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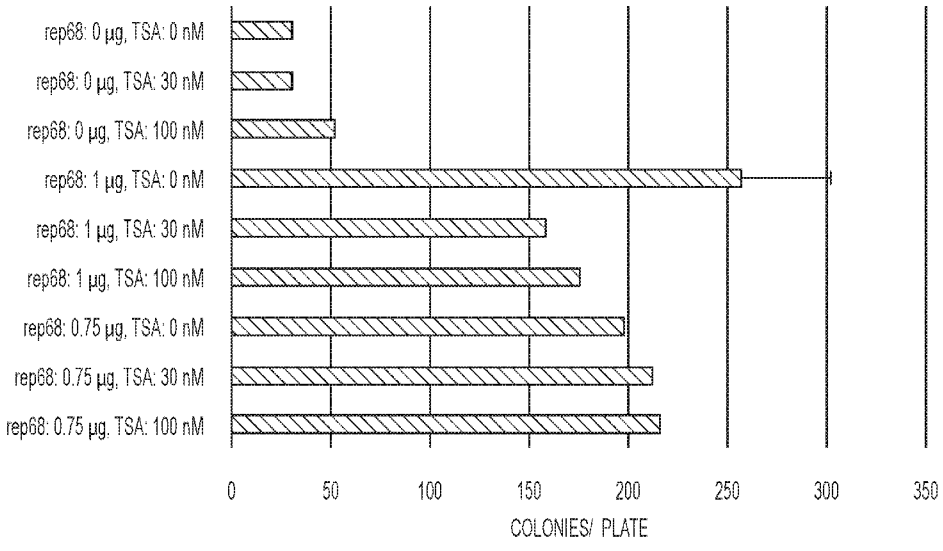
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(54) **Title:** CELL CULTURE METHODS INVOLVING HDAC INHIBITORS OR REP PROTEINS

FIG. 1



(57) **Abstract:** The invention relates to methods of culturing cells, generating cell lines, and delivering polynucleotides to cells involving the use of HDAC inhibitors and/or adeno-associated virus (AAV) rep proteins.

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CELL CULTURE METHODS INVOLVING HDAC INHIBITORS OR REP PROTEINS

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of and priority to U.S. application number 62/475,112 filed March 22, 2017, the entire content of which is hereby incorporated by reference in its entirety for all purposes.

FIELD OF THE INVENTION

[0002] The invention relates to cell culture methods involving the use of HDAC inhibitors or adeno-associated virus (AAV) rep proteins.

BACKGROUND

[0003] Adeno-associated virus (AAV) is a non-pathogenic, replication-defective parvovirus. Recombinant AAV (rAAV) vectors have many unique features that make them attractive as vectors for gene therapy. In particular, rAAV vectors can deliver therapeutic genes to dividing and non-dividing cells, and these genes can persist for extended periods without integrating into the genome of the targeted cell. Given the widespread therapeutic applications of rAAV, it is useful to produce large quantities of the virus in a cost-effective manner. Stable eukaryotic cell lines can be generated by transfecting them with rAAV vectors, which preferentially integrate into the host genome on chromosome 19. However, typically, thousands of cell clones must be screened from multiple transfection runs to identify one producer cell line with a rAAV yield high enough for developing a cost-effective manufacturing process. Accordingly, there is a need for improved methods of generating stable cell lines that produce high yield of rAAV.

[0004] Wildtype AAV genomes encode four regulatory proteins called rep78, rep68, rep52, and rep40. These proteins cumulatively include site-specific DNA binding, ATP-dependent site-specific endonuclease, helicase, and ATPase activity, which orchestrate aspects of the viral life cycle. Typically, rAAV vectors are devoid of DNA sequences encoding rep proteins, and are composed of foreign DNA flanked by the 145 nucleotide-long AAV inverted terminal repeats (ITR). Often, rAAV are produced by means of a helper virus such as adenovirus.

SUMMARY OF THE INVENTION

[0005] It has now been discovered that delivery of an AAV rep mRNA can be used, for example, to enhance generation of rAAV stable cell lines and the titer of rAAV vectors harvested from these cells. Accordingly, one aspect of the invention provides a method of increasing rAAV vector titer or enhancing rAAV yield, *e.g.,* from a eukaryotic or a host cell culture. The method includes co-delivering a rAAV vector with a rep mRNA (*e.g.,* rep68 and/or rep78 mRNA) to a eukaryotic or host cell culture and harvesting rAAV from the cell culture.

[0006] In some embodiments, the co-delivery methods include liposome-based transfection. In some embodiments, the co-delivery methods include chemical-based transfection, such as methods using calcium phosphate, cationic polymers, DEAE-dextran, or activated dendrimers. In other embodiments, the co-delivery methods involve microinjection, electroporation, magnetic beads, nanoparticles, or cell squeezing.

[0007] Another aspect of the invention provides a method of generating or developing a cell line, *e.g.,* a stable cell line for producing recombinant AAV (rAAV). The method includes co-delivering a rAAV vector with a rep mRNA (*e.g.,* rep68 and/or rep78 mRNA) to a eukaryotic or host cell culture, followed by identifying a eukaryotic or host cell that contains the rAAV vector sequence integrated into the eukaryotic or host cell genome (e.g., by selecting cell colonies that have the rAAV vector integrated into the eukaryotic or host cell genome) and, optionally, harvesting the rAAV from the cell culture. In some embodiments, co-delivery of the rAAV with the rep mRNA enhances the titer of the harvested rAAV

[0008] In some embodiments, the co-delivery methods include liposome-based transfection. In some embodiments, the co-delivery methods include chemical-based transfection, such as methods using calcium phosphate, cationic polymers, DEAE-dextran, or activated dendrimers. In other embodiments, the co-delivery methods involve microinjection, electroporation, magnetic beads or nanoparticles.

[0009] It has also been discovered that an HDAC inhibitor can be used, for example, to enhance integration of a recombinant polynucleotide into a eukaryotic or host cell genome, and/or increase yield of a gene product or viral vector from the eukaryotic or host cell. Accordingly, one aspect of the invention provides a method including co-delivering a recombinant polynucleotide with an HDAC inhibitor such as trichostatin A (TSA) to a

eukaryotic or a host cell culture. In some embodiments, integration of the polynucleotide into a eukaryotic or host cell genome is detected and/or a stable eukaryotic or host cell line is generated. In some embodiments, one or more gene products are expressed in the eukaryotic or host cell culture. The gene products can be encoded by the recombinant polynucleotide or indirectly dependent on the presence of the recombinant polynucleotide. The gene products can be, for example, ribonucleic acids, peptides, or proteins. In some embodiments, the recombinant polynucleotide is a viral vector, including but not limited to a rAAV vector. Co-delivery of a recombinant viral vector with an HDAC inhibitor may enhance the integration of the viral vector into the host cell genome and increase the yield of viral vector harvested from the host cell.

[0010] In some embodiments, the co-delivery methods involve liposome-based transfection or chemical-based transfection, such as methods utilizing calcium phosphate, cationic polymers, DEAE-dextran, or activated dendrimers. In other embodiments, the co-delivery methods involve microinjection, electroporation, magnetic beads, nanoparticles, or cell squeezing.

[0011] These and other aspects and features of the invention are described in the following detailed description and claims.

BRIEF DESCRIPTION OF THE DRAWINGS

[0012] **FIG. 1** is a quantification of HeLa S3 cell colonies that contain integrated rAAV genome after co-transfection of rAAV vectors with rep68 mRNA and/or TSA by electroporation. Results represent the number of colonies grown on day 10 after transfection on a 96 well plate. The number of colonies under the transfection condition of 1 μg of rep68 mRNA without TSA was an average from ten 96 well plates (+/- Standard Deviation)

[0013] **FIG. 2** shows quantification of DNase-Resistant Particles by qPCR. rAAV was produced by Hela S3 colonies established after co-delivery of rAAV vectors with rep68 mRNA and/or TSA by electroporation. Lower C_t value indicates higher yield of rAAV produced from the host cells. Mean C_t values and distributions of C_t values are shown.

[0014] **FIG. 3** shows amplified rAAV genome copy (GC) assayed by qPCR in four cell lines derived from HeLa cells after co-transfection of rAAV vectors with rep68 mRNA and/or TSA: 1 μg rep68 mRNA - 8G3 clone, 30 nM TSA - 12B5 clone, and 0.75 μg rep68 mRNA + 100 nM TSA - 16D2 clone. Cell line 10G8 was established after transfection of rAAV vectors alone. After infection with an adenoviral helper virus, cells were collected on

days 0, 1, 2, 3, and 4 (D0, D1, D2, D3, D4) post-infection, from which genomic DNA was isolated for the assay. Error bars indicate standard deviations from triplicates of qPCR.

[0015] **FIG. 4** shows rAAV titers produced in the same four cell lines described in FIG.3. After the helper virus infection, supernatants were collected from the cells, and DNase-Resistant Particles (DRP) were quantified by qPCR giving rAAV genome copy per ml (GC/ml) of supernatant.

DETAILED DESCRIPTION

[0016] The invention is based, in part, on the discovery that an HDAC inhibitor such as trichostatin A can be co-delivered with a recombinant polynucleotide to a eukaryotic cell, which can, for example, promote integration and/or expression of the polynucleotide in the eukaryotic cell. The invention is also based, in part, on the discovery that, a rep mRNA (*e.g.,* rep68 and rep78) and/or an HDAC inhibitor can be co-delivered with a rAAV vector to a host cell and, for example, enhance delivery and integration of the rAAV into the host cell, amplification of the rAAV in the host cell and/or rAAV titer produced by the host cell.

[0017] Various features and aspects of the invention are discussed in more detail below.

I. INCREASING PRODUCTION OF rAAV

[0018] One aspect of the invention provides a method of increasing rAAV titer or enhancing rAAV yield, *e.g.,* from a eukaryotic or a host cell culture. The method includes co-delivering a rAAV vector with a rep mRNA (*e.g.,* rep68 and/or rep78 mRNA) to a eukaryotic or host cell culture and harvesting rAAV from the cell culture. In certain embodiments, the method includes co-delivering a rAAV vector with a rep mRNA (*e.g.,* rep68 and/or rep78 mRNA) to a eukaryotic or host cell culture, culturing the eukaryotic or host cell culture under conditions that allow for generation of rAAV, and harvesting the rAAV from the cell culture.

[0019] Another aspect of the invention provides a method of increasing rAAV vector titer or enhancing rAAV yield, *e.g.,* from a eukaryotic or a host cell culture. The method includes co-delivering a rAAV vector with an HDAC inhibitor (*e.g.,* TSA) to a eukaryotic or host cell culture and harvesting rAAV from the cell culture. In some embodiments, the method includes co-delivering a rAAV vector with an HDAC inhibitor (*e.g.,* TSA) to a eukaryotic or host cell culture, culturing the eukaryotic or host cell culture under conditions that allow for generation of further rAAV, and harvesting the rAAV from the cell culture.

[0020] Another aspect of the invention provides a method of increasing rAAV vector titer or enhancing rAAV yield, *e.g.,* from a eukaryotic or a host cell culture. The method includes co-delivering a rAAV vector with a rep mRNA (*e.g.,* rep68 and/or rep78 mRNA) and an HDAC inhibitor (*e.g.,* TSA) to a eukaryotic or host cell culture and harvesting rAAV from the cell culture. In some embodiments, the method includes co-delivering a rAAV vector with a rep mRNA (*e.g.,* rep68 and/or rep78 mRNA) and an HDAC inhibitor (*e.g.,* TSA) to a eukaryotic or host cell culture, culturing the eukaryotic or host cell culture under conditions that allow for generation of further rAAV, and harvesting the rAAV from the cell culture.

[0021] Adeno-associated virus (AAV) is a small, nonenveloped icosahedral virus of the genus Dependoparvovirus and family Parvovirus. It is capable of infecting both dividing and quiescent cells of several tissue types, with different AAV serotypes exhibiting different tissue tropism. AAV is non-autonomously replicating, and has a life cycle with a latent phase and an infectious phase. In the latent phase, after a cell is infected with an AAV, the AAV integrates into the host's genome as a provirus. In human cells, the preferential integration site resides on a region of roughly 2-kb on the long arm of chromosome 19 (19q13.3-qter). The infectious phase does not occur unless the cell is also infected with a helper virus (for example, adenovirus or herpes simplex virus), which activates AAV gene expression leading to the excision of the provirus DNA from the host cell chromosome, followed by replication and packaging of the viral genome. Upon helper virus-induced cell lysis, the newly assembled viruses are released.

[0022] Wild-type AAV has a single-stranded linear DNA genome of approximately 4.7 kb, which contains two 145 nucleotide-long inverted terminal repeats (ITRs) at the termini flanking the two viral genes – rep and cap. The rep gene, through the use of two promoters and alternative splicing, encodes four regulatory proteins - rep78, rep68, rep52 and rep40. These proteins are involved in AAV genome replication. The cap gene, through alternative splicing and initiation of translation, gives rise to three capsid proteins, VP1, VP2 and VP3. These proteins form the capsid of the AAV particle.

[0023] Given that the AAV ITRs contain all *cis*-acting elements involved in genome rescue, replication and packaging, and that the AAV ITRs are segregated from the viral encoding regions, *i.e.*, rep and cap gene regions, some or all of the AAV 4.3 kb internal genome could be replaced with foreign DNA, for example, an expression cassette for an exogenous protein of interest to develop recombinant AAV (rAAV) vectors. rAAV vectors may have single-stranded genomes that are 4.7 kb in size, or are larger or smaller than 4.7 kb,

including oversized genomes that are as large as 5.2 kb, or as small as 3.0 kb. Further, vector genomes may be substantially self-complementary, so that within the virus the genome is substantially double stranded. rAAV vectors containing genomes of all types are suitable for use in the method of the current invention.

[0024] AAV includes numerous serologically distinguishable types including serotypes AAV-1 to AAV-12, as well as more than 100 serotypes from nonhuman primates (See, *e.g.*, Srivastava, J. Cell Biochem., 105(1): 17–24 (2008), and Gao *et al.*, J. Virol., 78(12), 6381–6388 (2004)). AAV of any serotype may be used in the present invention, and a person of skill in the art will be able to identify AAV types suitable for the production of their desired rAAV vectors.

[0025] rAAV vectors can be co-delivered with a rep mRNA (*e.g.,* rep68 mRNA and/or rep78 mRNA) to a cell culture, *e.g.,* a host or eukaryotic cell culture, using any suitable method known in the art. rAAV vectors can be co-delivered an HDAC inhibitor to a cell culture, *e.g.,* a host or eukaryotic cell culture, using any suitable method known in the art. rAAV vectors can be co-delivered with a rep mRNA (*e.g.,* rep68 mRNA and/or rep78 mRNA) and an HDAC inhibitor to a cell culture, *e.g.,* a host or eukaryotic cell culture, using any suitable method known in the art.

[0026] As used herein, a eukaryotic cell culture can be any cell or cells capable of producing a rAAV. In some embodiments, the host cell is a mammalian cell, for example, a HeLa cell, COS cell, HEK293 cell, A549 cell, BHK cell, or Vero cell. In other embodiments, the host cell is an insect cell, for example, a Sf9 cell, Sf-21 cell, Tn-368 cell, or BTI-Tn-5B1-4 (High-Five) cell (See *e.g.* U.S. Pat. Nos. 6,156,303, 5,387,484, 5,741,683, 5,691,176, 5,688,676, 8,163,543, U.S. Publication No. 20020081721, PCT Publication Nos. WO00/47757, WO00/24916, and WO96/17947). Unless otherwise indicated, the terms "cell" or "cell line" are understood to include modified or engineered variants of the indicated cell or cell line.

[0027] Rep mRNA (*e.g.,* rep68 and/or rep78 mRNA can be produced, for example, by *in vitro* transcription (Howden et al, J Gene Med 2008; 10:42-50).

[0028] HDAC inhibitors as used herein can be inhibitors of any one of the four classes of HDACs. Exemplary HDAC inhibitors include trichostatin A (TSA), trapxin B, benzamides, phenylbutyraet, valproic acid, hydroxamic acid vorinostate (SAHA), belinostat, LAQ824,

panobinostat, entinostat, CI994, mocetinostat, nicotinamide, dihydrocoumarin, naphthopyranone, and 2-hydroxynaphthaldehydes.

[0029] Methods of co-delivery vary depending on the target cells. Suitable methods include, for example: liposome-based transfection; chemical-based transfection, such as methods utilizing calcium phosphate, cationic polymers, DEAE-dextran, or activated dendrimers; microinjection; electroporation; magnetic beads; nanoparticles; or cell squeezing (see, for example, Ding *et al.* (2017) Nat. Biomed. Eng. 1:0039). A person of skill in the art will be able to identify suitable co-delivery methods and optimize the conditions according to the types of cells they intend to deliver rAAV vectors to.

[0030] In some embodiments, rAAV vectors are co-delivered to HelaS3 cells with rep68 mRNA using electroporation. In some embodiments, rAAV vectors are co-delivered to HelaS3 cells with an HDAC inhibitor, *e.g.,* TSA, using electroporation. In some embodiments, rAAV vectors are co-delivered to HelaS3 cells with both rep68 mRNA and TSA using electroporation.

[0031] After delivery to eukaryotic cell cultures, rAAV vectors may be produced by the cells by many methods known in the art. To allow for production of rAAV, the host cell must be provided with AAV inverted terminal repeats (ITRs) (which may, for example, flank a heterologous nucleotide sequence of interest or an expression cassette encoding an exogenous protein of interest), AAV rep and cap gene functions, as well as additional helper functions. These can be provided to the host cell using a variety of appropriate plasmids or vectors. Additional helper functions can be provided by, for example, an adenovirus (AV) infection, by a plasmid that carries all of the required AV helper function genes, or by other viruses such as herpes simplex virus (HSV) or baculovirus. Any genes, gene functions, or other genetic material necessary for rAAV production by the host cell may transiently exist within the host cell, or be stably inserted into the host cell genome. In some embodiments, the host cell is a producer cell comprising AAV rep and cap gene functions and a rAAV vector genome. In some embodiments, the host cell is a packaging cell comprising AAV rep and cap gene functions, which at the time of production is provided a rAAV vector genome by a separate recombinant virus. rAAV production methods suitable for use with the methods of the current invention include those disclosed in Clark *et al.*, Human Gene Therapy 6:1329–1341 (1995), Martin *et al.*, Human Gene Therapy Methods 24:253–269 (2013), Thorne *et al.*, Human Gene Therapy 20:707–714 (2009), Fraser Wright, Human Gene Therapy 20:698–706 (2009), and Virag *et al.*, Human Gene Therapy 20:807–817 (2009).

[0032] rAAV particles may be obtained from host cells by lysing the cells. Lysis of AAV-infected cells can be accomplished by methods that chemically or enzymatically treat the cells in order to release infections viral particles. These methods include the use of nucleases such as benzonase or DNAse, proteases such as trypsin, or detergents or surfactants. Physical disruption, such as homogenization or grinding, or the application of pressure via a microfluidizer pressure cell, or freeze-thaw cycles may also be used. Alternatively, supernatant may be collected from AAV-infected cells without the need for cell lysis.

[0033] It may be necessary to purify the sample containing rAAV and helper virus particles to remove, for example, the cellular debris resulting from cell lysis. Methods of minimal purification of helper virus and AAV particles are known in the art, and any appropriate method can be used to prepare samples containing both AAV and helper virus particles for use in the methods of the present invention. Two exemplary purification methods are cesium chloride (CsCl)- and iodixanol-based density gradient purification. Both methods are described in Strobel *et al.*, Human Gene Therapy Methods., 26(4): 147–157 (2015). Minimal purification can also be accomplished using affinity chromatography using, for example AVB Sepharose affinity resin (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Methods of AAV purification using AVB Sepharose affinity resin are described in, for example, Wang *et al.*, Mol Ther Methods Clin Dev., 2:15040 (2015).

[0034] It may be necessary to inactivate helper virus by heat. Heat inactivation techniques are based on the different thermal stabilities of AAV and helper virus particles. For example, AAV particles can be heated to temperatures as high as 56°C and still remain intact, while AV particles are rendered inactive. Conway *et al.*, Gene Therapy 6, 986–993, 1999, describes differential heat inactivation of HSV in AAV containing samples. Heat inactivation may be accomplished by any known methodology. In the examples described below, heat inactivation was accomplished using a thermocycler to rapidly heat and cool sample volumes of 300 µL or less. This system was chosen because it relies on heat transfer that is primarily conductive, making it a viable model for both continuous-flow systems and for larger batch systems that employ active mixing. Examples of continuous-flow systems include passage of the sample through a continuous-flow heat exchanger, such as the DHX™ Single-Use Heat Exchanger for Bio-therapeutic Manufacturing (Thermo Fisher Scientific, Millersburg, PA). Such systems allow the operator to control the heat inactivation process by controlling the flow rate of the sample through the heat exchanger, thus controlling the

duration of the heating process, and the temperature of the heat exchanger, thus controlling the temperature of heat inactivation.

[0035] Alternatively, heat inactivation may be accomplished using batch systems of various sizes. For example, heat inactivation may be accomplished at the 1L scale, by placing the AAV containing sample in a 1L PETG bottle and placing the bottle in a water bath set at the desired inactivating temperature for the desired period of time, with mixing, for example, the samples may be heated to 47°C for 20 minutes. At a larger scale, heat inactivation may be accomplished by placing the rAAV containing sample in a 5L bioprocessing bag on a temperature controlled rocking platform set at the desired inactivating temperature, for the desired period of time. For example, the rocking platform may be set to 49°C at a rocking speed of 30 RPM, with a 12° angle of mixing for 40 minutes.

[0036] Heat inactivation may occur at any temperature where there is a sufficient difference in stability between rAAV particles and helper virus particles that helper virus particles are substantially inactivated while active rAAV particles remain. A person of skill in the art will understand that higher temperatures may be required to achieve greater levels of AV reduction.

[0037] Once heat inactivation has been accomplished, it may be necessary or desirable to determine the efficiency of inactivation. The efficacy of an inactivation protocol is determined by assays that detect the presence of replication competent helper virus, such as a plaque assay. Plaque assays for helper virus are well known to those in the art, including plaque assays for AV, HSV, baculovirus, and others. Plaque assays of adenovirus may be conducted using any appropriate cell type, for example HeLa or HEK293 cells. Standard plaque assay protocols are described in, for example, Current Protocols in Human Genetics, 2003. Alternative assays for measuring adenoviral titers include those that allow the identification of infected cells in culture by detecting viral proteins, such as hexon proteins, using immunocytochemical staining. Such assays include the QuickTiter™ Adenovirus Titer Immunoassay Kit (Cell Biolabs, San Diego, CA). The efficiency of inactivation is generally reported as the log reduction of virus (LRV).

[0038] Quantification of rAAV particles is complicated by the fact that AAV infection does not result in cytopathic effect *in vitro*, and therefore plaque assays cannot be used to determine infectious titers. AAV particles can be quantified using a number of methods, however, including quantitative polymerase chain reaction (qPCR) (Clark *et al.*, Hum. Gene

Ther. 10, 1031–1039 (1999)) or dot-blot hybridization (Samulski *et al.*, J. Virol. 63, 3822–3828 (1989)), or by optical density of highly purified vector preparations (Sommer *et al.*, Mol. Ther. 7, 122–128 (2003)). DNase-resistant particles (DRP) can be quantified by real-time quantitative polymerase chain reaction (qPCR) (DRP-qPCR) in a thermocycler (for example, an iCycler iQ 96-well block format thermocycler (Bio-Rad, Hercules, CA)). Samples containing AAV particles are incubated in the presence of DNase I (100 U/ml; Promega, Madison, WI) at 37°C for 60 min, followed by proteinase K (Invitrogen, Carlsbad, CA) digestion (10 U/ml) at 50°C for 60 min, and then denatured at 95°C for 30 min. The primer–probe set used should be specific to a non-native portion of the AAV vector genome, for example, the poly(A) sequence of the protein of interest. The PCR product can be amplified using any appropriate set of cycling parameters, based on the length and composition of the primers, probe, and amplified sequence. Alternative protocols are disclosed in, for example, Lock *et al.*, Human Gene Therapy Methods 25(2): 115–125 (2014).

[0039] The infectivity of rAAV particles can be determined using a $TCID_{50}$ (tissue culture infectious dose at 50%) assay, as described for example in Zhen *et al.*, Human Gene Therapy 15:709–715 (2004). In this assay, AAV vector particles are serially diluted and used to co-infect a rep/cap-expressing cell line along with AV particles in 96-well plates. 48 hours post-infection, total cellular DNA from infected and control wells is extracted. AAV vector replication is then measured using qPCR with transgene-specific probe and primers. $TCID_{50}$ infectivity per milliliter ($TCID_{50}$/ml) is calculated with the Kärber equation, using the ratios of wells positive for AAV at 10-fold serial dilutions.

II. GENERATING STABLE CELL LINES

[0040] Another aspect of the invention provides a method of generating or developing a cell line, *e.g.,* a stable cell line for producing recombinant AAV (rAAV). The method includes co-delivering a rAAV vector with a rep mRNA (*e.g.,* rep68 and/or rep78 mRNA) to a eukaryotic or host cell culture, followed by identifying a eukaryotic or host cell that contains the rAAV vector sequence integrated into the eukaryotic or host cell genome (e.g., by selecting cell colonies that have the rAAV vector integrated into the eukaryotic or host cell genome) and, optionally, harvesting the rAAV from the cell culture. In some embodiments, the method includes co-delivering a rAAV vector with a rep mRNA to a eukaryotic or host cell culture, culturing the eukaryotic or host cell culture under conditions that allow for integration of the rAAV vector into the eukaryotic or host cell genome, identifying a eukaryotic or host cell that contains the rAAV vector sequence integrated into

the eukaryotic or host cell genome, culturing the eukaryotic or host cell under conditions that allow for generation of further rAAV, and, optionally, harvesting the rAAV from the cell culture. In some embodiments, co-delivery of the rAAV with the rep mRNA enhances integration of rAAV vector into the eukaryotic or host cell genome. In some embodiments, co-delivery of the rAAV with the rep mRNA enhances the titer of the harvested rAAV.

[0041] Another aspect of the invention provides a method of generating or developing a cell line, *e.g.,* a stable cell line for producing recombinant AAV (rAAV). The method includes co-delivering a rAAV vector with an HDAC inhibitor (*e.g.,* TSA) to a eukaryotic or host cell culture, followed by identifying a eukaryotic or host cell that contains the rAAV vector sequence integrated into the eukaryotic or host cell genome (e.g., by selecting cell colonies that have the rAAV vector integrated into the eukaryotic or host cell genome) and, optionally, harvesting the rAAV from the cell culture. In some embodiments, the method includes co-delivering a rAAV vector with an HDAC inhibitor (*e.g.,* TSA) to a eukaryotic or host cell culture, culturing the eukaryotic or host cell culture under conditions that allow for integration of the rAAV vector into the eukaryotic or host cell genome, identifying a eukaryotic or host cell that contains the rAAV vector sequence integrated into the eukaryotic or host cell genome, culturing the eukaryotic or host cell under conditions that allow for generation of further rAAV, and, optionally, harvesting the rAAV from the cell culture. In some embodiments, co-delivery of the rAAV with the HDAC inhibitor (*e.g.,* TSA) enhances integration of rAAV vector into the eukaryotic or host cell genome. In some embodiments, co-delivery of the rAAV with the HDAC inhibitor (*e.g.,* TSA) enhances the titer of the harvested rAAV.

[0042] Another aspect of the invention provides a method of generating or developing a cell line, *e.g.,* a stable cell line for producing recombinant AAV (rAAV). The method includes co-delivering a rAAV vector with a rep mRNA (*e.g.,* rep68 and/or rep78 mRNA) and an HDAC inhibitor (*e.g.,* TSA) to a eukaryotic or host cell culture, followed by identifying a eukaryotic or host cell that contains the rAAV vector sequence integrated into the eukaryotic or host cell genome (e.g., by selecting cell colonies that have the rAAV vector integrated into the eukaryotic or host cell genome) and, optionally, harvesting the rAAV from the cell culture. In some embodiments, the method includes co-delivering a rAAV vector with a rep mRNA (*e.g.,* rep68 and/or rep78 mRNA) and an HDAC inhibitor (*e.g.,* TSA) to a eukaryotic or host cell culture, culturing the eukaryotic or host cell culture under conditions that allow for integration of the rAAV vector into the eukaryotic or host cell genome,

identifying a eukaryotic or host cell that contains the rAAV vector sequence integrated into the eukaryotic or host cell genome, culturing the eukaryotic or host cell under conditions that allow for generation of further rAAV, and, optionally, harvesting the rAAV from the cell culture. In some embodiments, co-delivery of the rAAV with the rep mRNA (*e.g.,* rep68 and/or rep78 mRNA) and the HDAC inhibitor (*e.g.,* TSA) enhances integration of rAAV vector into the eukaryotic or host cell genome. In some embodiments, co-delivery of the rAAV with the rep mRNA (*e.g.,* rep68 and/or rep78 mRNA) and the HDAC inhibitor (*e.g.,* TSA) enhances the titer of the harvested rAAV.

[0043] Another aspect of the invention provides a method of generating or developing a cell line, *e.g.,* a stable cell line for producing recombinant AAV (rAAV). The method includes co-delivering a rAAV vector with a rep mRNA (*e.g.,* rep68 and/or rep78 mRNA) and an HDAC inhibitor (*e.g.,* TSA) to a eukaryotic or host cell culture, followed by identifying a eukaryotic or host cell that contains the rAAV vector sequence integrated into the eukaryotic or host cell genome (e.g., by selecting cell colonies that have the rAAV vector integrated into the eukaryotic or host cell genome) and, optionally, harvesting the rAAV from the cell culture.

[0044] After transfection, integration of the rAAV into the host genome can be assayed by various methods, such as antibiotic selection, fluorescence-activated cell sorting, southern blot, PCR based detection, fluorescence in situ hybridization as described by Nakai et al, Nature Genetics (2003) 34, 297-302; Philpott et al, Journal of Virology (2002) 76(11):5411-5421, and Howden et al, J Gene Med 2008; 10:42-50. Furthermore, stable cell lines can be established according to protocols well known in the art, such as those described in Clark, Kidney International Vol 61 (2002):S9-S15, and Yuan et al, Human Gene Therapy 2011 May;22(5):613-24.

III. CO-DELIVERING RECOMBINANT POLYNUCLEOTIDES WITH HDAC INHIBITORS

[0045] Another aspect of the invention provides a method including co-delivering a recombinant polynucleotide with an HDAC inhibitor such as trichostatin A (TSA) to a eukaryotic or a host cell culture. In some embodiments, the method includes co-delivering a recombinant polynucleotide with an HDAC inhibitor to a eukaryotic or a host cell culture, culturing the eukaryotic or host cell culture under conditions that allow for integration of the polynucleotide into the eukaryotic or host cell genome, detecting the integration of the

polynucleotide into the eukaryotic or host cell genome, and/or generating a stable eukaryotic or host cell line.

[0046] In some embodiments, one or more gene products are expressed in the eukaryotic or host cell culture. The gene products can be encoded by the recombinant polynucleotide or indirectly dependent on the presence of the recombinant polynucleotide. The gene products can be, for example, ribonucleic acids, peptides, or proteins.

[0047] In some embodiments, the recombinant polynucleotide is a viral vector, for example, a rAAV vector. Co-delivery of a recombinant viral vector with an HDAC inhibitor may enhance the integration of the viral vector into the host cell genome and increase the yield of virus harvested from the host cell.

[0048] Recombinant polynucleotides as used herein can be DNA, RNA, hybrids, and chimeras thereof. They may be single-stranded or double-stranded. In some embodiments, they may be an expression vector in which a nucleotide sequence encoding a desired protein is integrated downstream of the nucleotide sequence encoding the signal peptide, and is capable of high-level expression of the protein downstream of the nucleotide sequence of the signal peptide. The present invention is suitable for any vectors such as commonly used plasmids and viruses. Examples of such vectors may include pUC19, pUC18, pBR322, pHSG299, pHSG298, pHSG399, pHSG398, RSF1010, pMW119, pMW118, pMW219, pMW218, and vectors based on adeno-associated virus, adenovirus, lentivirus, and retrovirus. The recombinant polynucleotide of the present invention may further incorporate other nucleotide sequences commonly used in expression vectors, such as a promoter, terminator, Kozak sequence, multicloning site, drug-resistant gene, and other nucleotide sequences.

[0049] HDAC inhibitors as used herein can be inhibitors acting on any one of the four classes of HDACs. Examples include trichostatin A, trapxin B, benzamides, phenylbutyraet, valproic acid, hydroxamic acids vorinostat (SAHA), belinostat (PXD101), LAQ824, panobinostat (LBH589), entinostat (MS-275), CI994, mocetinostat (MGCD0103), nicotinamide, dihydrocoumarin, naphthopyranone, and 2-hydroxynaphthaldehydes.

[0050] The HDAC inhibitor and the recombinant polynucleotide could be coadministered to any eukaryotic cell culture, including cell lines of mammalian origin, insect origin or yeast origin; primary cells or stem cells capable of growth in culture. Co-delivery methods of polynucleotides and an HDAC inhibitor depend largely on the target cell types and polynucleotides. They can include: liposome-based transfection; chemical-based

transfection, such as methods utilizing calcium phosphate, cationic polymers, DEAE-dextran, or activated dendrimers; microinjection; electroporation; magnetic beads; nanoparticles; or cell squeezing. A person of skill in the art will be able to identify suitable co-delivery methods according to the types of cells to which they intend to deliver the polynucleotides.

[0051] After co-delivering polynucleotides with an HDAC inhibitor into a eukaryotic cell culture, integration of the polynucleotides into the host genome can be assayed by various methods well known in the art, such as antibiotic selection, fluorescence-activated cell sorting, PCR based method, fluorescence in situ hybridization as descried by Wang et al, *Gene Therapy* (2004) 11, 711–721; Philpott et al, Journal of Virology (2002) 76(11):5411-5421; Howden et al, J Gene Med 2008; 10:42-50. In addition, stable cell lines can be established according to protocols described in Longo et al. Methods Enzymol 2013; 529: 209-226; and Yuan et al, Human Gene Therapy 2011 May;22(5):613-24. In some embodiments, one or more gene products in the host cells are expressed. The gene products can be encoded by the recombinant polynucleotides or indirectly dependent on the presence of the recombinant polynucleotides introduced into the host cells. The gene products which vary from ribonucleic acids, peptides, to proteins can be detected by methods well established in the art, such as RT-PCR, Northern blot, Western blot, Southern blot, immunofluorescence and ELISA. In some embodiments, the polynucleotides are viral vectors, and their production, isolation, and quantification are known in the art.

[0052] Throughout the description, where compositions are described as having, including, or comprising specific components, or where processes and methods are described as having, including, or comprising specific steps, it is contemplated that, additionally, there are compositions of the present invention that consist essentially of, or consist of, the recited components, and that there are processes and methods according to the present invention that consist essentially of, or consist of, the recited processing steps.

[0053] In the application, where an element or component is said to be included in and/or selected from a list of recited elements or components, it should be understood that the element or component can be any one of the recited elements or components, or the element or component can be selected from a group consisting of two or more of the recited elements or components.

[0054] Further, it should be understood that elements and/or features of a composition or a method described herein can be combined in a variety of ways without departing from the

spirit and scope of the present invention, whether explicit or implicit herein. For example, where reference is made to a particular compound, that compound can be used in various embodiments of compositions of the present invention and/or in methods of the present invention, unless otherwise understood from the context. In other words, within this application, embodiments have been described and depicted in a way that enables a clear and concise application to be written and drawn, but it is intended and will be appreciated that embodiments may be variously combined or separated without parting from the present teachings and invention(s). For example, it will be appreciated that all features described and depicted herein can be applicable to all aspects of the invention(s) described and depicted herein.

[0055] It should be understood that the expression "at least one of" includes individually each of the recited objects after the expression and the various combinations of two or more of the recited objects unless otherwise understood from the context and use. The expression "and/or" in connection with three or more recited objects should be understood to have the same meaning unless otherwise understood from the context.

[0056] The use of the term "include," "includes," "including," "have," "has," "having," "contain," "contains," or "containing," including grammatical equivalents thereof, should be understood generally as open-ended and non-limiting, for example, not excluding additional unrecited elements or steps, unless otherwise specifically stated or understood from the context.

[0057] Where the use of the term "about" is before a quantitative value, the present invention also includes the specific quantitative value itself, unless specifically stated otherwise. As used herein, the term "about" refers to a ±10% variation from the nominal value unless otherwise indicated or inferred.

[0058] It should be understood that the order of steps or order for performing certain actions is immaterial so long as the present invention remain operable. Moreover, two or more steps or actions may be conducted simultaneously.

[0059] The use of any and all examples, or exemplary language herein, for example, "such as" or "including," is intended merely to illustrate better the present invention and does not pose a limitation on the scope of the invention unless claimed. No language in the specification should be construed as indicating any non-claimed element as essential to the practice of the present invention.

[0060] Practice of the invention will be more fully understood from the foregoing examples, which are presented herein for illustrative purposes only, and should not be construed as limiting the invention in any way.

EXAMPLES

Colony formation

[0061] HeLa S3 cells cultured in suspension were plated one day before transfection to allow them to adapt to adherent culture condition. On the day of transfection, 15 µg of rAAV vectors were mixed with 0, 0.75 or 1 µg of rep68 mRNA in combination with 0, 30 nM or 100 nM trichostatin A (TSA) and then transfected into 10^6 cells by electroporation at 100 V for 30 ms in 0.2 cm gap cuvettes. After electroporation, cells were incubated at room temperature for 5 min before being plated on a 96 well plate with DMEM media containing 10% FBS. 24 hours after transfection, media containing 400 ng/ml puromycin were used to select cells that have rAAV vectors integrated into their genome. The number of colonies grown on day 10 after transfection was counted on each plate. FIG. 1 shows quantification of cell colonies after co-delivery of rAAV vectors with various amounts of rep68 mRNA and/or TSA. Results represent the number of colonies grown after plating on a 96 well plate and antibiotic selection. The number of colonies under the transfection condition of 1 µg of rep68 without TSA was an average from ten 96 well plates (+/- Standard Deviation). Results show that co-transfection of rAAV vectors with rep68 mRNA significantly enhanced colony formation, and TSA also moderately increased colony formation.

rAAV production

[0062] On day 15 after the co-transfection described above, colonies formed on the 96 well plate were infected with adenovirus 5 (Ad5) at a Multiplicity of Infection (MOI) of 30 to enable formation of viral particles. On day 18, the infected cells were lysed for 2 hours at 37˚C in lysis buffer containing sodium deoxycholate and Benzonase®. Supernatants from the samples were sequentially digested with DNase I and then Proteinase K to liberate rAAV genomic DNA. TaqMan qPCR amplifying the BGH-PolyA coding region of the rAAV vector was then used to determine the C_t value of each sample. FIG. 2 shows distributions of the C_t values of colonies and the mean C_t value under each co-transfection condition. Co-delivery of rAAV with 1 µg rep68 mRNA + 30 nM TSA, or 0.75 µg rep68 mRNA + 100 nM TSA led to decreased C_t values, indicating enhanced rAAV production from the host cells under these transfection conditions.

Generation of stable cell lines

[0063] Stable cell lines were generated from different rAAV co-transfection conditions as described above: 1 μg rep68 mRNA (8G3 clone), 30 nM TSA (12B5 clone), or 0.75 μg rep68 mRNA + 100 nM TSA (16D2 clone) together with a control cell line generated by transfection of rAAV vector without rep mRNA or TSA (10G8 clone). After infection with Ad5 at an MOI of 50, cells were collected on days 0, 1, 2, 3, and 4 (D0, D1, D2, D3, D4) post-infection, from which genomic DNA was isolated. TaqMan qPCR was used to amplify the rAAV genome as well as an endogenous gene - RNase P. Quantification of rAAV genome copy number per cell (GC/cell) was accomplished by normalization with RNase P gene, which is present at approximately three copies in each HeLa cell. FIG. 3 shows increased rAAV genome copy in the stable cell lines derived by co-transfection of rAAV vectors and rep68 mRNA and/or TSA compared with the cell line transfected with rAAV alone, indicating that rep68 mRNA and/or TSA improved integration of rAAV genome into host cells and stable host cell line generation.

rAAV yield from stable cell lines

[0064] rAAV yield was assayed from the control and three stable cell lines described above. Cells were seeded in duplicate flasks at $1.5x10^6$ cells/mL in 100 mL of Production Medium (90 mL Suspension DMEM + 10 mL EX-CELL) and infected with Ad5 at an MOI of 50. Four days after infection, cells were lysed for 2 hours at 37˚C in lysis buffer containing sodium deoxycholate and Benzonase®. Supernatant from the samples were sequentially digested with DNase I and then Proteinase K to liberate rAAV genomic DNA. TaqMan qPCR amplifying the BGH-PolyA coding region of the rAAV genome was then used to determine the rAAV genome copy number (GC) based on a rAAV plasmid standard curve. FIG. 4 shows enhanced production of rAAV from stable cell lines established by co-delivery of rAAV vectors and rep68 mRNA and/or TSA, suggesting that co-delivery of rAAV vectors with rep68 mRNA and/or TSA to host cells improved rAAV production from the host cells.

NUMBERED EMBODIMENTS

[0065] 1. A method of increasing recombinant AAV (rAAV) vector titer, the method comprising:

a) co-delivering a rAAV vector with a rep mRNA to a eukaryotic cell culture; and

b) harvesting rAAV from the eukaryotic cell culture, wherein the co-delivery with the rep mRNA increases the titer of the harvested rAAV.

[0066] 2. The method of embodiment 1, wherein the rep mRNA is selected from the group consisting of rep68 mRNA and rep78 mRNA.

[0067] 3. The method of embodiment 1, wherein the co-delivering comprises electroporation.

[0068] 4. The method of embodiment 1, wherein the co-delivering involves magnetic beads.

[0069] 5. The method of embodiment 1, wherein the co-delivering involves nanoparticles.

[0070] 6. The method of embodiment 1, wherein the co-delivering comprises microinjection.

[0071] 7. The method of embodiment 1, wherein the co-delivering comprises cell squeezing.

[0072] 8. The method of embodiment 1, wherein the co-delivering comprises chemical-based transfection.

[0073] 9. The method of embodiment 8, wherein the chemical-based transfection comprises exposure to lipids.

[0074] 10. The method of embodiment 8, wherein the chemical-based transfection comprises exposure to calcium phosphate.

[0075] 11. The method of embodiment 8, wherein the chemical-based transfection comprises exposure to cationic polymers.

[0076] 12. The method of embodiment 8, wherein the chemical-based transfection comprises exposure to DEAE-dextran.

[0077] 13. The method of embodiment 8, wherein the chemical-based transfection comprises exposure to activated dendrimers.

[0078] 14. A method of developing a cell line for producing recombinant AAV (rAAV), the method comprising:

a) co-delivering a rAAV vector with a rep mRNA to eukaryotic cells;

b) identifying a eukaryotic cell that contains the rAAV vector sequence integrated into the eukaryotic cell genome; and

c) optionally harvesting rAAV from a culture comprising the eukaryotic cell, wherein the co-delivery with the rep mRNA enhances the titer of the harvested rAAV.

[0079] 15. The method of embodiment 14, wherein the rep mRNA is selected from the group consisting of rep68 mRNA and rep78 mRNA.

[0080] 16. The method of embodiment 14, wherein the co-delivering comprises electroporation.

[0081] 17. The method of embodiment 14, wherein the co-delivering involves magnetic beads.

[0082] 18. The method of embodiment 14, wherein the co-delivering involves nanoparticles.

[0083] 19. The method of embodiment 14, wherein the co-delivering comprises microinjection.

[0084] 20. The method of embodiment 14, wherein the co-delivering comprises cell squeezing.

[0085] 21. The method of embodiment 14, wherein the co-delivering comprises chemical-based transfection.

[0086] 22. The method of embodiment 21, wherein the chemical-based transfection comprises exposure to lipids.

[0087] 23. The method of embodiment 21, wherein the chemical-based transfection comprises exposure to calcium phosphate.

[0088] 24. The method of embodiment 21, wherein the chemical-based transfection comprises exposure to cationic polymers.

[0089] 25. The method of embodiment 21, wherein the chemical-based transfection comprises exposure to DEAE-dextran.

[0090] 26. The method of embodiment 21, wherein the chemical-based transfection comprises exposure to activated dendrimers.

[0091] 27. A method comprising co-delivering an HDAC inhibitor and a recombinant polynucleotide to eukaryotic cells.

[0092] 28. The method of embodiment 27, further comprising the step of detecting integration of the recombinant polynucleotide within a chromosome of one of the eukaryotic cells.

[0093] 29. The method of embodiment 27 or 28, further comprising the step of establishing a stable cell line with the recombinant polynucleotide integrated into the eukaryotic cell genome.

[0094] 30. The method of any one of embodiments 27-29, further comprising a step of expressing a gene product dependent on the presence of the recombinant polynucleotide within the eukaryotic cell.

[0095] 31. The method of embodiment 30, wherein the gene product is a ribonucleic acid.

[0096] 32. The method of embodiment 30, wherein the gene product is a polypeptide.

[0097] 33. The method of embodiment 30, wherein the polynucleotide encodes the gene product.

[0098] 34. The method of embodiment 30, wherein the polynucleotide comprises a recombinant viral vector.

[0099] 35. The method of embodiment 34, wherein the recombinant viral vector is an adeno-associated viral vector (AAV vector).

[00100] 36. The method of embodiment 34, further comprising the step of harvesting the recombinant virus from a culture of the eukaryotic cells.

[00101] 37. The method of embodiment 35, further comprising the step of harvesting the recombinant AAV from a culture of the eukaryotic cells.

[00102] 38. The method of embodiment 27, wherein the co-delivering comprises electroporation.

[00103] 39. The method of embodiment 27, wherein the co-delivering involves magnetic beads.

[00104] 40. The method of embodiment 27, wherein the co-delivering involves nanoparticles.

[00105] 41. The method of embodiment 27, wherein the co-delivering comprises microinjection.

[00106] 42. The method of embodiment 27, wherein the co-delivering comprises cell squeezing.

[00107] 43. The method of embodiment 27, wherein the co-delivering comprises chemical-based transfection.

[00108] 44. The method of embodiment 43, wherein the chemical-based transfection comprises exposure to lipids.

[00109] 45. The method of embodiment 43, wherein the chemical-based transfection comprises exposure to calcium phosphate.

[00110] 46. The method of embodiment 43, wherein the chemical-based transfection comprises exposure to cationic polymers.

[00111] 47. The method of embodiment 43, wherein the chemical-based transfection comprises exposure to DEAE-dextran.

[00112] 48. The method of embodiment 43, wherein the chemical-based transfection comprises exposure to activated dendrimers.

[00113] 49. The method according to any one of embodiments 27-48, wherein the HDAC inhibitor is trichostatin A.

INCORPORATION BY REFERENCE

[00114] The entire disclosure of each of the patent and scientific documents referred to herein is incorporated by reference for all purposes.

EQUIVALENTS

[00115] The invention may be embodied in other specific forms without departing from the spirit or essential characteristics thereof. The foregoing embodiments are therefore to be considered in all respects illustrative rather than limiting on the invention described herein. Scope of the invention is thus indicated by the appended claims rather than by the foregoing description, and all changes that come within the meaning and range of equivalency of the claims are intended to be embraced therein.

CLAIMS

WHAT IS CLAIMED IS:

1. A method of increasing recombinant AAV (rAAV) vector titer, the method comprising:

    a) co-delivering a rAAV vector with a rep mRNA to a eukaryotic cell culture; and

    b) harvesting rAAV from the eukaryotic cell culture, wherein the co-delivery with the rep mRNA increases the titer of the harvested rAAV.

2. The method of claim 1, wherein the rep mRNA is selected from the group consisting of rep68 mRNA and rep78 mRNA.

3. The method of claim 1, wherein the co-delivering comprises electroporation.

4. The method of claim 1, wherein the co-delivering involves magnetic beads.

5. The method of claim 1, wherein the co-delivering involves nanoparticles.

6. The method of claim 1, wherein the co-delivering comprises microinjection.

7. The method of claim 1, wherein the co-delivering comprises cell squeezing.

8. The method of claim 1, wherein the co-delivering comprises chemical-based transfection.

9. The method of claim 8, wherein the chemical-based transfection comprises exposure to lipids.

10. The method of claim 8, wherein the chemical-based transfection comprises exposure to calcium phosphate.

11. The method of claim 8, wherein the chemical-based transfection comprises exposure to cationic polymers.

12. The method of claim 8, wherein the chemical-based transfection comprises exposure to DEAE-dextran.

13. The method of claim 8, wherein the chemical-based transfection comprises exposure to activated dendrimers.

14. A method of developing a cell line for producing recombinant AAV (rAAV), the method comprising:

    a) co-delivering a rAAV vector with a rep mRNA to a eukaryotic cell;

    b) identifying a eukaryotic cell that contains the rAAV vector sequence integrated into the eukaryotic cell genome; and

    c) optionally harvesting rAAV from a culture comprising the eukaryotic cell, wherein the co-delivery with the rep mRNA enhances the titer of the harvested rAAV.

15. The method of claim 14, wherein the rep mRNA is selected from the group consisting of rep68 mRNA and rep78 mRNA.

16. The method of claim 14, wherein the co-delivering comprises electroporation.

17. The method of claim 14, wherein the co-delivering involves magnetic beads.

18. The method of claim 14, wherein the co-delivering involves nanoparticles.

19. The method of claim 14, wherein the co-delivering comprises microinjection.

20. The method of claim 14, wherein the co-delivering comprises cell squeezing.

21. The method of claim 14, wherein the co-delivering comprises chemical-based transfection.

22. The method of claim 21, wherein the chemical-based transfection comprises exposure to lipids.

23. The method of claim 21, wherein the chemical-based transfection comprises exposure to calcium phosphate.

24. The method of claim 21, wherein the chemical-based transfection comprises exposure to cationic polymers.

25. The method of claim 21, wherein the chemical-based transfection comprises exposure to DEAE-dextran.

26. The method of claim 21, wherein the chemical-based transfection comprises exposure to activated dendrimers.

27. A method comprising co-delivering an HDAC inhibitor and a recombinant polynucleotide to eukaryotic cells.

28. The method of claim 27, further comprising the step of detecting integration of the recombinant polynucleotide into genome of one of the eukaryotic cells.

29. The method of claim 27 or 28, further comprising the step of establishing a stable cell line with the recombinant polynucleotide integrated into the eukaryotic cell genome.

30. The method of any one of claims 27-29, further comprising a step of expressing a gene product dependent on the presence of the recombinant polynucleotide within the eukaryotic cell.

31. The method of claim 30, wherein the gene product is a ribonucleic acid.

32. The method of claim 30, wherein the gene product is a polypeptide.

33. The method of claim 30, wherein the polynucleotide encodes the gene product.

34. The method of claim 30, wherein the polynucleotide comprises a recombinant viral vector.

35. The method of claim 34, wherein the recombinant viral vector is a recombinant adeno-associated viral vector (rAAV vector).

36. The method of claim 34, further comprising the step of harvesting the recombinant virus from a culture of the eukaryotic cells.

37. The method of claim 35, further comprising the step of harvesting the recombinant AAV from a culture of the eukaryotic cells.

38. The method of claim 27, wherein the co-delivering comprises electroporation.

39. The method of claim 27, wherein the co-delivering involves magnetic beads.

40. The method of claim 27, wherein the co-delivering involves nanoparticles.

41. The method of claim 27, wherein the co-delivering comprises microinjection.

42. The method of claim 27, wherein the co-delivering comprises cell squeezing.

43. The method of claim 27, wherein the co-delivering comprises chemical-based transfection.

44. The method of claim 43, wherein the chemical-based transfection comprises exposure to lipids.

45. The method of claim 43, wherein the chemical-based transfection comprises exposure to calcium phosphate.

46. The method of claim 43, wherein the chemical-based transfection comprises exposure to cationic polymers.

47. The method of claim 43, wherein the chemical-based transfection comprises exposure to DEAE-dextran.

48. The method of claim 43, wherein the chemical-based transfection comprises exposure to activated dendrimers.

49. The method according to any one of claims 27-48, wherein the HDAC inhibitor is trichostatin A.

FIG. 1

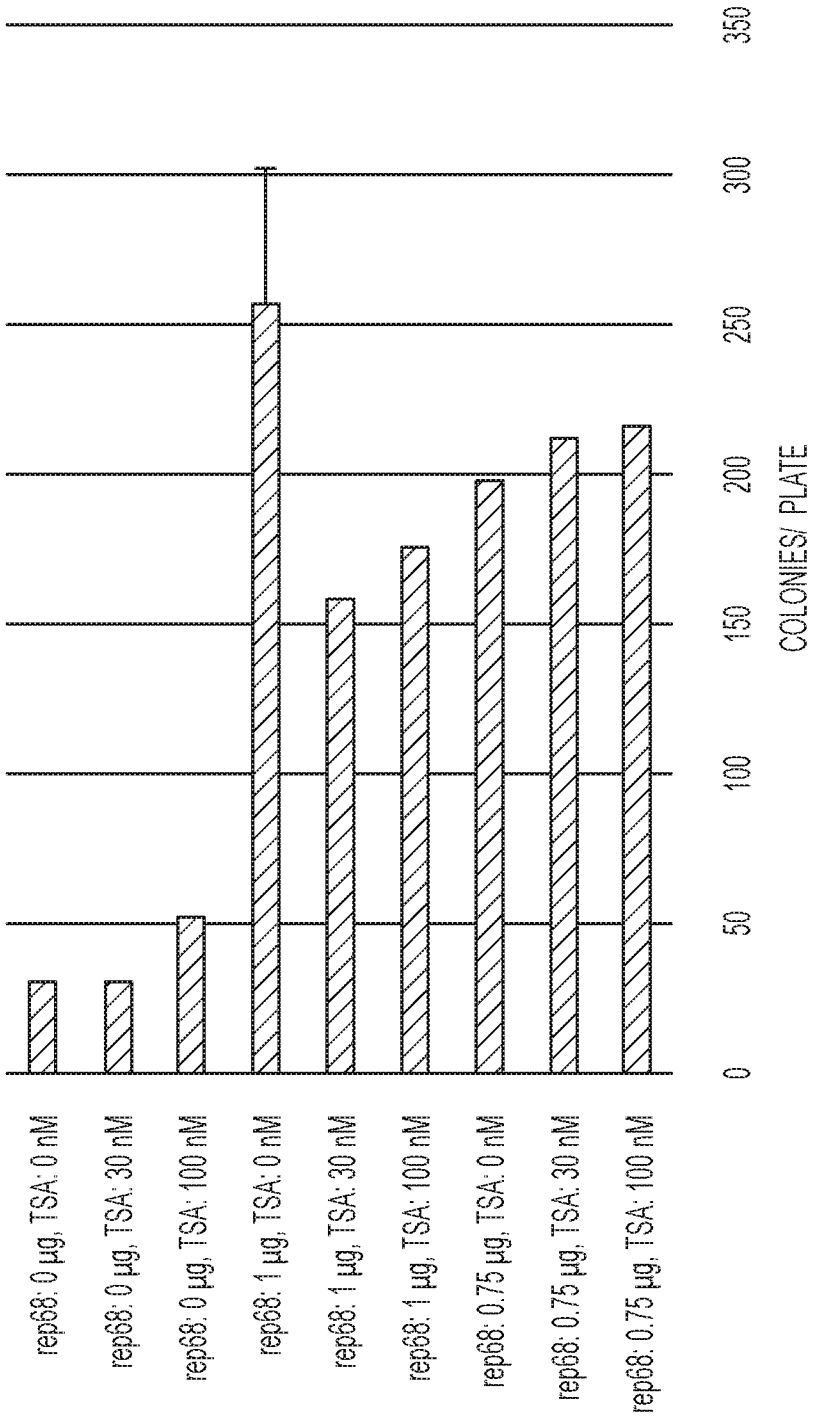

0
50
100
150
200
250
300
350
COLONIES/ PLATE
rep68: 0 µg, TSA: 0 nM
rep68: 0 µg, TSA: 30 nM
rep68: 0 µg, TSA: 100 nM
rep68: 1 µg, TSA: 0 nM
rep68: 1 µg, TSA: 30 nM
rep68: 1 µg, TSA: 100 nM
rep68: 0.75 µg, TSA: 0 nM
rep68: 0.75 µg, TSA: 30 nM
rep68: 0.75 µg, TSA: 100 nM

FIG. 2

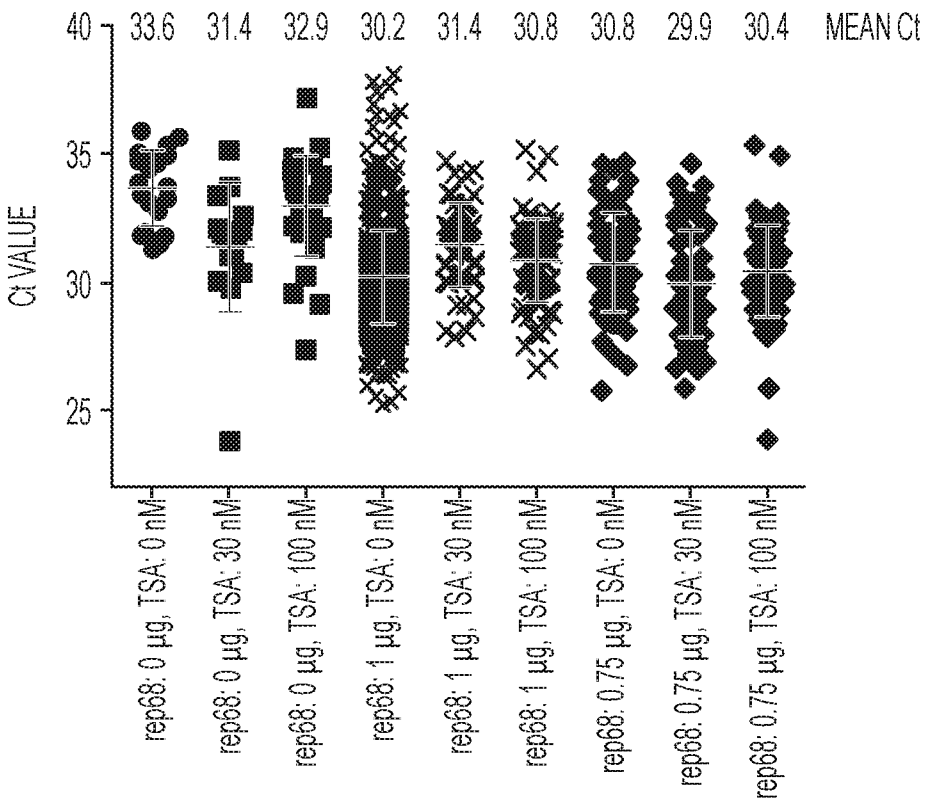

40
35
30
25
Ct VALUE
33.6
31.4
32.9
30.2
31.4
30.8
30.8
29.9
30.4
MEAN Ct
rep68: 0 µg, TSA: 0 nM
rep68: 0 µg, TSA: 30 nM
rep68: 0 µg, TSA: 100 nM
rep68: 1 µg, TSA: 0 nM
rep68: 1 µg, TSA: 30 nM
rep68: 1 µg, TSA: 100 nM
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rep68: 0.75 µg, TSA: 100 nM

FIG. 3

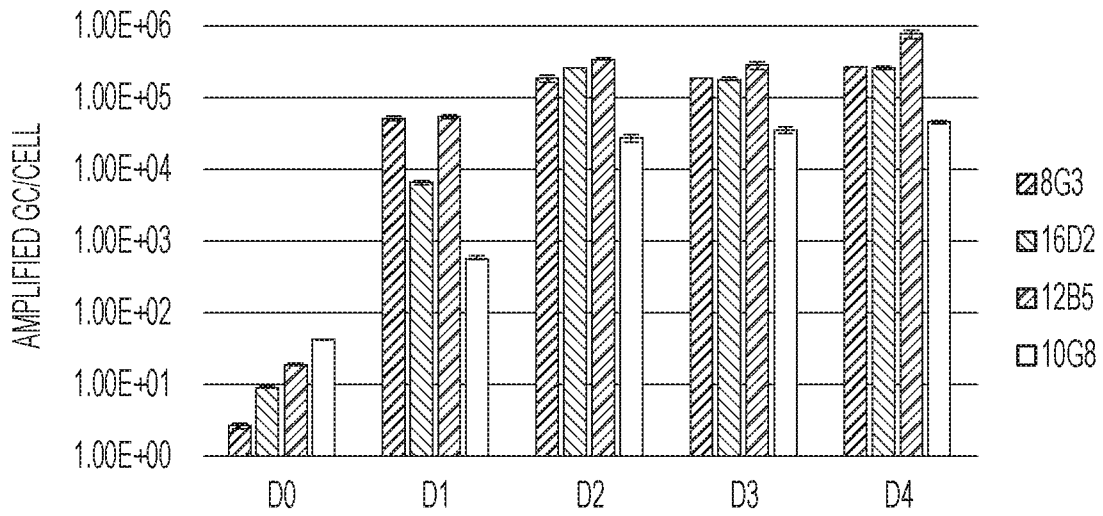
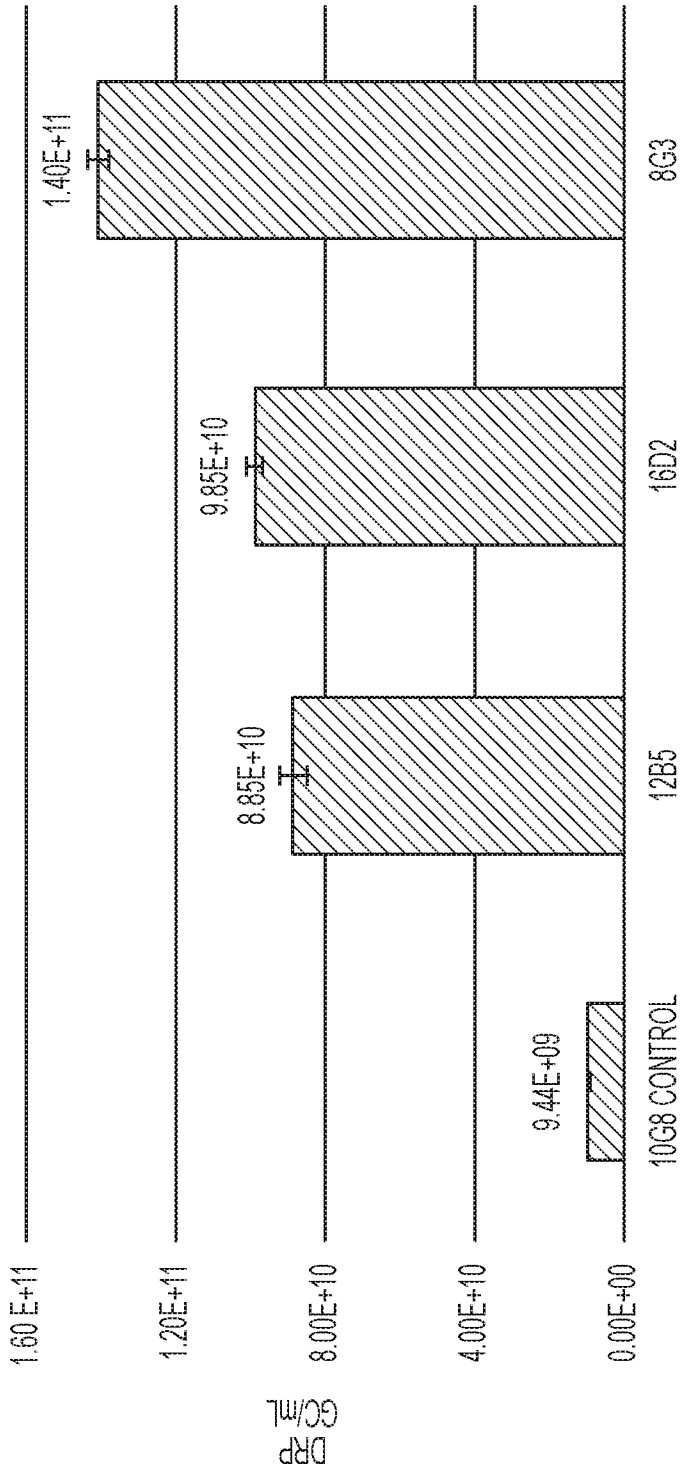

AMPLIFIED GC/CELL
1.00E+06
1.00E+05
1.00E+04
1.00E+03
1.00E+02
1.00E+01
1.00E+00
D0
D1
D2
D3
D4
8G3
16D2
12B5
10G8

FIG. 4


1.60 E+11
1.20E+11
8.00E+10
4.00E+10
0.00E+00
DRP GC/mL
1.40E+11
9.85E+10
8.85E+10
9.44E+09
8G3
16D2
12B5
10G8 CONTROL

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 18/23841

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.: 30-37, 49
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/US 18/23841

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C12N 15/86, A61K 48/00, C12N 7/00 (2018.01)
CPC - C12N 2750/14111, C12N 2750/1414, C12N 2750/14143

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 ---- Y	US 2014/0359799 A1 (CASE WESTERN RESERVE UNIVERSITY) 04 December 2014 (04.12.2014) abstract; para [0027], [0079], [0085], [0089], [0090]-[0091], [0094].	27-29, 38, 41, 43-44 ---------- 39-40, 42, 45-48
X ---- Y	US 2002/0115189 A1 (NATSOULIS et al) 22 August 2002 (22.08.2002) abstract; para [0014], [0026], [0039], [0044], [0053]-[0054], [0056], [0070], [0075], [0079], [0086].	1-3, 5-6, 8-10, 14-16, 18-19, 21-23 ---------- 4, 7, 11-13, 17, 20, 24-26
Y	US 2011/0251547 A1 (XING et al) 13 October 2011 (13.10.2011) abstract; para [0064].	4, 17, 39-40
Y	SHAREI et al., Cell Squeeing as a Robust, Microfluidic Intracellular Delivery Platform, J. Vis. Exp., 07 November 2013, Vol. 81, pages 1-7. Especially abstract; pg 6, para 2.	7, 20, 42
Y	US 2012/0058917 A1 (GAKEN et al) 08 March 2012 (08.03.2012) abstract; para [0012], [0035].	11-13, 24-26, 45-48

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

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
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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
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
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Date of the actual completion of the international search	Date of mailing of the international search report
14 May 2018	07 JUN 2018

Name and mailing address of the ISA/US	Authorized officer:
Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774