHDAd-PB-TR (used to make HDAd-PB-TR), was included in the PCR assay as a control and, as expected, yielded only the unexcised 4.1 kb PCR product (**FIGS. 6B, lane 6**). As expected, infection with HDAd-PB-TR alone does not result in excision as evident by the presence of the 4.1 kb PCR product and the absence of the 1.8 kb PCR product (**FIG. 6B, lane 4**). In contrast, the 1.8 kb PCR product, indicative of hyPBase-mediated excision, is only present following co-infection of cells with HDAd-CAG-hyPB-VAI and HDAd-PB-TR (**FIG. 6B, lane 2**). However, the unexcised 4.1 kb PCR product remains visible following co-infection with HDAd-CAG-hyPB-VAI and HDAd-PB-TR (**FIG. 6B, lane 2**) and this may be due to inaccessibility of a fraction of the HDAd-PB-TR genomes to hyPBase (such as some genomes remaining encapsidated) and/or that hyPBase-mediated excision is not 100% efficient and/or some cells were infected with HDAd-PB-TR only. Nevertheless, these results confirm that HDAd-CAG-hyPB-VAI does indeed express functional hyPBase. Subsequently, we have used HDAd-CAG-hyPB-VAI to successfully excise DNA segments flanked by PB TRs in other applications.

Significance of Certain Embodiments

[0035] During production, the HDAd genome, along with its transgene expression cassette, is replicated to very high copy numbers in the producer cell. The extraordinarily high transgene copy numbers, exacerbated by the use of strong promoter/enhancers, result in very high quantities of transgene product in the producer cells during vector production. At such high amounts, a transgene product that is otherwise benign, may have toxic effects on the producer cells and this could lead to a selection for rearranged vectors with no or reduced transgene expression. HDAd expressing hyPBase from the strong CAG promoter is an example of such a

vector; hyBPase is not toxic to mammalian cells[13] but our repeated attempts to produce this vector were unsuccessful resulting in HDAd and HV genome rearrangements.

[0036] The inventors have overcome this obstacle by exploiting the fact that the HV expresses VA RNAI, a short non-coding RNA that is processed in functional miRNAs, called mivaRNA, by the endogenous cellular RNAi pathway in the producer cells to downregulate transgene expression from the HDAd. This was accomplished by simply inserting the mivaRNAI target sequence into the 3' UTR of the HDAd's expression cassette. This simple modification allowed for repeated and robust production of an HDAd expressing the functional hyPBase from the strong CAG promoter. This strategy is straightforward and universal; it does not require the use of a special producer cell line, or drugs to suppress or induce transgene expression, and no special DNA expression control elements need be included in the expression cassette. Once the HDAd is produced and purified, transduction of the target cell results in unimpeded transgene expression from the HDAd because of the absence of the HV.

[0037] Recently, Saydaminova et al[17] reported a miRNA-mediated method of downregulating transgene expression from a HDAd during its production. In this case, by miRNA expression profiling, Saydaminova et al. identified two endogenous cellular miRNAs, hsa-miR183-5p and hsa-miR218-5p, that were strongly expressed in the producer 293-Cre cells but not in their intended human CD34+ target cells. Thus by inserting the target sequence for hsa-miR183-5p and hsa-miR218-5p into the 3' UTR of the expression cassette, transgene expression was suppressed during vector production by hsa-miR183-5p and hsa-miR218-5p present in the 293-Cre producer cells. However, transgene expression was unimpeded following HDAd transduction of CD34+ cells due to the absence of hsa-miR183-5p and hsa-miR218-5p in these cells. However, this strategy would be ineffective for target cells, unlike CD34+ cells, that expressed either hsa-miR183-5p or hsa-miR218-5p.

[0038] In summary, the inventors have developed a simple and universal strategy to downregulate transgene expression from HDAd during vector production. This permits production of HDAd that otherwise could not be produced because of transgene product-mediated cellular toxicity. Indeed, the inventors have subsequently used this strategy to produce other recalcitrant HDAds that could not previously produce despite multiple attempts (not shown).

Examples of Materials and Methods

[0039] An expression cassette containing the CAG promoter, hyPBase coding sequence[13] and the SV40 polyadenylation signal was inserted into the AscI site of the HDAd genomic plasmid pΔ28E4[18] and the resulting plasmid was used to produce HDAd-CAG-hyPB as described below. The VA RNAI target sequence, created by annealing two oligonucleotide (sequence shown in Figure 5), was inserted into the NotI site in the 3' UTR of hyPBase expression cassette which was then inserted into the AscI site of pΔ28E4 and the resulting plasmid was used to produce HDAd-CAG-hyPB-VAI as described below. The expression cassette in HDAd-CAG-LacZ is identical to the one in HDAd-CAG-hyPB except that the hyPB coding sequence was replaced with the *E. coli* beta-galactosidase coding sequence.

[0040] Amplification of HDAd was performed as described in detail elsewhere[14,15]. Briefly, 20 μg of the plasmid form of the HDAd was digested with PmeI and transfected into a confluent 60 mm dish of 116 cells[14] by calcium phosphate co-precipitation (Promega, Madison, WI) and then the cells were infected with the HV AdNG163[19] at an MOI of 2000 vp/cell (serial passage 0). The HDAd titer was increased by serial coinfections as follows; for each serial coinfection (called a passage), a confluent 60 mm dish of 116 cells was coinfected with HV (200 vp/cell) and 20% of the crude viral lysate containing the HDAd from the previous passage. Total DNA was extracted from each serial passage, digested with ApaLI and visualized by ethidium bromide staining following agarose gel electrophoresis. Serial passages containing peak titers of HDAd were identified by the visible presence of HDAd-specific bands in the agarose gel. The earliest serial passage containing the most intensely visible HDAd-specific bands was used to initial large-scale vector production as follows; 20% of the crude viral lysate from the aforementioned passage was used to co-infect a single 150 mm dish of 116 cells along with AdNG163. 48 hours later, 100% of the crude viral lysate from this single 150 mm dish was used to co-infect 2 L of 116 cells ($1x10^9$ cells total) along with AdNG163 at an MOI of 200 vp/cell. HDAd was purified from the co-infected 116 cells 48 later by triple CsCl ultracentrifugation; one step gradient followed by two continuous gradients.

[0041] hyPBase-mediated DNA excision was analyzed as follows; feeder free human iPS cells[16] were maintained in mTeSR 1 (STEMCELL Technologies, Vancouver, Canada) on Matrigel (Corning, Tewksbury, MA) coated plates. The iPS cells were infected as follows; $2x10^6$ cells were resuspended in 1 ml mTeSR 1 supplemented with Y27632 (Reagents Direct,

Encinitas, CA) to 10 μM in a 1.5 ml microfuge tube and infected with HDAd at an MOI of 350 vp/cell for 1 hour at 37°C with gentle rocking. Following infection, cells were washed twice with 1 ml mTeSR 1 supplemented with Y27632 to 10 μM and plated in a single Matrigel coated well of a 6 well plate in mTeSR 1 supplemented with Y27632 to 10 μM. The next day, DNA was extracted from the infected cells for PCR analysis. PCR was performed with PrimeStar GXL (Takara-Clonetech, Mountain View, CA) using primers 5' CTCAGTTTTCCTGGATTATGCCTGGCACC (SEQ ID NO:10) and 5' GCCTGACCAACATGGAGAAACCCCATCTC (SEQ ID NO:11). Thermocycling conditions were as follows; 1 min at 94°C, followed by 30 cycles of 98°C for 10 sec and 72°C for 10 min, and a final extension of 10 min at 72°C.

REFERENCES

[0042] All patents and publications mentioned in the specification are indicative of the level of those skilled in the art to which the invention pertains. All patents and publications are herein incorporated by reference to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference.

1. Brunetti-Pierri N. and Ng P. (2015). Helper-dependent adenoviral vectors for Gene Therapy. In: Templeton, Nancy Smyth (ed). *Gene and Cell Therapy: Therapeutic Mechanisms and Strategies*, 4th edn. CRC Press: Boca Raton, FL pp. 47-84

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[0043] Although the present invention and its advantages have been described in detail, it should be understood that various changes, substitutions and alterations can be made herein without departing from the spirit and scope of the invention as defined by the appended claims. Moreover, the scope of the present application is not intended to be limited to the particular embodiments of the process, machine, manufacture, composition of matter, means, methods and steps described in the specification. As one of ordinary skill in the art will readily appreciate from the disclosure of the present invention, processes, machines, manufacture, compositions of matter, means, methods, or steps, presently existing or later to be developed that perform substantially the same function or achieve substantially the same result as the corresponding embodiments described herein may be utilized according to the present invention. Accordingly, the appended claims are intended to include within their scope such processes, machines, manufacture, compositions of matter, means, methods, or steps.

CLAIMS

What is claimed is:

1. A composition comprising a vector polynucleotide comprising an expression cassette, said expression cassette comprising:

a) a coding sequence; and

b) one or more untranslated sequences operably linked to the coding sequence, said untranslated sequences comprising a target sequence for a microRNA produced from the adenoviral viral associated RNA I (VA RNAI) (mivaRNAI) and/or a microRNA produced from the adenoviral VA RNAII (mivaRNAII).

2. The composition of claim 1, wherein the vector is a viral vector.

3. The composition of claim 2, wherein the viral vector is a helper-dependent adenoviral vector (HDAd), adeno-associated viral vector, lentiviral vector, or retroviral vector.

4. The composition of claim 1, 2, or 3, wherein the untranslated sequence is 5'-untranslated sequence, 3'-untranslated sequence, or both.

5. The composition of any one of claims 1-4, wherein the coding sequence encodes a therapeutic gene product.

6. The composition of any one of claims 1-4, wherein the coding sequence encodes a diagnostic gene product.

7. The composition of any one of claims 1-6, wherein the coding sequence encodes a gene product that is not a reporter gene product.

8. The composition of any one of claims 1-7, wherein the mivaRNAI target sequence comprises

a) the sequence 5'-AAAGGAGCACTCCCCCGTTGTCTG-3' (SEQ ID NO:1);

b) a sequence that is at least 70, 75, 80, 85, 90, 95, 96, 97, 98, or 99% identical to 5'-AAAGGAGCACTCCCCCGTTGTCTG-3' (SEQ ID NO:1); or

c) a sequence that is complementary to the sequence in a) or b).

9. The composition of claim 8, wherein the 5'-terminal end of the sequence further comprises 1, 2, 3, 4, 5, or more nucleotides.

10. The composition of claim 9, wherein the 1, 2, 3, 4, 5, or more nucleotides are A.

11. The composition of claim 8, wherein the 5'-terminal end of the sequence lacks 1, 2, 3, 4, 5, or more nucleotides of the sequence.

12. The composition of claim 8, 9, 10, or 11, wherein the 3'-terminal end of the sequence further comprises 1, 2, 3, 4, 5, or more nucleotides.

13. The composition of claim 8, 9, 10, 11 or 12, wherein the 3'-terminal end of the sequence lacks 1, 2, 3, 4, 5, or more nucleotides of the sequence.

14. The composition of any one of claims 1-7, wherein the mivaRNAI target sequence comprises:

a) the sequence 5'-AAAAGGAGCACTCCCCCGTTGTCTG-3' (SEQ ID NO:1);

b) a sequence that is at least 70, 75, 80, 85, 90, 95, 96, 97, 98, or 99% identical to 5'-AAAAGGAGCACTCCCCCGTTGTCTG-3' (SEQ ID NO:1); or

c) a sequence that is complementary to the sequence in a) or b).

15. The composition of claim 14, wherein the 5'-terminal end of the sequence further comprises 1, 2, 3, 4, 5, or more nucleotides.

16. The composition of claim 15, wherein the 1, 2, 3, 4, 5, or more nucleotides are A.

17. The composition of claim 14, wherein the 5'-terminal end of the sequence lacks 1, 2, 3, 4, 5, or more nucleotides of the sequence.

18. The composition of claim 14, 15, 16, or 17, wherein the 3'-terminal end of the sequence further comprises 1, 2, 3, 4, 5, or more nucleotides.

19. The composition of claim 14, 15, 16, or 17, wherein the 3'-terminal end of the sequence lacks 1, 2, 3, 4, 5, or more nucleotides of the sequence.

20. The composition of any one of claims 1-7, wherein the mivaRNAII target sequence comprises:

a) the sequence 5'-AGGGGCTCGTCCCTGTTTCCG-3' (SEQ ID NO:1);

b) a sequence that is at least 70, 75, 80, 85, 90, 95, 96, 97, 98, or 99% identical to 5'-AGGGGCTCGTCCCTGTTTCCG-3' (SEQ ID NO:1); or

c) a sequence that is complementary to the sequence in a) or b).

21. The composition of claim 20, wherein the 5'-terminal end of the sequence further comprises 1, 2, 3, 4, 5, or more nucleotides.

22. The composition of claim 21, wherein the 1, 2, 3, 4, 5, or more nucleotides are A.

23. The composition of claim 20, wherein the 5'-terminal end of the sequence lacks 1, 2, 3, 4, 5, or more nucleotides of the sequence.

24. The composition of claim 20, 21, 22 or 23, wherein the 3'-terminal end of the sequence further comprises 1, 2, 3, or more nucleotides.

25. The composition of claim 20, 21, 22, or 23, wherein the 3'-terminal end of the sequence lacks 1, 2, 3, 4, 5, or more nucleotides of the sequence.

26. The composition of any one of claims 1-25, wherein the vector is a helper-dependent adenoviral vector that derives from adenovirus serotype 1, adenovirus serotype 2, adenovirus serotype 5, adenovirus serotype 6, adenovirus serotype 35, or a chimera thereof.

27. A cell comprising the composition of any one of claims 1-26.

28. The cell of claim 27, wherein the cell comprises a helper virus that encodes VA RNAI, VA RNAII, or both.

29. The cell of claim 28, wherein 1) the helper virus (HV) is an E1-deleted adenovirus comprising a packaging signal flanked by loxP sites; 2) the HV comprises a packaging signal flanked by frt sites; 3) the HV comprises a pIX deletion so that only HDAd of a certain size can be packaged, 3) the HV comprises a mutated, inefficient packaging signal; or 4) the HV comprises an insertion of φC31 phage into it to delay its life cycle relative to the HDAd.

30. The cell of any one of claims, 27, 28, or 29, wherein the cell expresses Cre.

31. A method of producing the composition of any one of claims 1-26, comprising the steps of:

a) providing, obtaining, or generating an expression cassette comprising a coding sequence operably linked to a 3' untranslated region; and

b) modifying the 3' untranslated sequence to comprise a target sequence for a microRNA produced from the adenoviral viral associated RNA I (VA RNAI) (mivaRNAI) and/or a microRNA produced from the adenoviral VA RNAII (mivaRNAII).

32. The method of claim 31, further comprising the step of incorporating the expression cassette into a vector.

33. The method of claim 32, wherein the expression cassette is comprised in a helper-dependent adenoviral vector, an adenoviral associated vector, a lentiviral vector, or a retroviral vector.

34. The method of claim 32 or 33, comprising the step of transducing a cell with the vector.

35. The method of claim 34, wherein when the vector is a helper-dependent adenoviral vector or adeno-associated viral vector, the cell comprises a helper virus.

36. The method of claim 35, further comprising the step of transducing the cell with a helper virus.

37. The method of claim 35 or 36, wherein the helper virus encodes VA RNAI, VA RNAII, or both.

38. The method of claim 35, 36, or 37, wherein the helper virus is an E1-deleted adenovirus comprising a packaging signal flanked by loxP sites.

39. A method of producing a vector in a cell, comprising the steps of:

a) providing, obtaining, or generating in the cell a vector that includes an expression cassette that comprises a coding sequence operably linked to at least one untranslated region that comprises a target sequence for mivaRNAI and/or a target sequence for mivaRNAII; and

b) exposing the vector to the mivaRNAI and/or mivaRNAII under suitable conditions such that expression of the coding sequence is inhibited upon binding of the mivaRNAI and/or mivaRNAII to their respective target sequences; and

c) producing the vector.

40. The method of claim 29, wherein the vector is a helper-dependent adenoviral vector or adeno-associated viral vector.

41. The method of claim 29, wherein the mivaRNAI and/or mivaRNAII are expressed from a vector in the cell.

42. The method of claim 41, wherein the mivaRNAI and/or mivaRNAII are expressed from a helper virus.

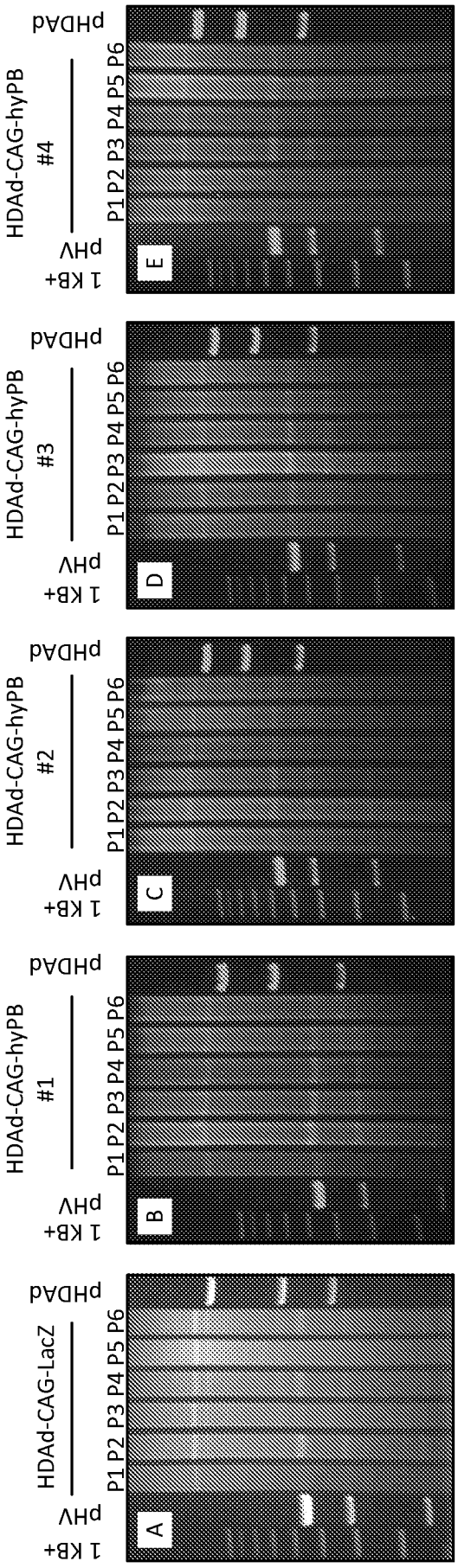

FIG. 1A

FIG. 1B

FIG. 1C

FIG. 1D

FIG. 1E

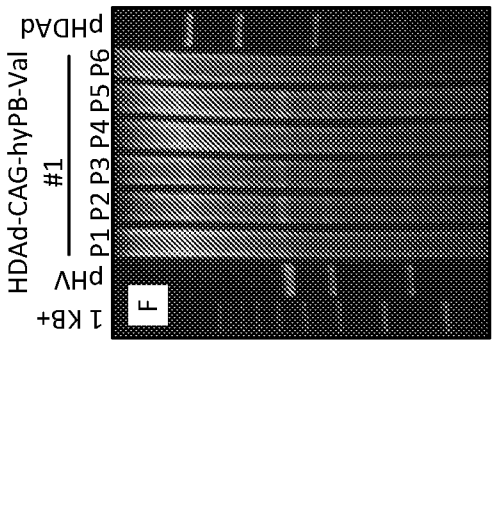

FIG. 1F

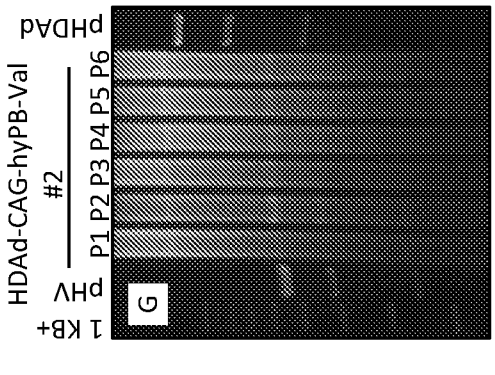

FIG. 1G

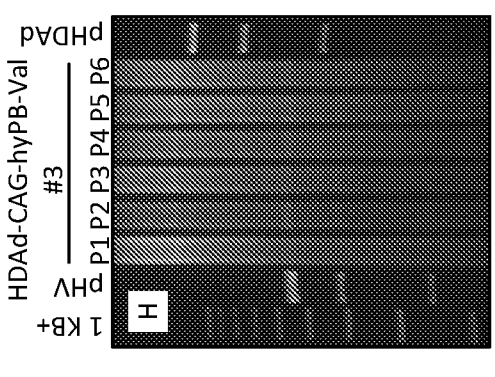

FIG. 1H

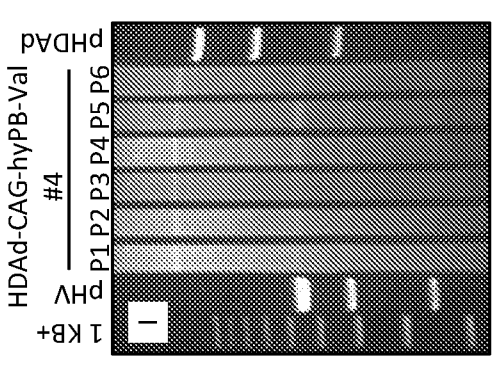

FIG. 1I

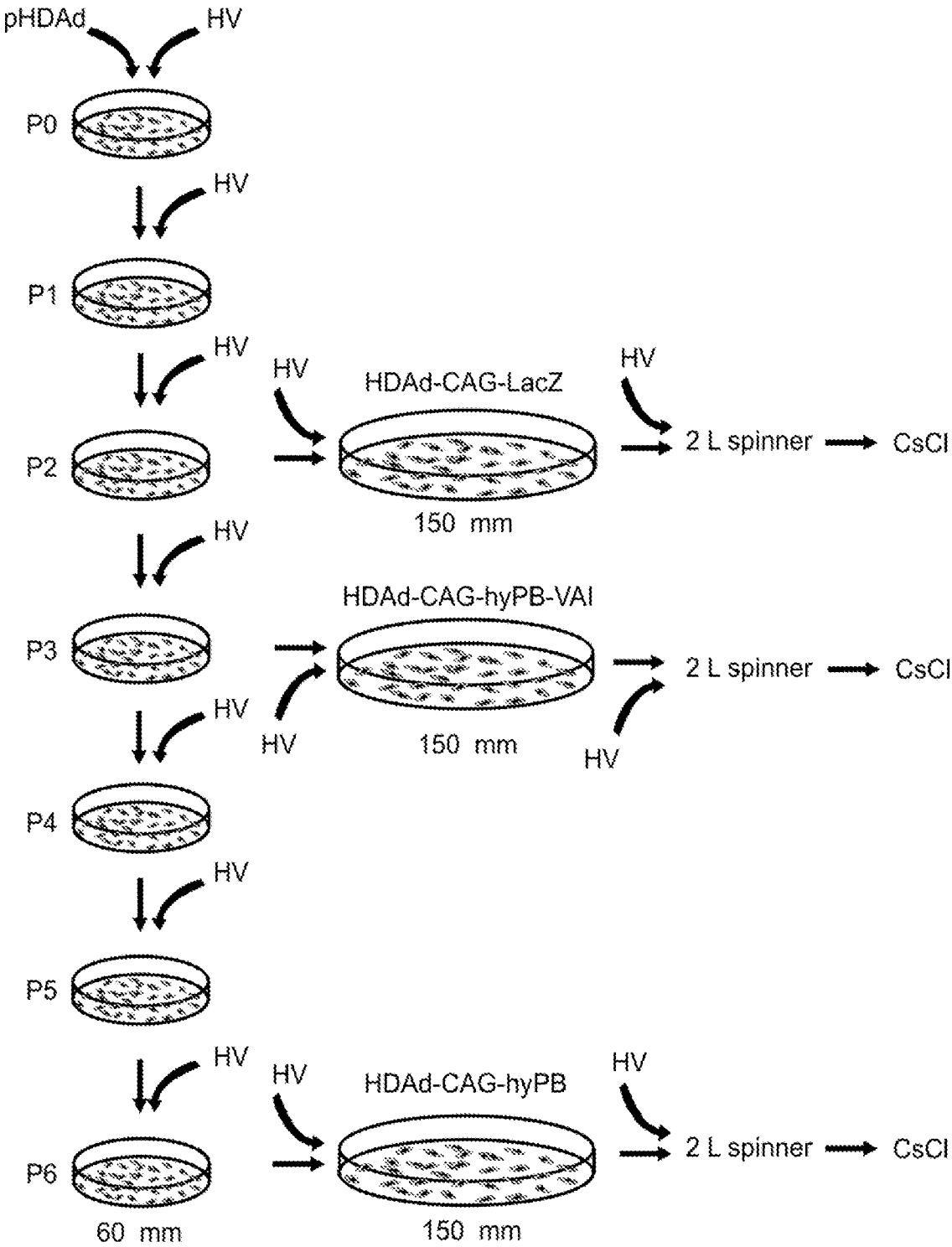
pHDAd
HV
P0
HV
P1
HV
P2
HV
HDAd-CAG-LacZ
HV
2 L spinner
CsCl
150 mm
HV
P3
HDAd-CAG-hyPB-VAI
HV
HV
2 L spinner
CsCl
150 mm
HV
P4
HV
P5
HV
HV
HDAd-CAG-hyPB
HV
P6
2 L spinner
CsCl
60 mm
150 mm

FIG. 2

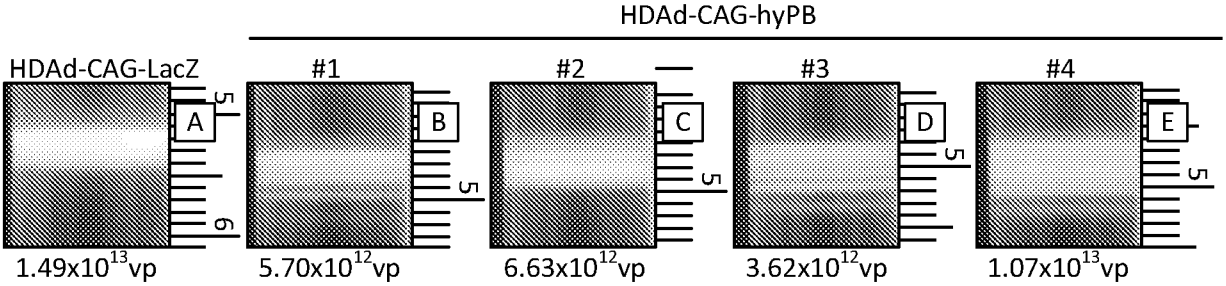

FIG. 3A FIG. 3B FIG. 3C FIG. 3D FIG. 3E

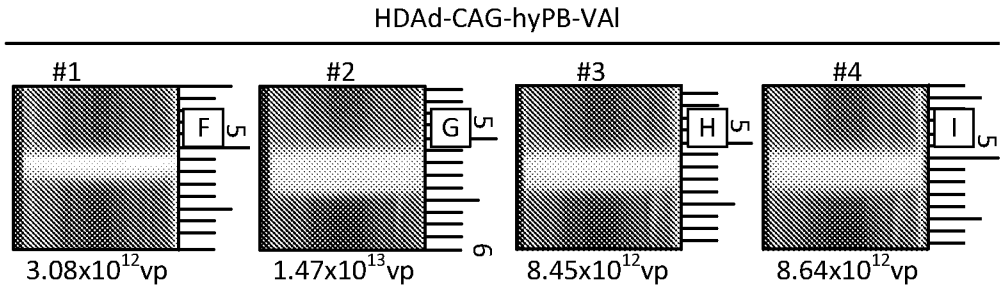

FIG. 3F FIG. 3G FIG. 3H FIG. 3I

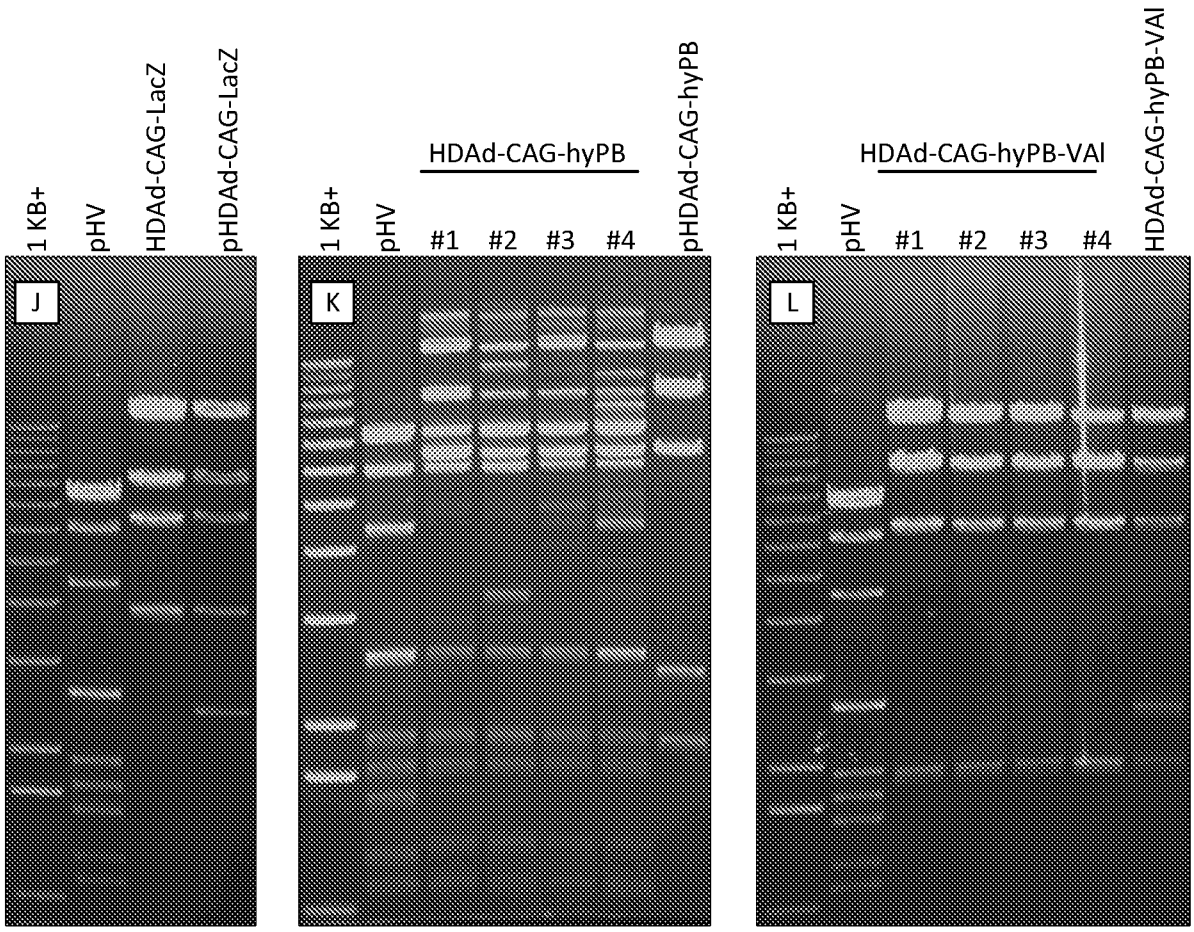

FIG. 3J FIG. 3K FIG. 3L

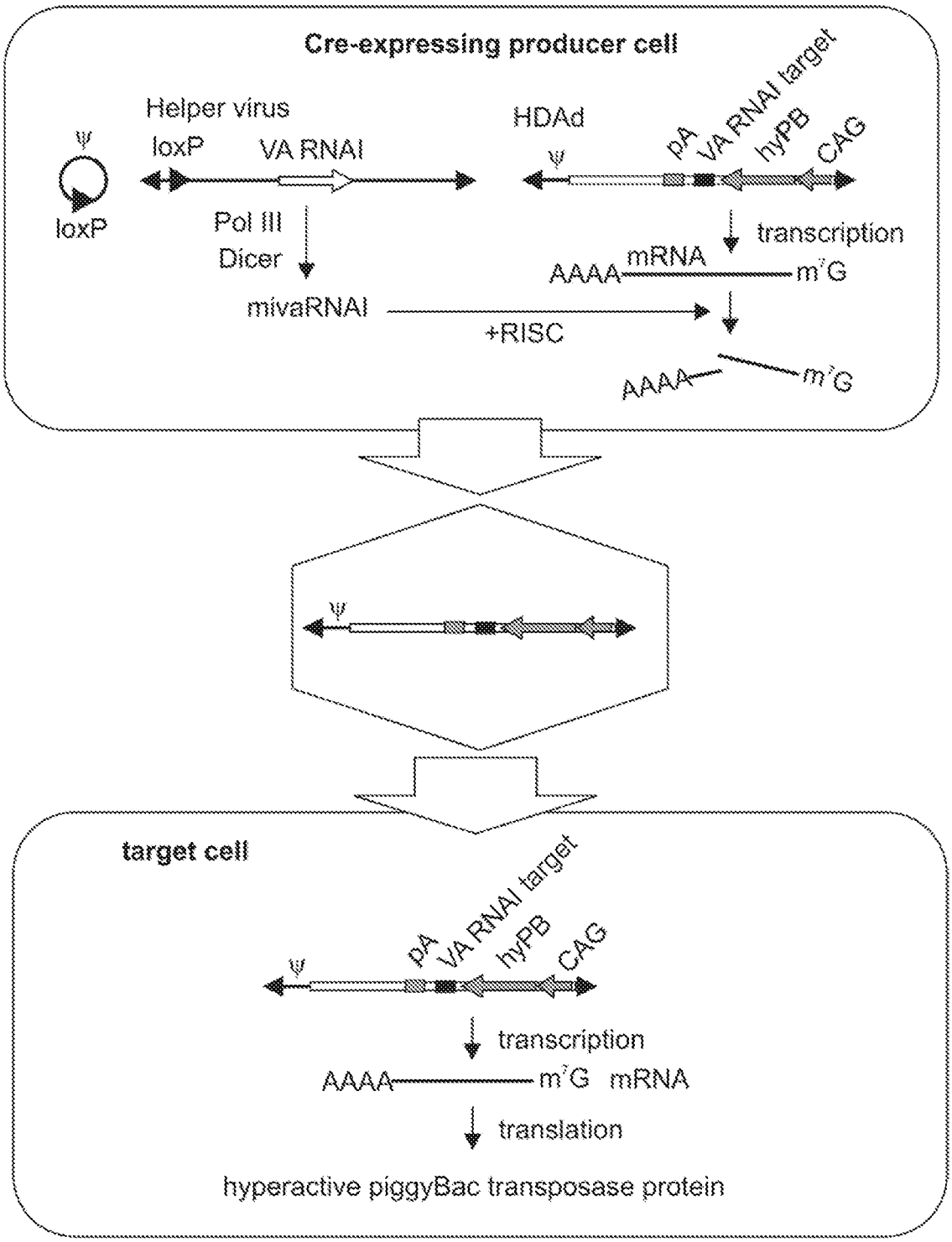
Cre-expressing producer cell
Helper virus
loxP
VA RNAI
Ψ
loxP
Pol III
Dicer
mivaRNAI
+RISC
HDAd
Ψ
pA
VA RNAI target
hyPB
CAG
transcription
AAAA
mRNA
m7G
AAAA
m7G
Ψ
target cell
Ψ
pA
VA RNAI target
hyPB
CAG
transcription
AAAA
m7G mRNA
translation
hyperactive piggyBac transposase protein

FIG. 4

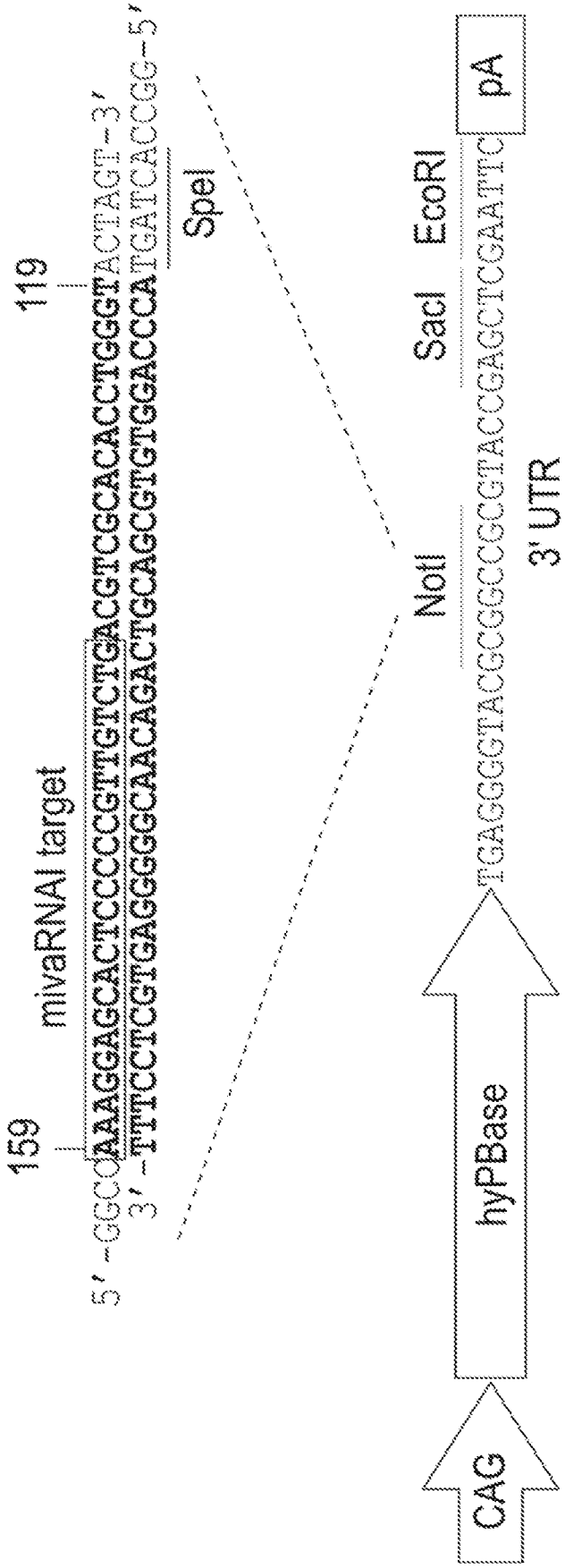
159
mivaRNAI target
119
5′ -GGCCAAAGGAGCACTCCCCGTTGTCTGACGTCGCACACCTGGGTACTAGT-3′
3′ -TTTCCTCGTGAGGGGCAACAGACTGCAGCGTGTGGACCCATGATCACCGG-5′
SpeI
NotI
SacI
EcoRI
pA
3' UTR
TGAGGGGTACGGCGGCGTACCGAGCTCGAATTC
hyPBase
CAG

FIG. 5

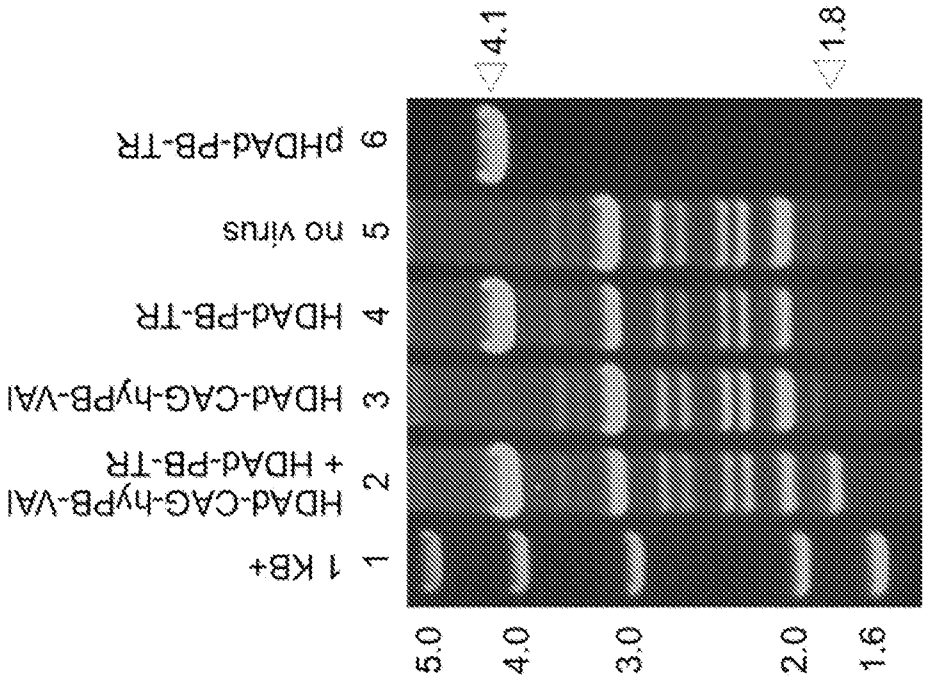

FIG. 6B

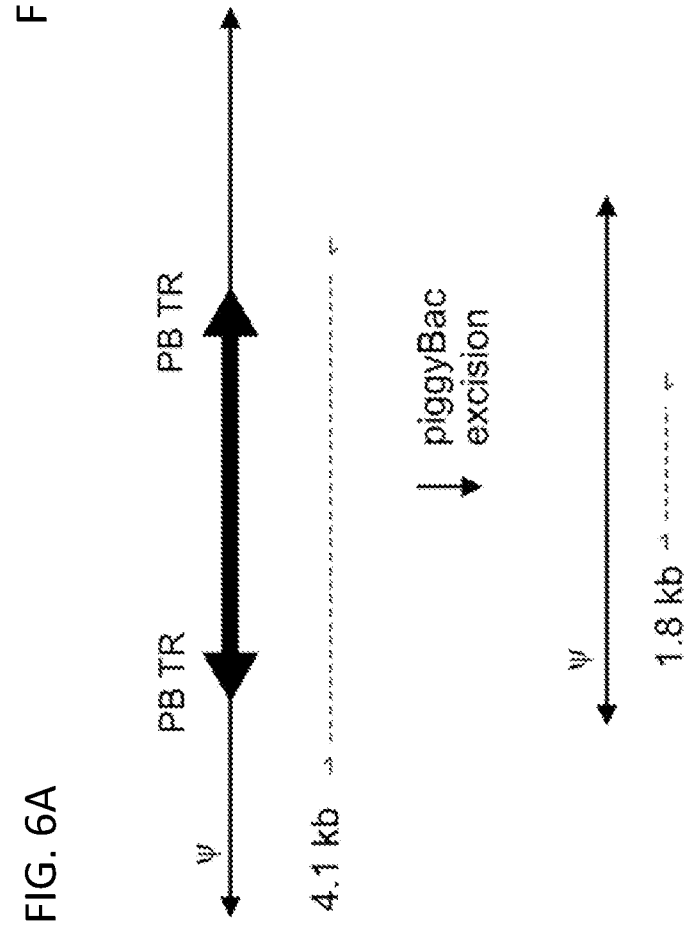

FIG. 6A

FIGS. 6A-6B

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US17/29933

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

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

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 5-38
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US17/29933

A. CLASSIFICATION OF SUBJECT MATTER

IPC - A61K 31/713, 48/005; C07H 21/04; C12N 15/113, 15/861, 15/63; G01N 33/50, 33/53 (2017.01)

CPC - A61K 31/713, 38/465, 48/005; C07H 21/04; C12N 15/113, 15/907, 15/86, 15/102, 15/869, 15/867, 15/864, 15/63, 15/64, 15/8695, 15/8676, 15/8673, 15/1082, 15/79; C12Q 1/00; G01N 33/50, 33/53, 33/574

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
See Search History document

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	(BELLUTTI, F et al.) Identification of RISC-associated adenoviral microRNAs, a subset of their direct targets, and global changes in the targetome upon lytic adenovirus 5 infection. Journal of Virology. February 2015, Vol. 89, Issue. 3; pages1608–1627; Figure 2; page 1609, 1st column, 4th and 5th paragraphs; page 1609, 2nd column, 3rd paragraph; page 1610, 1st column, 3rd paragraph; page 1614, 1st column, 1st paragraph; page 1616, 2nd column, 2nd paragraph; DOI: 10.1128/JVI.02336-14.	1-3, 4/1-3, 39-42
A	(APARICIO, O et al.) Adenovirus Virus-Associated RNA Is Processed to Functional Interfering RNAs Involved in Virus Production. Journal of Virology. February 2006, Vol. 80, Issue. 3; pages 1376–1384; DOI: 10.1128/JVI.80.3.1376–1384.2006	1-3, 4/1-3, 39-42
P, X	(PALMER, DJ et al.) Helper virus-mediated downregulation of transgene expression permits production of recalcitrant helper-dependent adenoviral vector. Methods & Clinical Development. 8 June 2016, Vol. 3, No. 16039; DOI: 10.1038/mtm.2016.39	1-3, 4/1-3, 39-42

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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

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

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

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

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
17 July 2017 (17.07.2017)	02 AUG 2017
Name and mailing address of the ISA/ Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer Shane Thomas PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774