DESCRIPTION

CAPSID-MODIFIED, RAAV3 VECTOR COMPOSITIONS AND METHODS OF USE IN GENE THERAPY OF HUMAN LIVER CANCER

BACKGROUND OF THE INVENTION

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] The present international patent application claims priority to U.S. Patent Appl. No. 13/899,481, filed May 21, 2013 (pending; Atty. Dkt. No. 36689.331), which was a continuation-in-part of U.S. Patent Appl. No. 12/595,196, filed December 31, 2009 (now U.S. Patent No. 8,445,267; Atty. Docket No. 36689.305), which was the U.S. national-stage filing of PCT Intl. Patent Appl. No. PCT/US2008/059647 filed April 8, 2008 (nationalized; Atty. Docket No. 36689.272), which claimed priority to U.S. Provisional Patent Appl. No. 60/910,798, filed April 9, 2007 (expired; Atty. Docket No. 36689.266). The content of each of the aforementioned applications is hereby incorporated in its entirety by express reference thereto.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

[0002] This invention was made with government support under Grant Nos. R01-HL-097088 and R21-EB-015684 awarded by the National Institutes of Health. The government has certain rights in the invention.

NAMES OF THE PARTIES TO A JOINT RESEARCH AGREEMENT

[0003] Not Applicable.

FIELD OF THE INVENTION

[0004] The present invention relates generally to the fields of molecular biology and virology, and in particular, to the development of gene delivery vehicles. Also disclosed are improved rAAV vector compositions useful in delivering a variety of nucleic acid segments, including those encoding therapeutic proteins polypeptides, peptides, antisense oligonucleotides, and ribozyme constructs to selected host cells for use in various diagnostic and/or therapeutic regimens. Methods are also provided for preparing and using these modified rAAV-based vector constructs in a variety of viral-based gene therapies, and in particular, for the diagnosis, prevention, treatment and/or amelioration of symptoms of human diseases, disorders, dysfunctions, trauma, or injury. The invention also provides mutated rAAV-based viral vector delivery systems with increased transduction efficiency and/or improved viral infectivity of selected mammalian host cells. In particular, the invention provides improved rAAV vectors and virions having particles having amino acid substitutions in one or more surface-exposed residues of a viral capsid protein.

DESCRIPTION OF RELATED ART

[0005] Major advances in the field of gene therapy have been achieved by using viruses to deliver

therapeutic genetic material. The adeno-associated virus (AAV) has attracted considerable attention as a highly effective viral vector for gene therapy due to its low immunogenicity and ability to effectively transduce non-dividing cells. AAV has been shown to infect a variety of cell and tissue types, and significant progress has been made over the last decade to adapt this viral system for use in human gene therapy.

[0006] In its normal "wild type" form, recombinant AAV (rAAV) DNA is packaged into the viral capsid as a single stranded molecule about 4600 nucleotides (nt) in length. Following infection of the cell by the virus, the molecular machinery of the cell converts the single DNA strand into a double-stranded form. Only the double-stranded DNA form is useful to the polypeptides of the cell that transcribe the contained gene or genes into RNA.

[0007] AAV has many properties that favor its use as a gene delivery vehicle: 1) the wild type virus is not associated with any pathologic human condition; 2) the recombinant form does not contain native viral coding sequences; and 3) persistent transgenic expression has been observed in many applications.

[0008] The transduction efficiency of recombinant adeno-associated virus 2 (AAV) vectors varies greatly in different cells and tissues in *vitro* and *in vivo,* which has limited the usefulness of many of them in potential gene therapy regimens. Systematic studies have been performed to elucidate the fundamental steps in the life cycle of AAV. For example, it has been documented that a cellular protein, FKBP52, phosphorylated at tyrosine residues by epidermal growth factor receptor protein tyrosine kinase (EGFR-PTK), inhibits AAV second-strand DNA synthesis and consequently, transgene expression *in vitro* as well as *in vivo.* It has also been demonstrated that EGFR-PTK signaling modulates the ubiquitin/proteasome pathway-mediated intracellular trafficking as well as FKBP52-mediated second-strand DNA synthesis of AAV vectors. In those studies, inhibition of EGFR-PTK signaling led to decreased ubiquitination of AAV capsid proteins, which in turn, facilitated nuclear transport by limiting proteasome-mediated degradation of AAV vectors, implicating EGFR-PTK-mediated phosphorylation of tyrosine residues on AAV capsids. What is lacking in the prior art are improved rAAV viral vectors that have enhanced transduction efficiency for infecting selected mammalian cells, and for targeted gene delivery to human cells in particular.

BRIEF SUMMARY OF THE INVENTION

[0009] The present invention overcomes limitations and deficiencies inherent in the prior art by providing novel improved rAAV-based genetic constructs that encode one or more therapeutic agents useful in the preparation of medicaments for the prevention, treatment, and/or amelioration of one or more diseases, disorders or dysfunctions resulting from a deficiency in one or more of such polypeptides. In particular, the invention provides VP3 capsid-protein-modified rAAV-based genetic constructs encoding one or more selected molecules, such as, for example, one or more diagnostic or therapeutic agents (including, *e.g.,* proteins, polypeptides, peptides, antibodies, antigen binding

fragments, siRNAs, RNAis, antisense oligo- and poly-nucleotides, ribozymes, and variants and/or active fragments thereof), for use in the diagnosis, prevention, treatment, and/or amelioration of symptoms of a variety of mammalian diseases, disorders, dysfunctions, trauma, injury, and such like.

[0010] The present invention provides mutated AAV VP3 capsid proteins that include modification of one or more surface-exposed amino acid resides (including, *e.g.*, without limitation, lysine, serine, threonine, and/or tyrosine residues) as compared to wildtype. Also provided are infectious rAAV virions that comprise the mutated AAV capsid proteins of the present invention, as well as nucleic acid molecules and rAAV vectors encoding the mutant AAV capsid proteins of the present invention, and nucleic acids encoding one or more selected diagnostic and/or therapeutic agents for delivery to a selected population of mammalian cells.

[0011] Advantageously, the novel rAAV vectors, express constructs, and infectious virions and viral particles comprising them as disclosed herein preferably have an improved efficiency in transducing one or more of a variety of cells, tissues and organs of interest, when compared to wild-type, unmodified, expression constructs, and to the corresponding rAAV vectors and virions comprising them.

[0012] The improved rAAV vectors provided hererin transduce one or more selected host cells at higher-efficiencies (and often much higher efficiencies) than conventional, wild type (*i.e.*, "unmodified") rAAV vectors. By performing extensive analysis and detailed experiments involving the site-directed mutagenesis of various individual and/or combinations of two, three, four, five, or even six or more surface-exposed amino acid residues on various AAV capsid proteins from a variety of AAV serotypes, the inventors have developed a large collection of single- or multi-mutated rAAV vectors that possess improved transduction efficiencies. The inventors have demonstrated in a number of different AAV serotypes that the substitution of one or more virion surface-presenting amino acid residues results in improved viral vectors, which are capable of higher-efficiency transduction than that of the corresponding, non-substituted vectors from which the mutants were prepared.

[0013] The development of these new capsid-mutant rAAV viral vectors dramatically reduces the number of viral particles needed for conventional gene therapy regimens. In addition to having improved transduction efficiencies for various mammalian cells, the surface-exposed amino acid-modified rAAV vectors described herein are more stable, less immunogenic, and can be produced at much lower cost than the traditional viral vectors currently employed in mammalian gene therapy regimens.

[0014] In a particular embodiment the invention provides a modified rAAV VP3 capsid protein, that includes: (a) a non-tyrosine amino acid residue at one or more positions corresponding to Y252, Y272, Y444, Y701, Y705, and Y731 of the wild-type AAV3 capsid protein as set forth in SEQ ID NO:3; (b) a non-serine amino acid residue at each of one or more positions corresponding to S459 or S663, of the wild-type AAV3 capsid protein as set forth in SEQ ID NO:3; (c) a non-threonine

amino acid residue at each of one or more positions corresponding to T251or T492 of the wild-type AAV3 capsid protein as set forth in SEQ ID NO:3; (d) a non-lysine amino acid residue at each of one or more positions corresponding to K528, K533,or K545 of the wild-type AAV3 capsid protein as set forth in SEQ ID NO:3; (e) (i) a non-tyrosine amino acid residue at position Y701, or Y705; and (ii) a non-tyrosine amino acid residue at position Y705 or Y731, or a non-serine amino acid residue at position S663 of the wild-type AAV3 capsid protein as set forth in SEQ ID NO:3; (f) a combination of three or more amino acid substitutions listed in (a), (b), (c), and (d); each with a non-native amino acid; (g) a combination of four or more amino acid substitutions listed in (a), (b), (c), and (d); each with a non-native amino acid; or (h) a combination of five or more amino acid substitutions listed in (a), (b), (c), and (d); each with a non-native amino acid; or alternatively, wherein each of the amino acid substitutions is at an equivalent amino acid position corresponding thereto in any one of the other wild-type vector serotypes selected from the group consisting of AAV1, AAV2, AAV4, AAV5, AAV7, AAV8, AAV9, and AAV10, as set forth in SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, and SEQ ID NO:10, respectively.

[0015] Exemplary multi-mutated proteins of the present invention include, but are not limited to, combinations of non-native amino acid substitutions at each of three or more distinct surface-exposed amino acid residues on the AAV3 capsid. These mult-mutant vectors include, without limitation:

(a) Y701F, Y705F, and Y731F;

(b) Y705F, Y731F, and S663V;

(c) Y705F, Y731F, and T492V;

(d) Y705F, Y731F, K533R;

(e) S663V, T492V, and K533R;

(f) Y705F, Y731F, S663V, and T492V; and

(g) Y705F, Y731F, S663V, T492V, and K533R substitutions at the denoted amino acid residues of the wild-type AAV3 capsid protein as set forth in SEQ ID NO:3, or at the equivalent surface-exposed amino acid residues in any one of the corresponding wild-type AAV1, AAV2, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, or AAV10 capsid proteins, as set forth in SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, or SEQ ID NO:10, respectively, or in any combination thereof.

[0016] In the practice of the invention, the substituted non-native amino acids may include a substitution of one or more amino acids not normally present at a particular residue in the corresponding wild-type protein, and preferably include one or more non-native amino acid substitutions selected from the group consisting of phenylalanine (F), valine (V), histidine (H), isoleucine (I), alanine (A), leucine (L) aspartic acid (D), asparagine (N). glutamic acid (E), arginine (R), serine (S), and isoleucine (I).

[0017] The invention also provides isolated and purified polynucleotides that encode one or more of the disclosed capsid protein-mutated variants as described herein, as well as recombinant adeno-

associated viral (rAAV) vectors that comprise one or more such polynucleotides. Preferably, the vector constructs of the present invention further include at least one nucleic acid segment that encodes a diagnostic or therapeutic molecule operably linked to a promoter capable of expressing the nucleic acid segment in a suitable host cell comprising the vector. In the practice of the invention, the transduction efficiency of a virion comprising the modified AAV VP3 capsid protein will be higher than that of the corresponding, unmodified, wild-type protein, and as such, will preferably possess a transduction efficiency in a mammalian cell that is at least 2-fold, at least about 4-fold, at least about 6-fold, at least about 8—fold, at least about 10-fold, or at least about 12-fold or higher in a selected mammalian host cell than that of a virion that comprises a corresponding, unmodified, capsid protein. In certain embodiments, the transduction efficiency of the rAAV vectors provided herein will be at least about 15-fold higher, at least about 20-fold higher, at least about 25-fold higher, at least about 30-fold higher, or at least about 40, 45, or 50-fold or more greater than that of a virion that comprises a corresponding, unmodified, capsid protein. Moreover, the infectious virions of the present invention that include one or more modified AAV VP3 capsid proteins are preferably less susceptible to ubiquitination when introduced into a mammalian cell than that of a virion that comprises a corresponding, unmodified, capsid protein.

[0018] The present invention also concerns rAAV vectors, wherein the nucleic acid segment further comprises a promoter, an enhancer, a post-transcriptional regulatory sequence, a polyadenylation signal, or any combination thereof, operably linked to the nucleic acid segment that encodes the selected polynucleotide of interest.

[0019] Preferably, the promoter is a heterologous promoter, a tissue-specific promoter, a cell-specific promoter, a constitutive promoter, an inducible promoter, or any combination thereof.

[0020] In certain embodiments, the nucleic acid segments cloned into the novel rAAV expression vectors described herein will express or encode one or more polypeptides, peptides, ribozymes, peptide nucleic acids, siRNAs, RNAis, antisense oligonucleotides, antisense polynucleotides, antibodies, antigen binding fragments, or any combination thereof.

[0021] As noted herein, the therapeutic agents useful in the invention may include one or more agonists, antagonists, anti-apoptosis factors, inhibitors, receptors, cytokines, cytotoxins, erythropoietic agents, glycoproteins, growth factors, growth factor receptors, hormones, hormone receptors, interferons, interleukins, interleukin receptors, nerve growth factors, neuroactive peptides, neuroactive peptide receptors, proteases, protease inhibitors, protein decarboxylases, protein kinases, protein kinase inhibitors, enzymes, receptor binding proteins, transport proteins or one or more inhibitors thereof, serotonin receptors, or one or more uptake inhibitors thereof, serpins, serpin receptors, tumor suppressors, diagnostic molecules, chemotherapeutic agents, cytotoxins, or any combination thereof.

[0022] While the inventors particularly contemplate the use of the rAAV3 vectors denoted in FIG. 41 in methods for the gene therapy of one or more mammalian liver cancers, capsid-mutated vectors may be prepared and packaged within virions of any known AAV serotype, including, for

examples, AAV serotype 1 (AAV1), AAV serotype 2 (AAV2), AAV serotype 4 (AAV4), AAV serotype 5 (AAV5), AAV serotype 6 (AAV6), AAV serotype 7 (AAV7), AAV serotype 8 (AAV8), AAV serotype 9 (AAV9), AAV serotype 10 (AAV10), AAV serotype 11 (AAV11), or AAV serotype 12 (AAV12).

[0023] The invention further provides populations and pluralities of such capsid-mutated rAAV vectors, as well as virions, infectious viral particles, and mammalian host cells that include one or more nucleic acid segments encoding them.

[0024] Preferably, the mammalian host cells will be human host cells, including, for example blood cells, stem cells, hematopoietic cells, $CD34^+$ cells, liver cells, cancer cells, vascular cells, pancreatic cells, neural cells, ocular or retinal cells, epithelial or endothelial cells, dendritic cells, fibroblasts, or any other cell of mammalian origin, including, without limitation, hepatic (*i.e.,* liver) cells, lung cells, cardiac cells, pancreatic cells, intestinal cells, diaphragmatic cells, renal (*i.e.,* kidney) cells, neural cells, blood cells, bone marrow cells, or any one or more selected tissues of a mammal for which viral-based gene therapy is contemplated.

[0025] The invention further provides composition and formulations that include one or more of the proteins nucleic acid segments viral vectors, host cells, or viral particles of the present invention together with one or more pharmaceutically-acceptable buffers, diluents, or excipients. Such compositions may be included in one or more diagnostic or therapeutic kits, for diagnosing, preventing, treating or ameliorating one or more symptoms of a mammalian disease, injury, disorder, trauma or dysfunction.

[0026] The invention further includes a method for providing a mammal in need thereof with a diagnostically- or therapeutically-effective amount of a selected biological molecule, the method comprising providing to a cell, tissue or organ of a mammal in need thereof, an amount of an rAAV vector; and for a time effective to provide the mammal with a diagnostically- or a therapeutically-effective amount of the selected biological molecule.

[0027] The invention further provides a method for diagnosing, preventing, treating, or ameliorating at least one or more symptoms of a disease, a disorder, a dysfunction, an injury, an abnormal condition, or trauma in a mammal. In an overall and general sense, the method includes at least the step of administering to a mammal in need thereof one or more of the disclosed rAAV vectors, in an amount and for a time sufficient to diagnose, prevent, treat or ameliorate the one or more symptoms of the disease, disorder, dysfunction, injury, abnormal condition, or trauma in the mammal. In the case of rAAV3-based vectors, such abnormal conditions preferably include one or more diseases or dysfunctions of the mammalian liver, including, for example, HCC; in the case of rAAV8-based vectors, such abnormal conditions preferably include one or more diseases or dysfunctions of the mammalian eye; or, in the case of rAAV6 vectors, one or more diseases of stem cells, blood cells, hematopoietic cells, or $CD35^+$ cells, including for example, sickle cell disease, β-thalassemia, and such like.

[0028] The invention also provides a method of transducing a population of mammalian cells. In an overall and general sense, the method includes at least the step of introducing into one or more cells of the population, a composition that comprises an effective amount of one or more of the rAAV vectors disclosed herein.

[0029] In a further embodiment, the invention also provides isolated nucleic acid segments that encode one or more of the VP3 mutant capsid proteins as described herein, and provides recombinant vectors, virus particles, infectious virions, and isolated host cells that comprise one or more of the improved vector sequences described and tested herein.

[0030] Additionally, the present invention provides compositions, as well as therapeutic and/or diagnostic kits that include one or more of the disclosed vectors or AAv compositions, formulated with one or more additional ingredients, or prepared with one or more instructions for their use.

[0031] The invention also demonstrates methods for making, as well as methods of using the disclosed improved rAAV capsid-mutated vectors in a variety of ways, including, for example, *ex situ*, *in vitro* and *in vivo* applications, methodologies, diagnostic procedures, and/or gene therapy regimens. Because many of the improved vectors described herein are also resistant to proteasomal degradation, they possess significantly increased transduction efficiencies *in vivo* making them particularly well suited for viral vector-based human gene therapy regimens, and in particular, for delivering one or more genetic constructs to selected mammalian cells *in vivo* and/or *in vitro*.

[0032] In one aspect, the invention provides compositions comprising recombinant adeno-associated viral (AAV) vectors, virions, viral particles, and pharmaceutical formulations thereof, useful in methods for delivering genetic material encoding one or more beneficial or therapeutic product(s) to mammalian cells and tissues. In particular, the compositions and methods of the invention provide a significant advancement in the art through their use in the treatment, prevention, and/or amelioration of symptoms of one or more mammalian diseases. It is contemplated that human gene therapy will particularly benefit from the present teachings by providing new and improved viral vector constructs for use in the treatment of a number of diverse diseases, disorders, and dysfunctions.

[0033] In another aspect, the invention concerns modified rAAV vector that encode one or more mammalian therapeutic agents for the prevention, treatment, and/or amelioration of one or more disorders in the mammal into which the vector construct is delivered.

[0034] In particular, the invention provides rAAV-based expression constructs that encode one or more mammalian therapeutic agent(s) (including, but not limited to, for example, protein(s), polypeptide(s), peptide(s), enzyme(s), antibodies, antigen binding fragments, as well as variants, and/or active fragments thereof, for use in the treatment, prophylaxis, and/or amelioration of one or more symptoms of a mammalian disease, dysfunction, injury, and/or disorder.

[0035] In one embodiment, the invention provides an rAAV vector that comprises at least a first capsid protein comprising at least a first amino acid substitution to a non-native amino acid at one or more surface exposed amino acid residues in an rAAV capid protein, and wherein the vector further

additionally includes at least a first nucleic acid segment that encodes at least a first diagnostic or therapeutic agent operably linked to a promoter capable of expressing the segment in a host cell that contains the expression vector construct.

[0036] The surface-exposed amino acid-modified rAAV vectors of the present invention may optionally further include one or more enhancer sequences that are each operably linked to the nucleic acid segment. Exemplary enhancer sequences include, but are not limited to, one or more selected from the group consisting of a CMV enhancer, a synthetic enhancer, a liver-specific enhancer, an vascular-specific enhancer, a brain-specific enhancer, a neural cell-specific enhancer, a lung-specific enhancer, a muscle-specific enhancer, a kidney-specific enhancer, a pancreas-specific enhancer, and an islet cell-specific enhancer.

[0037] Exemplary promoters useful in the practice of the invention include, without limitation, one or more heterologous, tissue-specific, constitutive or inducible promoters, including, for example, but not limited to, a promoter selected from the group consisting of a CMV promoter, a β-actin promoter, an insulin promoter, an enolase promoter, a BDNF promoter, an NGF promoter, an EGF promoter, a growth factor promoter, an axon-specific promoter, a dendrite-specific promoter, a brain-specific promoter, a hippocampal-specific promoter, a kidney-specific promoter, an elafin promoter, a cytokine promoter, an interferon promoter, a growth factor promoter, an α_1-antitrypsin promoter, a brain cell-specific promoter, a neural cell-specific promoter, a central nervous system cell-specific promoter, a peripheral nervous system cell-specific promoter, an interleukin promoter, a serpin promoter, a hybrid CMV promoter, a hybrid β-actin promoter, an EF1 promoter, a U1a promoter, a U1b promoter, a Tet-inducible promoter, a VP16-LexA promoter, or any combination thereof. In exemplary embodiments, the promoter may include a mammalian or avian β-actin promoter.

[0038] The first nucleic acid segment may also further include one or more post-transcriptional regulatory sequences or one or more polyadenylation signals, including, for example, but not limited to, a woodchuck hepatitis virus post-transcription regulatory element, a polyadenylation signal sequence, or any combination thereof.

[0039] Exemplary diagnostic or therapeutic agents deliverable to host cells by the present vector constructs include, but are not limited to, an agent selected from the group consisting of a polypeptide, a peptide, an antibody, an antigen binding fragment, a ribozyme, a peptide nucleic acid, a siRNA, an RNAi, an antisense oligonucleotide, an antisense polynucleotide, and any combination thereof.

[0040] In exemplary embodiments, the improved rAAV vectors of the invention will preferably encode at least one diagnostic or therapeutic protein or polypeptide selected from the group consisting of a molecular marker, an adrenergic agonist, an anti-apoptosis factor, an apoptosis inhibitor, a cytokine receptor, a cytokine, a cytotoxin, an erythropoietic agent, a glutamic acid decarboxylase, a glycoprotein, a growth factor, a growth factor receptor, a hormone, a hormone receptor, an interferon, an interleukin, an interleukin receptor, a kinase, a kinase inhibitor, a nerve growth factor, a netrin, a

neuroactive peptide, a neuroactive peptide receptor, a neurogenic factor, a neurogenic factor receptor, a neuropilin, a neurotrophic factor, a neurotrophin, a neurotrophin receptor, an N-methyl-D-aspartate antagonist, a plexin, a protease, a protease inhibitor, a protein decarboxylase, a protein kinase, a protein kinsase inhibitor, a proteolytic protein, a proteolytic protein inhibitor, a semaphorin,, a semaphorin receptor, a serotonin transport protein, a serotonin uptake inhibitor, a serotonin receptor, a serpin, a serpin receptor, a tumor suppressor, and any combination thereof.

[0041] In certain applications, the capsid-modified rAAV vectors of the present invention may include one or more nucleic acid segments that encode a polypeptide selected from the group consisting of BDNF, CNTF, CSF, EGF, FGF, G-SCF, GM-CSF, gonadotropin, IFN, IFG-1, M-CSF, NGF, PDGF, PEDF, TGF, TGF-B2, TNF, VEGF, prolactin, somatotropin, XIAP1, IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-10(I87A), viral IL-10, IL-11, IL-12, IL-13, IL-14, IL-15, IL-16, IL-17, IL-18, and any combination thereof.

[0042] In another embodiment, the invention concerns genetically-modified inproved transduction-efficiency rAAV vectors that include at least a first nucleic acid segment that encodes one or more therapeutic agents that alter, inhibit, reduce, prevent, eliminate, or impair the activity of one or more endogenous biological processes in the cell. In particular embodiments, such therapeutic agents may be those that selectively inhibit or reduce the effects of one or more metabolic processes, dysfunctions, disorders, or diseases. In certain embodiments, the defect may be caused by injury or trauma to the mammal for which treatment is desired. In other embodiments, the defect may be caused the over-expression of an endogenous biological compound, while in other embodiments still; the defect may be caused by the under-expression or even lack of one or more endogenous biological compounds.

[0043] When the use of such vectors is contemplated for introduction of one or more exogenous proteins, polypeptides, peptides, ribozymes, siRNAs, and/or antisense oligonucleotides, to a particular cell transfected with the vector, one may employ the modified AAV vectors disclosed herein by incorporating into the vector at least a first exogenous polynucleotide operably positioned downstream and under the control of at least a first heterologous promoter that expresses the polynucleotide in a cell comprising the vector to produce the encoded therapeutic agent, including for example, peptides, proteins, polypeptides, antibodies, ribozymes, siRNAs, and antisense oligo- or polynucleotides.

[0044] The genetically-modified rAAV vectors and expression systems of the present invention may also further optionally include a second distinct nucleic acid segment that comprises, consists essentially of, or consists of, one or more enhancers, one or more regulatory elements, one or more transcriptional elements, or any combination thereof, that alter, improve, regulate, and/or affect the transcription of the nucleotide sequence of interest expressed by the modified rAAV vectors.

[0045] For example, the rAAV vectors of the present invention may further include a second nucleic acid segment that comprises, consists essentially of, or consists of, a CMV enhancer, a

synthetic enhancer, a cell-specific enhancer, a tissue-specific enhancer, or any combination thereof. The second nucleic acid segment may also further comprise, consist essentially of, or consist of, one or more intron sequences, one or more post-transcriptional regulatory elements, or any combination thereof.

[0046] The improved vectors and expression systems of the present invention may also optionally further include a polynucleotide that comprises, consists essentially of, or consists of, one or more polylinkers, restriction sites, and/or multiple cloning region(s) to facilitate insertion (cloning) of one or more selected genetic elements, genes of interest, or therapeutic or diagnostic constructs into the rAAV vector at a selected site within the vector.

[0047] In further aspects of the present invention, the exogenous polynucleotide(s) that may be delivered into suitable host cells by the improved, capsid-modified, rAAV vectors disclosed herein are preferably of mammalian origin, with polynucleotides encoding one or more polypeptides or peptides of human, non-human primate, porcine, bovine, ovine, feline, canine, equine, epine, caprine, or lupine origin being particularly preferred.

[0048] The exogenous polynucleotide(s) that may be delivered into host cells by the disclosed capsid-modified viral vectors may, in certain embodiments, encode one or more proteins, one or more polypeptides, one or more peptides, one or more enzymes, or one or more antibodies (or antigen-binding fragments thereof), or alternatively, may express one or more siRNAs, ribozymes, antisense oligonucleotides, PNA molecules, or any combination thereof. When combinational gene therapies are desired, two or more different molecules may be produced from a single rAAV expression system, or alternatively, a selected host cell may be transfected with two or more unique rAAV expression systems, each of which may comprise one or more distinct polynucleotides that encode a therapeutic agent.

[0049] In other embodiments, the invention also provides capsid-modified rAAV vectors that are comprised within an infectious adeno-associated viral particle or a virion, as well as pluralities of such virions or infectious particles. Such vectors and virions may be comprised within one or more diluents, buffers, physiological solutions or pharmaceutical vehicles, or formulated for administration to a mammal in one or more diagnostic, therapeutic, and/or prophylactic regimens. The vectors, virus particles, virions, and pluralities thereof of the present invention may also be provided in excipient formulations that are acceptable for veterinary administration to selected livestock, exotics, domesticated animals, and companion animals (including pets and such like), as well as to non-human primates, zoological or otherwise captive specimens, and such like.

[0050] The invention also concerns host cells that comprise at least one of the disclosed capsid protein-modified rAAV expression vectors, or one or more virus particles or virions that comprise such an expression vector. Such host cells are particularly mammalian host cells, with human host cells being particularly highly preferred, and may be either isolated, in cell or tissue culture. In the case of genetically modified animal models, the transformed host cells may even be comprised within

the body of a non-human animal itself.

[0051] In certain embodiments, the creation of recombinant non-human host cells, and/or isolated recombinant human host cells that comprise one or more of the disclosed rAAV vectors is also contemplated to be useful for a variety of diagnostic, and laboratory protocols, including, for example, means for the production of large-scale quantities of the rAAV vectors described herein. Such virus production methods are particularly contemplated to be an improvement over existing methodologies including in particular, those that require very high titers of the viral stocks in order to be useful as a gene therapy tool. The inventors contemplate that one very significant advantage of the present methods will be the ability to utilize lower titers of viral particles in mammalian transduction protocols, yet still retain transfection rates at a suitable level.

[0052] Compositions comprising one or more of the disclosed capsid-modified, improved transduction-efficiency rAAV vectors, expression systems, infectious AAV particles, or host cells also form part of the present invention, and particularly those compositions that further comprise at least a first pharmaceutically-acceptable excipient for use in therapy, and for use in the manufacture of medicaments for the treatment of one or more mammalian diseases, disorders, dysfunctions, or trauma. Such pharmaceutical compositions may optionally further comprise one or more diluents, buffers, liposomes, a lipid, a lipid complex; or the tyrosine-modified rAAV vectors may be comprised within a microsphere or a nanoparticle.

[0053] Pharmaceutical formulations suitable for intramuscular, intravenous, or direct injection into an organ or tissue or a plurality of cells or tissues of a human or other mammal are particularly preferred, however, the compositions disclosed herein may also find utility in administration to discreet areas of the mammalian body, including for example, formulations that are suitable for direct injection into one or more organs, tissues, or cell types in the body. Such injection sites include, but are not limited to, the brain, a joint or joint capsule, a synovium or subsynovium tissue, tendons, ligaments, cartilages, bone, peri-articular muscle or an articular space of a mammalian joint, as well as direct administration to an organ such as the heart, liver, lung, pancreas, intestine, brain, bladder, kidney, or other site within the patient's body, including, for example , introduction of the viral vectors *via* intraabdominal, intrathorascic, intravascular, or intracerebroventricular delivery.

[0054] Other aspects of the invention concern recombinant adeno-associated virus virion particles, compositions, and host cells that comprise, consist essentially of, or consist of, one or more of the capsid-modified, improved transduction efficiency, rAAV vectors disclosed herein, such as for example pharmaceutical formulations of the vectors intended for administration to a mammal through suitable means, such as, by intramuscular, intravenous, intra-articular, or direct injection to one or more cells, tissues, or organs of a selected mammal. Typically, such compositions may be formulated with pharmaceutically-acceptable excipients as described hereinbelow, and may comprise one or more liposomes, lipids, lipid complexes, microspheres or nanoparticle formulations to facilitate administration to the selected organs, tissues, and cells for which therapy is desired.

[0055] Kits comprising one or more of the disclosed capsid-modified rAAV vectors (as well as one or more virions, viral particles, transformed host cells or pharmaceutical compositions comprising such vectors); and instructions for using such kits in one or more therapeutic, diagnostic, and/or prophylactic clinical embodiments are also provided by the present invention. Such kits may further comprise one or more reagents, restriction enzymes, peptides, therapeutics, pharmaceutical compounds, or means for delivery of the composition(s) to host cells, or to an animal (*e.g.*, syringes, injectables, and the like). Exemplary kits include those for treating, preventing, or ameliorating the symptoms of a disease, deficiency, dysfunction, and/or injury, or may include components for the large-scale production of the viral vectors themselves, such as for commercial sale, or for use by others, including *e.g.*, virologists, medical professionals, and the like.

[0056] Another important aspect of the present invention concerns methods of use of the disclosed rAAV vectors, virions, expression systems, compositions, and host cells described herein in the preparation of medicaments for diagnosing, preventing, treating or ameliorating at least one or more symptoms of a disease, a dysfunction, a disorder, an abnormal condition, a deficiency, injury, or trauma in an animal, and in particular, in a vertebrate mammal. Such methods generally involve administration to a mammal in need thereof, one or more of the disclosed vectors, virions, viral particles, host cells, compositions, or pluralities thereof, in an amount and for a time sufficient to diagnose, prevent, treat, or lessen one or more symptoms of such a disease, dysfunction, disorder, abnormal condition, deficiency, injury, or trauma in the affected animal. The methods may also encompass prophylactic treatment of animals suspected of having such conditions, or administration of such compositions to those animals at risk for developing such conditions either following diagnosis, or prior to the onset of symptoms.

[0057] As described above, the exogenous polynucleotide will preferably encode one or more proteins, polypeptides, peptides, ribozymes, or antisense oligonucleotides, or a combination of these. In fact, the exogenous polynucleotide may encode two or more such molecules, or a plurality of such molecules as may be desired. When combinational gene therapies are desired, two or more different molecules may be produced from a single rAAV expression system, or alternatively, a selected host cell may be transfected with two or more unique rAAV expression systems, each of which will provide unique heterologous polynucleotides encoding at least two different such molecules.

[0058] Compositions comprising one or more of the disclosed rAAV vectors, expression systems, infectious AAV particles, host cells also form part of the present invention, and particularly those compositions that further comprise at least a first pharmaceutically-acceptable excipient for use in the manufacture of medicaments and methods involving therapeutic administration of such rAAV vectors. Such pharmaceutical compositions may optionally further comprise liposomes, a lipid, a lipid complex; or the rAAV vectors may be comprised within a microsphere or a nanoparticle. Pharmaceutical formulations suitable for intramuscular, intravenous, or direct injection into an organ or tissue of a human are particularly preferred.

[0059] Another important aspect of the present invention concerns methods of use of the disclosed vectors, virions, expression systems, compositions, and host cells described herein in the preparation of medicaments for treating or ameliorating the symptoms of various polypeptide deficiencies in a mammal. Such methods generally involve administration to a mammal, or human in need thereof, one or more of the disclosed vectors, virions, host cells, or compositions, in an amount and for a time sufficient to treat or ameliorate the symptoms of such a deficiency in the affected mammal. The methods may also encompass prophylactic treatment of animals suspected of having such conditions, or administration of such compositions to those animals at risk for developing such conditions either following diagnosis, or prior to the onset of symptoms.

BRIEF DESCRIPTION OF THE DRAWINGS

[0060] For promoting an understanding of the principles of the invention, reference will now be made to the embodiments, or examples, illustrated in the drawings and specific language will be used to describe the same. It will, nevertheless be understood that no limitation of the scope of the invention is thereby intended. Any alterations and further modifications in the described embodiments, and any further applications of the principles of the invention as described herein are contemplated as would normally occur to one of ordinary skill in the art to which the invention relates.

[0061] The following drawings form part of the present specification and are included to demonstrate certain aspects of the present invention. The invention may be better understood by reference to the following description taken in conjunction with the accompanying drawings, in which like reference numerals identify like elements, and in which:

[0062] **FIG. 1A, FIG. 1B, and FIG. 1C** show the effect of NF-κB pathway inhibitors and activator on AAV vector-mediated EGFP expression in HeLa cells *in vitro*. Cells were pre-treated with various concentrations of inhibitors and activators for 12 hrs and transduced with 2×10^3 AAV-EGFP vgs per cell. **FIG. 1A** shows the transgene expression was detected by fluorescence microscopy 48 hrs post-infection. Representative images are shown. **FIG. 1B** shows the quantitative analyses of the data from **FIG. 1A**. Images from five visual fields were analyzed as described. $^*P < 0.001$. **FIG. 1C** is a Western blot analysis of HeLa cell extracts transduced with scAAV vectors and in the presence of NF-κB modulators. The samples were analyzed by using anti-p65 and anti-IκB antibodies [classical pathway], anti-p100/p52 antibody [non-canonical pathway] for detection NF-κB signaling in response to AAV exposure. These results are representative of two independent experiments;

[0063] **FIG. 2A and FIG. 2B** show AAV-EGFP vector-mediated transduction of primary human monocytes-derived dendritic cells in the presence of NF-κB modulators. **FIG. 2A** shows the transgene expression was detected by flow cytometry 48 hrs post-transduction. **FIG. 2B** is a Western blot analysis for components of classical and non-canonical pathway of NF-κB activation in nuclear

extracts from dendritic cells, mock-transduced or transduced with 2,000 vgs/cell of scAAV vectors and in the presence of NF-κB modulators;

[0064] **FIG. 3A and FIG. 3B** show AAV vector-induced innate immune and NF-κB response in mice *in vivo*. Gene expression profiling of innate immune mediators (**FIG. 3A**) or NF-κB activation (**FIG. 3B**) was performed as described. The data for fold changes in gene expression at the 2-hr time-point comparing AAV vectors with Bay11 (hatched or open bars) with AAV vectors without Bay11 (black or grey bars) are shown. The minimal threshold fold-increase (horizontal black line) was 2.5 (FIG. 3A) or 3.0 (FIG. 3B) by measuring the variability of duplicate ΔCT (compared to GAPDH, $2^{\wedge-\Delta CT(variability)}$);

[0065] **FIG. 4A and FIG. 4B** illustrate transgene expression in murine hepatocytes 10 days post-injection of 1×10^{11} vgs each of WT-scAAV-EGFP or TM-scAAV-EFGP vectors/animal *via* the tail-vein. **FIG. 4A** shows representative images are shown. Original magnification: ×400. **FIG. 4B** shows the quantitative analyses of the data from **FIG. 4A**. Images from five visual fields were analyzed quantitatively as described in the legend to **FIG. 1A**;

[0066] **FIG. 5** demonstrates that AAV genome contains putative binding sites for NF-κB-responsive transcription factors within the inverted terminal repeats (ITRs). The putative NF-κB-responsive transcription factor-binding sites in the AAV-ITRs were identified by *in silico* analysis using the web-based TRANSFAC database. The binding sites for p300, TFIIB, and SpII transcriptions factors are denoted by green, red, and blue underlined fonts, respectively. The boxed sequence represents the 20-nucleotide, single-stranded D-sequence within the ITR;

[0067] **FIG. 6A, FIG. 6B, FIG. 6C, FIG. 6D, and FIG. 6E** show the effect of NF-κB activators and inhibitors on transgene expression from an AAV2-EGFP vector in HeLa cells *in vitro*. Cells were either mock-treated or pretreated with various combinations of inhibitors and activators for 12 hr. Washed cells were infected with 2×10^3 vg/cell of scAAV2-EGFP (**FIG. 6A**), ssAAV2-EGFP (**FIG. 6B**), or TM-scAAV2-EGFP (**FIG. 6C**). Transgene expression was detected by fluorescence microscopy 48-hrs' postinfection. Representative images are shown; Western blot analysis of cytoplasmic (**FIG. 6D**) and nuclear (**FIG. 6E**) extracts from HeLa cells transduced with scAAV vectors and in the presence of NF-κB modulators. The samples were analyzed by using anti-p100/p52 antibody for detection of NF-κB signaling. Anti-GAPDH and lamin B antibodies were used as appropriate controls. These results are representative of two independent experiments;

[0068] **FIG. 7** shows a Western blot analysis of liver homogenates from mice following mock-injections (n = 2), or injections with scAAV vectors, with and without prior administration of Bay11 (n = 3 each). The samples were analyzed by using anti-p52 antibody for detection NF-κB signaling in response to AAV exposure. Anti-β-actin antibody was used as a loading control;

[0069] **FIG. 8A, FIG. 8B, FIG. 8C, FIG. 8D, FIG. 8E, and FIG. 8F** show fold changes in gene expression of various cytokines/chemokines from total mRNA collected from liver samples from animals injected with the WT-AAV or the TM-AAV vectors, following PBS- or Bay11-pre-treatment.

FIG. 8A: IL-1α; **FIG. 8B**: IL-6; **FIG. 8C**: TNF-α; **FIG. 8D**: IL-12α, **FIG. 8E**: KC; and **FIG. 8F**: RANTES. Values are significant above 2.6 and below 0.38; calculated by determining the variability in the 96-well plates used to measure specific gene expression;

[0070] **FIG. 9** demonstrates humoral response to AAV vectors in the absence or presence of NF-kB inhibitor. Anti-AAV2 IgG2a levels were determined in peripheral blood from mice at day 10 following injections with scAAV vectors, with and without prior administration of Bay11 (n = 4 each);

[0071] **FIG. 10** illustrate electrophoretic mobility-shift assays carried out with whole-cell extracts prepared from HeLa cells and ^{32}P-labeled single-stranded D[+]-sequence probe (lane 1), which interacted with a host cell protein (lane 3, arrowhead). Single- stranded D[-]-sequence (lane 2) probe was used as an appropriate control, which also interacted with a cellular protein, FKBP52 (lane 4, arrow). Binding assays were also carried out using biotin-labeled ssD[+]-sequence probe followed by selection with streptavidin-beads, and fractionation by SDS-polyacrylamide gel electrophoresis. The relevant protein band was visualized by silver staining, excised from the gel, and subjected to mass spectrometry, and one of the unique peptides was found to share homology with the NF-κB–repressing factor (NRF);

[0072] **FIG. 11A and FIG. 11B** show the analysis of AAV3-mediated transgene expression in T47D and T47D+hHGFR cells. **FIG. 11A** shows equivalent numbers of T47D and T47D+hHGFR cells were infected with various indicated multiplicity-of-infection (MOI) of scAAV3-CBAp-EGFP vectors under identical conditions. Transgene expression was determined by fluorescence microscopy 72 hrs post-infection. **FIG. 11B** shows T47D+hHGFR cells were transduced with 2,000 vgs/cell of scAAV3 vectors in the absence or the presence of 5 μg/mL of hHGF. Transgene expression was determined by fluorescence microscopy 72-hrs' post-infection;

[0073] **FIG. 12A, FIG. 12B and FIG. 12C** show the effect of BMS-777607 on AAV3-mediated transgene expression. **FIG. 12A** shows T47D+ hHGFR cells, either mock-treated or treated with various concentration of BMS-777607, that were infected with 2,000 vgs/cell of scAAV3-CBAp-EGFP vectors. Transgene expression was determined by fluorescence microscopy 72 hrs' post-infection. **FIG. 12B** illustrates T47D and T47D+hHGFR cells were infected with 10,000 vgs/cell of scAAV3-CBAp-EGFP vectors in the absence or the presence of 1 μM of BMS-777607. **FIG. 12C** shows T47D and T47D+hHGFR cells were mock-treated or pretreated with BMS-777607 for two hrs. Whole-cell lysates were prepared and analyzed on Western blots using various indicated primary antibodies. β-actin was used as a loading control;

[0074] **FIG. 13A and FIG. 13B** show the effect of BMS-777607 on various AAV serotype-mediated transgene expression. In **FIG. 13A,** T47D+ hHGFR cells, either mock-treated or treated with 1 μM of BMS-777607, were infected with 2,000 vgs/cell of either scAAV2-, scAAV3- or scAAV4-CBAp-EGFP vectors. In **FIG. 13B,** T47D+ hHGFR cells, either mock-treated or treated with 1 μM of BMS-777607, were infected with 2,000 vgs/cell of either scAAV5-, scAAV7-,

scAAV8- or scAAV9-CBAp-EGFP vectors. Transgene expression was determined by fluorescence microscopy 72 hrs post-infection;

[0075] **FIG. 14A, FIG. 14B, FIG. 14C and FIG. 14D** show the comparative analyses of AAV3-mediated transduction efficiency in Huh7 and Hep293TT cells with or without treatment with MG132. In **FIG. 14A,** HeLa cells, either mock-treated or treated with 5 μM of MG132, were infected with scAAV2-CBAp-EGFP vectors. In **FIG. 14B,** Huh7 and Hep293TT cells, either mock-treated or treated with various concentration of MG132, were infected with scAAV3-WT-CBAp-EGFP vectors. In **FIG. 14C,** HeLa cells, either mock-treated or treated with 200 μM of Tyr23, were infected by scAAV2-CBAp-EGFP vectors. In **FIG. 14D,** Hep293TT cells, either mock-treated or treated with Tyr23, were infected by scAAV3-CBAp-EGFP vectors. Transgene expression was determined 72 hrs' post-transduction;

[0076] **FIG. 15A, FIG. 15B and FIG. 15C** show the site-directed mutational analyses of surface-exposed tyrosine residues on AAV3 capsids. Huh7 cells were transduced with WT or F501Y scAAV3-CBAp-EGFP vectors under identical conditions, and transgene expression was determined 72 hrs' post-transduction. Transduction efficiency of WT (**FIG. 15A**) and various Y-F scAAV3-mediated transgene expression in Huh7 (**FIG. 15B**) and Hep293TT (**FIG. 15C**) cells are shown. Transgene expression was determined 72 hrs post-transduction;

[0077] **FIG. 16A, FIG. 16B, and FIG. 16C** illustrate the transduction efficiency of WT and single, double, and triple tyrosine-mutant AAV3 vectors. In **FIG. 16A,** Huh7 cells were transduced with WT or various indicated Y-F mutant scAAV3-CBAp-EGFP vectors under identical conditions. Transgene expression was determined 72-hrs' post-transduction. In **FIG. 16B,** Huh7 cells were transduced with 5,000 vgs/cell of WT or Y→F mutated scAAV3 vectors in the absence or the presence of 5 μg/mL of hHGF. **FIG. 16C** shows transgene expression was determined by fluorescence microscopy 72 hrs post-infection;

[0078] **FIG. 17A, FIG. 17B, FIG. 17C and FIG. 17D** show the transduction efficiency of AAV3 vectors *in vivo* following direct intra-tumor injections. Transduction efficiency of WT-AAV3 vectors in Huh7- (**FIG. 17A**) and Hep293TT- (**FIG. 17B**) derived tumors in NSG mice is shown. The transduction efficiency of WT- (**FIG. 17C**) and Y705+731F-AAV3 (**FIG. 17D**) vectors in Hep293TT-derived tumors are also shown in NSG mice. EGFP fluorescence (green) and DAPI staining (blue) of two representative tumor sections from each set of mice is shown;

[0079] **FIG. 18A, FIG. 18B and FIG. 18C** illustrate the transduction efficiency of WT- and Y705+731F-AAV3 vectors in Hep293TT-derived tumors in NSG mice following tail-vein injections. EGFP fluorescence (green) and DAPI staining (blue) of tumor in three representative tumor sections from each set of mice injected with PBS **(FIG. 18A),** WT-AAV3 (**FIG. 18B**), or Y705+731F-AAV3 **(FIG. 18C)** vectors is shown;

[0080] **FIG. 19A and FIG. 19B** show the effect of various kinase inhibitors on ssAAV and scAAV mediated EGFP expression in HEK293 cells. Cells were pretreated with inhibitors for 1 hr

before infection then transduced with 1×10^3 vgs/cell. In **FIG. 19A,** transgene expression was detected by fluorescence microscopy 48 hrs post infection. In **FIG. 19B**, images from three visual fields were analyzed as described. $*P < 0.005$, $**P < 0.001$ *vs.* WT AAV2;

[0081] **FIG. 20A and FIG. 20B** show the analysis of EGFP expression after transduction of HEK293 cells with individual site-directed scAAV2 capsid mutants. Each of the 15 surface-exposed serines (S) in the AAV2 capsid was substituted with valine (V), and then evaluated for its efficiency to mediate transgene expression. **FIG. 20A** shows the EGFP expression analysis at 48 hrs post-infection at an MOI of 1×10^3 vgs/cell. **FIG. 20B** shows the quantitation of transduction efficiency of each of the serine-mutant AAV2 vectors. $*P < 0.005$, $**P < 0.001$ *vs.* WT AAV2;

[0082] **FIG. 21 and FIG. 21B** illustrate the structure of AAV2. In **FIG. 21A**, a trimer of the AAV2 VP3 shown in ribbon representation and viewed down the icosahedral threefold axis (left) and rotated 90° (right) with VP monomers colored in blue, purple and light blue showing the location of serine residues 458, 492, and 662 in the yellow, green, and red spheres, respectively. The approximate positions of the icosahedral two-, three-, and five-fold axes are depicted by the filled oval, triangle, and pentagon, respectively. In **FIG. 21B**, the capsid surface of AAV2 shown in blue with serine residues 458, 492, and 662 highlighted in the same colors as shown previouisly in similar figures. The S458 and S492 residues are located adjacent to each other on the outer surface of the protrusions facing the depression surrounding the two-fold axes. S662 is located on the HI loop (colored white) (between the β-H and β-I strands of the core eight-stranded beta-barrel) which lie on the floor of the depression surrounding the icosahedral five-fold axes. The five-fold symmetry related DE loops (between the β-D and β-E strands), which form the channel at the icosahedral 5-fold axes, are shown in brown. The approximate positions of an icosahedral two-fold (2F), three-fold (3F), and five-fold (5F) axes are indicated by the open arrows;

[0083] **FIG. 22A and FIG. 22B** summarize the evaluation of the effect of serine substitution at position 662 in the scAAV2 capsid with different amino acids in mediating transgene expression. T he following eight serine mutants were generated with different amino acids: S662→Valine (V), S662→Alanine (A), S662→Asparagine (N), S662→Aspartic acid (D), S662→Histidine (H), S662→Isoleucine (I), S662→Leucine (L), and S662→Phenylalanine (F), and their transduction efficiency in 293 cells was analyzed. **FIG. 22A** shows the EGFP expression analysis at 48 h after infection of 293 cells at an MOI of 1×10^3 vgs/cell. **FIG. 22B** shows the quantitation of the transduction efficiency of each of the serine-mutant AAV2 vectors. $*P < 0.005$, $**P < 0.001$ *vs.* WT AAV2;

[0084] **FIG. 23A and FIG. 23B** show the analysis of correlation of transduction efficiency of scAAV2-S662V vectors with p38 MAPK activity in various cell types. **FIG. 23A** illustrates the quantitation of the transduction efficiency of WT- and S662V-AAV2 vectors in HEK293, HeLa, NIH3T3, H2.35 and moDCs. **FIG. 23B** is a Western blot analysis of lysates from different cell lines for p-p38 MAPK expression levels. Total p38 MAPK and GAPDH levels were measured and used as

loading controls. *$P < 0.005$, **$P < 0.001$ *vs.* WT AAV2;

[0085] **FIG. 24A, FIG. 24B, and FIG. 24C** illustrate scAAV vector-mediated transgene expression in monocyte-derived dendritic cells (moDCs). **FIG. 24A** illustrates the effect of JNK and p38 MAPK inhibitors, and site-directed substitution of the serine residue at position 662 on EGFP expression. **FIG. 24B** summarizes the quantitation of data from **FIG. 24A** at 48 hrs after infection and initiation of maturation. **FIG. 24C** is an analysis of expression of co-stimulatory markers such as CD80, CD83, CD86 in moDCs infected with scAAV2-S662V vectors at an MOI of 5×10^4 vgs/cell. iDCs – immature dendritic cells, and mDCs – mature dendritic cells, stimulated with cytokines, generated as described herein, were used as negative and positive controls, respectively. A representative example is shown. *$P < 0.005$, **$P < 0.001$ *vs.* WT AAV2;

[0086] **FIG. 25** illustrates analysis of hTERT-specific cytotoxic T-lymphocytes (CTLs) killing activity on K562 cells. CTLs were generated after transduction of moDCs by scAAV2-S662V vectors encoding the truncated human telomerase (hTERT). scAAV2-S662V-EGFP vector-traduced moDCs were used to generate non-specific CTLs. Pre-stained with 3,3-dioctadecyloxacarbocyanine (DiOC18(3)), a green fluorescent membrane stain, 1×10^5 target K562 cells were co-cultured overnight with different ratios of CTLs (80:1, 50:1, 20:1, 10:1, 5:1). Membrane-permeable nucleic acid counter-stain, propidium iodide, was added to label the cells with compromised plasma membranes. Percentages of killed, double stain-positive cells were analyzed by flow cytometry;

[0087] **FIG. 26A and FIG. 26B** show the analysis of EGFP expression after transduction of HEK293 cells with individual site-directed AAV2 capsid mutants. Each of the 17 surface-exposed threonine (T) residues in AAV2 capsid was substituted with valine (V) and evaluated for its efficiency to mediate transgene expression. In **FIG. 26A, E**GFP expression analysis at 48-hrs' post-infection is shown (MOI of 1×10^3 vg/cell). **FIG. 26B** shows the quantification of transduction efficiency of each of the threonine-mutant scAAV2 vectors. *$P < 0.005$, **$P < 0.001$ *vs.* WT AAV2;

[0088] **FIG. 27A and FIG. 27B** show the analysis of EGFP expression in HEK293 cells infected with multiple site-directed AAV2 capsid mutants. Several most efficient threonine mutations were combined on single AAV2 capsid to produce double- and triple-mutant and efficiency of each vector was evaluated. **FIG. 27A** illustrates EGFP expression analysis at 48 hrs' post-infection at MOI of 1×10^3 vg/cell. **FIG. 27B** shows the quantification of transduction efficiency of each of the threonine-mutant AAV2 vectors. *$P < 0.005$, **$P < 0.001$ *vs.* WT AAV2;

[0089] **FIG. 28A and FIG. 28B** demonstrate the evaluation of EGFP expression in H2.35 cell transduced with capsid optimized AAV2 vectors. The most efficient tyrosine, serine and threonine mutations were combined on single AAV2 capsid to produce several optimized AAV mutants. Efficiency of each vector was estimated on immortalized murine hepatocytes. **FIG. 28A** shows EGFP expression analysis at 48 hrs' post-infection at MOI of 1×10^3 vg/cell. **FIG. 28B** summarizes the quantification of transduction efficiency of each of the optimized scAAV2 vectors. *$P < 0.005$, **$P < 0.001$ *vs.* WT AAV2;

[0090] **FIG. 29A and FIG. 29B** illustrate the kinetics of EGFP expression in H2.35 cell mediated by capsid optimized AAV vectors. **FIG. 29A** shows EGFP expression analysis at 16, 24 and 48 hrs' post-infection at MOI of 1×10^3 vg/cell. **FIG. 29B** illustrates results from quantification of transduction efficiency of each of the optimized scAAV2 vectors. $^*P < 0.005$, $^{**}P < 0.001$ *vs.* WT AAV2;

[0091] **FIG. 30A and FIG. 30B** show the analysis of intracellular trafficking of AAV multiple mutant vectors to the nucleus. Nuclear and cytoplasmic fractions of H2.35 cell infected with AAV2-WT, AAV2-Y444F+Y500F+Y730F or the AAV2-Y444F+Y500F+Y730F+T491V multi-mutant were separated and qPCR analysis was performed to evaluate vector genome distribution within cells at 16 hrr (**FIG. 30A**) and 48 hrr (**FIG. 30B**) post infection. ** $P < 0.001$ *vs.* WT in nucleus was considered as significant;

[0092] **FIG. 31A and FIG. 31B** show the *in vivo* imaging of luciferase gene expression following tail vein injection of multiple site-directed AAV2 capsid mutants. C57BL/6 mice were injected with 1×10^{10} vg/animal of several most efficient mutant scAAV vectors carrying luciferase gene. Live images were taken to analyses difference in luciferase activity. The visual output represents the number of photons emitted/second/cm^2 as a false color image where the maximum is red and the minimum is blue (**FIG. 31A**) and relative signal intensity (**FIG. 31B**) $^*P < 0.005$ was considered as significant;

[0093] **FIG. 32A and FIG. 32B** illustrate the AAV2 capsid surface. **FIG. 32A** shows the capsid surface of AAV2 (grey) with the 17 surface threonine residues mutated in blue (251, 329, 330, 454, 503, 581, 592, 597, 660, 671, 701, 713, 716), green (455), yellow (491), brown (550), and pink (659). The surface location of T329, T330, T713 and T716 are indicated by arrows. The five-fold symmetry related DE loops (between the βD and βE strands) are colored in orange. The HI loops (between the βH and βI strands) are colored white and S662 located in this loop is in red. The white dashed triangle in **FIG. 32A** depicts a viral asymmetric unit bounded by a five-fold axis and two three-fold axes with a two-fold axis between the three-folds. Dashed ovals delineate the approximate footprints (2/60) of threonine residues that affect transduction when mutated. **FIG. 32B** shows a "roadmap" projection of the AAV2 capsid surface residues within a viral asymmetric unit. The areas covered by AAV2 surface threonines and S662 are colored as in **FIG. 32A**. The residues in the tyrosine triple mutant residues, 444, 500, and 730 are shown in shades of purple. Dashed ovals are as described in **FIG. 23A**. Dashed rectangle (blue) shows residues previously determined to be important in heparin sulfate receptor binding for AAV2 and AAV6 (Wu *et al.*, 2006; Opie *et al.*, 2003);

[0094] **FIG. 33A, FIG. 33B, and FIG. 33C** illustrate the amino acid alignment of the wild-type capsids from serotypes AAV1 through AAV10. **FIG. 33A** shows amino acid alignment of the wild-type AAV1-10 serotype capsids (SEQ ID NO:1 through SEQ ID NO:10). **FIG. 33B** shows amino acid alignment of the wild-type AAV1-AAV10 serotype capsids, as well as surface-exposed serine and threonine residues that are conserved in among AAV1-AAV10 capsids (conserved, surface-

exposed residues are shown in bold); and **FIG. 33C** shows conserved, surface-exposed tyrosine residues in the wild-type AAV1-AAV12 capsids, as well as embodiments of amino acid modifications. The tyrosine residues conserved among AAV1-AAV12 are shown in bold;

[0095] FIG. 34 show packaging and transduction efficiencies of various serine→valine mutated AAV2 vectors relative to that of WT AAV2 vectors and the amino acid alignment of wild-type AAV1-AAV10 capsids;

[0096] FIG. 35 depicts packaging and transduction efficiencies of serine-mutant vectors replaced with various samino acids relative to WT AAV2 vectors;

[0097] FIG. 36A, FIG. 36B, FIG. 36C, FIG. 36D, and FIG. 36E show transduction efficiency of rAAV3 and rAAV8 vectors in human HCC tumors in a murine xenograft model *in vivo*. Female and male Huh7 tumor-bearing NSG mice were used for tail-vein injection with either rAAV3-CBAp-FLuc or rAAV8-CBAp-FLuc vectors at 1×10^{11} vgs/mouse. $n = 4$ per group. **FIG. 36A** shows representative images of mouse whole body bioluminescent images at 3 days post-vector administration are shown. **FIG. 36B** illustrates the quantitative analysis of transgene expression data from whole body bioluminescent images of mice at 3-days' post-vector administration. **FIG. 36C** shows Huh7 tumor-bearing male NSG mice were used for direct intra-tumor injections with either rAAV3-CBAp-FLuc or rAAV8-CBAp-FLuc vectors at a lower dose of 1×10^{11} vgs/mouse (L) or at a higher dose of 1×10^{12} vgs/mouse (H). $n = 4$ per group. Representative images of lower dose at 7 days post-vector administration are shown. **FIG. 36D** presents the quantitative data for transgene expression in tumors and liver from mice injected with rAAV3 or rAAV8 vectors at 3 days or 7 days post-vector administration. **FIG. 36E** shows vector genome copy numbers persisting in the liver tissue samples from mice injected with higher dose of rAAV3 or rAAV8 vectors 7 days post-vector administration;

[0098] FIG. 37A, FIG. 37B, FIG. 37C, FIG. 37D, FIG. 37E, and FIG. 37F show transduction efficiency of WT and capsid-modified rAAV3 vectors in human liver cancer cells *in vitro*. **FIG. 37A** shows Huh7 cells were transduced with the indicated viral vectors carrying the CBAp-FLuc expression cassette at 5,000 vgs/cell. **FIG. 37B** shows HepG2 cells and **FIG. 37C** shows Hep293TT cells were transduced with the indicated viral vectors carrying the CBAp-EGFP expression cassette at 5,000 vgs/cell. **FIG. 37D** shows Huh7 cells were transduced with the indicated viral vectors carrying the CBAp-EGFP expression cassette at 5,000 vgs/cell either in the absence or presence of low (100 ng/mL) or high (100 μg/mL) doses of soluble heparin. **FIG. 37E** shows Huh7 cells were transduced with the indicated viral vectors carrying the CBAp-EGFP expression cassette at 5,000 vgs/cell in either the absence or presence of 5 μg/mL hHGF. **FIG. 37F** shows human T47D cells or T47D+hHGFR cells were transduced with indicated viral vectors carrying the CBAp-EGFP expression cassette at 5,000 vgs/cell. All transgene expression levels were determined 48 hrs post-transduction;

[0099] FIG. 38A, FIG. 38B, FIG. 38C, and FIG. 38D show transduction efficiency of exemplary

rAAV3 capsid-mutated vectors *in vivo*. **FIG. 38A** shows human Huh7- or Hep293TT liver tumor-bearing NSG mice were used for tail-vein injections with the indicated mutant viral vectors carrying the CBAp-FLuc expression cassette at 5×10^{10} vgs/mouse. $n = 4$ per each group. On Day 3, mouse whole-body bioluminescent images were obtained, followed by dissection of both growing tumor and normal liver. Representative images are shown. **FIG. 38B** shows quantitative data showing FLuc expression in Huh7- and Hep293TT-derived tumors. Vector genome copy numbers are shown in Huh7 tumors (**FIG. 38C**), and in normal livers (**FIG. 38D**);

[0100] **FIG. 39A, FIG. 39B and FIG. 39C** show exemplary embodiments of the present invention. **FIG. 39A** shows suppression of human liver tumorigenesis in a murine xenograft model by optimized rAAV3 vectors expressing the TCS gene; **FIG. 39B and FIG. 39C** show results of Huh7-FLuc tumor-bearing mice were used for tail-vein injections with the indicated viral vectors at 5×10^{10} vgs/mouse at Day 0, and tumor growth was monitored over time until Day 11. **FIG. 39B** depicts representative whole-body bioluminescence images of mice from both groups at Day 8. **FIG. 39C** summarizes serum activities of AST and ALT that were measured in rAAV3-TCS vector-injected and rAAV3-EGFP vector-injected mice at Day 11 by spectrophotometric methods. Data are presented as mean ± SD. (n = 5/group);

[0101] **FIG. 40A, FIG. 40B and FIG. 40C** show exemplary vector constructs and polynucleotide sequences useful in accordance with one aspect of the present invention. **FIG. 40A** shows the schematic structures of the rAAV vectors used in these studies. HP: hairpin; D: D-sequence in the AAV inverted terminal repeat (ITR); CBAp: CMV enhancer/chicken β-actin promoter; FLuc: firefly luciferase; hGH (A)n: human growth hormone polyA sequence; HP-: hairpin structure without the terminal resolution site (*trs)*; EGFP: enhanced green fluorescence protein; AFPp: human α-feto-protein promoter; TCS: Tricosanthin; **FIG. 40B** shows the nucleotide sequence of the original TCS gene. The start codon (ATG) and the stop codon (TAG) are shown in green and red fonts, respectively. **FIG. 40C** shows the nucleotide sequence of the FLAG-tagged TCS gene. The *Eco*RI and *Xho*I restriction enzyme sites used for cloning the chemically synthesized TCS gene are shown in bold italic font, respectively, and the FLAG-tag sequence containing the stop codon (TAA) is underlined; and

[0102] **FIG. 41** shows the transduction efficiency of various single-, double-, and multiple-mutant scAAV3-CBAp-EGFP vectors in human HCC cell line, Huh7.

BRIEF DESCRIPTION OF THE SEQUENCES

[0103] **SEQ ID NO:1** is an amino acid sequence of the capsid protein of the wild-type adeno-associated virus serotype 1 (AAV1);

[0104] **SEQ ID NO:2** is an amino acid sequence of the capsid protein of the wild-type adeno-associated virus serotype 2 (AAV2);

[0105] **SEQ ID NO:3** is an amino acid sequence of the capsid protein of the wild-type adeno-associated virus serotype 3 (AAV3);

[0106] **SEQ ID NO:4** is an amino acid sequence of the capsid protein of the wild-type adeno-associated virus serotype 4 (AAV4);

[0107] **SEQ ID NO:5** is an amino acid sequence of the capsid protein of the wild-type adeno-associated virus serotype 5 (AAV5);

[0108] **SEQ ID NO:6** is an amino acid sequence of the capsid protein of the wild-type adeno-associated virus serotype 6 (AAV6);

[0109] **SEQ ID NO:7** is an amino acid sequence of the capsid protein of the wild-type adeno-associated virus serotype 7 (AAV7);

[0110] **SEQ ID NO:8** is an amino acid sequence of the capsid protein of the wild-type adeno-associated virus serotype 8 (AAV8);

[0111] **SEQ ID NO:9** is an amino acid sequence of the capsid protein of the wild-type adeno-associated virus serotype 9 (AAV9);

[0112] **SEQ ID NO:10** is an amino acid sequence of the capsid protein of the wild-type adeno-associated virus serotype 10 (AAV10);

[0113] **SEQ ID NO:11** is an oligonucleotide primer sequence useful according to the present invention;

[0114] **SEQ ID NO:12** is an oligonucleotide primer sequence useful according to the present invention;

[0115] **SEQ ID NO:13** is an oligonucleotide primer sequence useful according to the present invention;

[0116] **SEQ ID NO:14** is an oligonucleotide primer sequence useful according to the present invention;

[0117] **SEQ ID NO:15** is an oligonucleotide primer sequence useful according to the present invention;

[0118] **SEQ ID NO:16** is an oligonucleotide primer sequence useful according to the present invention;

[0119] **SEQ ID NO:17** is an oligonucleotide primer sequence useful according to the present invention;

[0120] **SEQ ID NO:18** is an oligonucleotide primer sequence useful according to the present invention;

[0121] **SEQ ID NO:19** is an oligonucleotide primer sequence useful according to the present invention;

[0122] **SEQ ID NO:20** is an oligonucleotide primer sequence useful according to the present invention;

[0123] **SEQ ID NO:21** is an oligonucleotide primer sequence useful according to the present invention;

[0124] **SEQ ID NO:22** is a nucleic acid sequence containing the putatitve binding site for NF-kB-responsive transcription factors (See FIG. 5);

[0125] **SEQ ID NO:23** is a single-stranded nucleic acid sequence probe (see FIG. 10);

[0126] **SEQ ID NO:24** is a double-stranded nucleic acid sequence probe (see FIG. 10);

[0127] **SEQ ID NO:25** is a single-stranded nucleic acid sequence probe (see FIG. 10);

[0128] **SEQ ID NO:26** is the nucleic acid sequence of the TCS gene (see FIG. 40B); and

[0129] **SEQ ID NO:27** is the nucleic acid sequence of the FLAG-tagged TCS gene (see FIG. 40C).

DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

[0130] Illustrative embodiments of the invention are described below. In the interest of clarity, not all features of an actual implementation are described in this specification. It will of course be appreciated that in the development of any such actual embodiment, numerous implementation-specific decisions must be made to achieve the developers' specific goals, such as compliance with system-related and business-related constraints, which will vary from one implementation to another. Moreover, it will be appreciated that such a development effort might be complex and time-consuming, but would be a routine undertaking for those of ordinary skill in the art having the benefit of this disclosure.

[0131] Recombinant adeno-associated virus (AAV) vectors have been used successfully for *in vivo* gene transfer in numerous pre-clinical animal models of human disease, and have been used successfully for long-term expression of a wide variety of therapeutic genes (Daya and Berns, 2008; Niemeyer *et al.,* 2009; Owen *et al.,* 2002; Keen-Rhinehart *et al.,* 2005; Scallan *et al.,* 2003; Song *et al.,* 2004). AAV vectors have also generated long-term clinical benefit in humans when targeted to immune-privileged sites, *i.e.,* ocular delivery for Leber's congenital amaurosis (Bainbridge *et al.,* 2008; Maguire *et al.,* 2008; Cideciyan *et al.,* 2008). A major advantage of this vector is its comparatively low immune profile, eliciting only limited inflammatory responses and, in some cases, even directing immune tolerance to transgene products (LoDuca *et al.,* 2009). Nonetheless, the therapeutic efficiency, when targeted to non-immune privileged organs, has been limited in humans due to antibody and $CD8^+$ T cell responses against the viral capsid, while in animal models, adaptive responses to the transgene product have also been reported (Manno *et al.,* 2006; Mingozzi *et al.,* 2007; Muruve *et al.,* 2008; Vandenberghe and Wilson, 2007; Mingozzi and High, 2007). These results suggested that immune responses remain a concern for AAV vector-mediated gene transfer.

[0132] Based on pre-clinical data from murine models (Snyder *et al.,* 1999), AAV was considered as minimally immunogenic for years, due to absence of prior exposure of these antigens in these models and the presence of variety of tolerance-inducing mechanisms against the vector (Dobrzynski

et al., 2004; Cao *et al.*, 2007). This was best illustrated in gene transfer studies in murine and canine models of hemophilia B, which showed remarkable therapeutic efficiency (5-25% of F.IX levels) and long-term (2-8 years) and stable F.IX expression (Snyder *et al.*, 1999). In the first clinical trial using AAV to deliver the human F.IX gene to the liver in subjects with hemophilia B, therapeutic levels (~11.8%) of F.IX expression were observed at a high dose of vector (2×10^{12} vgs/kg body weight) (Manno *et al.*, 2006).

[0133] However, 4-6 weeks after gene transfer, an AAV capsid-specific T cell response was observed that coincided with a rise in liver transaminases and a drop in F.IX transgene expression to baseline levels. This $CD8^+$ T cell-mediated immune response was unexpected (Mingozzi *et al.*, 2007), as this had not been observed in any pre-clinical animal models. This study and several others have implicated the host inflammatory and innate immune responses for cytotoxic T-lymphocyte mediated elimination of transduced hepatocytes (Zhu *et al.*, 2009; Li *et al.*, 2009; Madsen *et al.*, 2009). Subsequently, a great deal of effort has been devoted to circumvent the host immune response to AAV vectors. These include the use of alternate naturally occurring AAV serotypes such as AAV1 (Brantly *et al.*, 2009; Cohn *et al.*, 2007) or AAV8 (Nathwani *et al.*, 2006), the use of shuffled capsids (Gray *et al.*, 2010), or surface-exposed tyrosine-mutant AAV2 (Markusic *et al.*, 2010) vectors. In addition, strategies to counter the risks associated with the immune response have included the use of transgene constructs which have targeted expression in the host tissue (Wang *et al.*, 2010), or the development of transient immune-suppression protocols (Jiang *et al.*, 2006).

[0134] Although such strategies have incrementally improved the safety of AAV gene transfer, their efficacy in humans remains to be seen. For example, immune suppression with cyclosporine and MMF was effective at lower AAV1 vector dose (3×10^{11} vg/kg) but failed to prevent IFN-α CD8+ T cell responses against capsid at high doses (1×10^{12} vg/kg) during muscle-directed gene transfer in patients with lipoprotein lipase deficiency (Ross *et al.*, 2006). These data underscore the importance of pursuing further studies on the biology of the virus-host cell interactions to identify the first "danger signal" in response to AAV infection. It was reasoned that understanding how the potential activity and the selectivity of proteins associated with inflammatory and innate immune response are regulated in host cells upon transduction with AAV might offer clues to address obstacles of the host immune response against the capsid and/or the transgene product. Although compared with other viral vectors, AAV vectors are inefficient in transducing professional APCs such as DCs, additional signals that activate NF-κB would lead to increased transgene expression in these cells, thereby increasing the risk of adaptive responses to the transgene product.

[0135] Recombinant vectors based on AAV serotype 2 are currently in use in a number of gene therapy clinical trials (Daya and Berns, 2008), and have recently shown remarkable efficacy in the treatment of Leber's congenital amaurosis (Bainbridge *et al.*, 2008; Cideciyan *et al.*, 2008; Maguire *et al.*, 2008). However, concerns have been raised with reference to the humoral response to AAV2 vectors based on the high prevalence of sero-positivity in the general population (~80 to 90%) (Boutin

et al., 2008; Mendell *et al.*, 2010; Manno *et al.*, 2006). The discovery of many novel AAV serotypes has prompted the development of AAV vectors to circumvent this potential problem (Muramatsu *et al.*, 1996; Chiorini *et al.*, 1997; Chiorini *et al.*, 199; Rutledge *et al.*, 1998; Gao *et al.*, 2002; Gao *et al.*, 2004).

[0136] For example, recombinant AAV8 vectors were recently reported to be therapeutic in a mouse model of liver cancer (Kato *et al.*, 2006). However, several groups have described various strategies to target human liver cancer cells in murine models using AAV2 vectors (Su *et al.*, 1996; Peng *et al.*, 2000; Su *et al.*, 2000; Ma *et al.*, 2005; Wang *et al.*, 2005; Tse *et al.*, 2008; Zhang *et al.*, 2008; Malecki *et al.*, 2009; Wang *et al.*, 2010). To identify the most efficient AAV serotype to target human liver cancer cells, three different human liver cancer cell lines were shown to be transduced extremely efficiently by AAV3 vectors (Glushakova *et al.*, 2009). Human hepatocyte growth factor receptor (hHGFR) was subsequently identified as a cellular co-receptor for AAV3 infection (Ling *et al.*, 2010). The precise role of hHGFR, especially the role of tyrosine kinase activity associated with the intracellular domain of hHGFR, in AAV3-mediated transduction initially remained unclear. Data in Example 5 (see below) provided a more-detailed explanation of AAV3-hHGFR interactions, and illustrated the development of optimized capsid-mutated AAV3 vectors for use in targeting human liver cancer cells.

rAAV Capsid Proteins

[0137] Supramolecular assembly of 60 individual capsid protein subunits into a non-enveloped, T-1 icosahedral lattice capable of protecting a 4.7-kb single-stranded DNA genome is a critical step in the life-cycle of the helper-dependent human parvovirus, adeno-associated virus2 (AAV2). The mature 20-nm diameter AAV2 particle is composed of three structural proteins designated VP1, VP2, and VP3 (molecular masses of 87, 73, and 62 kDa respectively) in a ratio of 1:1:18. Based on its symmetry and these molecular weight estimates, of the 60 capsid proteins comprising the particle, three are VP1 proteins, three are VP2 proteins, and fifty-four are VP3 proteins. The employment of three structural proteins makes AAV serotypes unique among parvoviruses, as all others known package their genomes within icosahedral particles composed of only two capsid proteins. The anti-parallel β-strand barreloid arrangement of these 60 capsid proteins results in a particle with a defined tropism that is highly resistant to degradation. Modification of one or more tyrosine residues in one or more of the capsid proteins has been shown by the inventors to achieve superior transfection at lower dose and lower cost than conventional protocols. By site-specifically modifying one or more tyrosine residues on the surface of the capsid, the inventors have achieved significant improvement in transduction efficiency.

Uses for Improved, Capsid-Modified rAAV Vectors

[0138] The present invention provides compositions including one or more of the disclosed tyrosine-modified rAAV vectors comprised within a kit for diagnosing, preventing, treating or ameliorating one or more symptoms of a mammalian disease, injury, disorder, trauma or dysfunction.

Such kits may be useful in diagnosis, prophylaxis, and/or therapy, and particularly useful in the treatment, prevention, and/or amelioration of one or more symptoms of cancer, diabetes, autoimmune disease, kidney disease, cardiovascular disease, pancreatic disease, intestinal disease, liver disease, neurological disease, neuromuscular disorder, neuromotor deficit, neuroskeletal impairment, neurological disability, neurosensory dysfunction, stroke, ischemia, eating disorder, α_1-antitrypsin (AAT) deficiency, Batten's disease, Alzheimer's disease, sickle cell disease, β-thalassamia, Huntington's disease, Parkinson's disease, skeletal disease, trauma, pulmonary disease, or any combination thereof.

[0139] The invention also provides for the use of a composition disclosed herein in the manufacture of a medicament for treating, preventing or ameliorating the symptoms of a disease, disorder, dysfunction, injury or trauma, including, but not limited to, the treatment, prevention, and/or prophylaxis of a disease, disorder or dysfunction, and/or the amelioration of one or more symptoms of such a disease, disorder or dysfunction. Exemplary conditions for which rAAV viral based gene therapy may find particular utility include, but are not limited to, cancer, diabetes, sickle cell disease, β-thalassamia, autoimmune disease, kidney disease, cardiovascular disease, pancreatic disease, diseases of the eye, intestinal disease, liver disease, neurological disease, neuromuscular disorder, neuromotor deficit, neuroskeletal impairment, neurological disability, neurosensory dysfunction, stroke, α_1-antitrypsin (AAT) deficiency, Batten's disease, ischemia, an eating disorder, Alzheimer's disease, Huntington's disease, Parkinson's disease, skeletal disease, pulmonary disease, and any combinations thereof.

[0140] The invention also provides a method for treating or ameliorating the symptoms of such a disease, injury, disorder, or dysfunction in a mammal. Such methods generally involve at least the step of administering to a mammal in need thereof, one or more of the tyrosine-modified rAAV vectors as disclosed herein, in an amount and for a time sufficient to treat or ameliorate the symptoms of such a disease, injury, disorder, or dysfunction in the mammal.

[0141] Such treatment regimens are particularly contemplated in human therapy, *via* administration of one or more compositions either intramuscularly, intravenously, subcutaneously, intrathecally, intraperitoneally, or by direct injection into an organ or a tissue of the mammal under care.

[0142] The invention also provides a method for providing to a mammal in need thereof, a therapeutically-effective amount of the rAAV compositions of the present invention, in an amount, and for a time effective to provide the patient with a therapeutically-effective amount of the desired therapeutic agent(s) encoded by one or more nucleic acid segments comprised within the rAAV vector. Preferably, the therapeutic agent is selected from the group consisting of a polypeptide, a peptide, an antibody, an antigen-binding fragment, a ribozyme, a peptide nucleic acid, an siRNA, an RNAi, an antisense oligonucleotide, an antisense polynucleotide, a diagnostic marker, a diagnostic molecule, a reporter molecule, and any combination thereof.

AAV VECTOR COMPOSITIONS

[0143] One important aspect of the present methodology is the fact that the improved rAAV vectors described herein permit the delivery of smaller titers of viral particles in order to achieve the same transduction efficiency as that obtained using higher levels of conventional, non-surface capsid modified rAAV vectors. To that end, the amount of AAV compositions and time of administration of such compositions will be within the purview of the skilled artisan having benefit of the present teachings. In fact, the inventors contemplate that the administration of therapeutically-effective amounts of the disclosed compositions may be achieved by a single administration, such as for example, a single injection of sufficient numbers of infectious particles to provide therapeutic benefit to the patient undergoing such treatment. Alternatively, in some circumstances, it may be desirable to provide multiple, or successive administrations of the AAV vector compositions, either over a relatively short, or over a relatively prolonged period, as may be determined by the medical practitioner overseeing the administration of such compositions. For example, the number of infectious particles administered to a mammal may be approximately 10^7, 10^8, 10^9, 10^{10}, 10^{11}, 10^{12}, 10^{13}, or even higher, infectious particles/mL, given either as a single dose (or divided into two or more administrations, *etc.*,) as may be required to achieve therapy of the particular disease or disorder being treated. In fact, in certain embodiments, it may be desirable to administer two or more different rAAV vector-based compositions, either alone, or in combination with one or more other diagnostic agents, drugs, bioactives, or such like, to achieve the desired effects of a particular regimen or therapy. In most rAAV-vectored, gene therapy-based regimens, the inventors contemplate that lower titers of infectious particles will be required when using the modified-capsid rAAV vectors described herein, as compared to the use of equivalent wild-type, or corresponding "un-modified" rAAV vectors.

[0144] As used herein, the terms "engineered" and "recombinant" cells are intended to refer to a cell into which an exogenous polynucleotide segment (such as DNA segment that leads to the transcription of a biologically active molecule) has been introduced. Therefore, engineered cells are distinguishable from naturally occurring cells, which do not contain a recombinantly introduced exogenous DNA segment. Engineered cells are, therefore, cells that comprise at least one or more heterologous polynucleotide segments introduced through the hand of man.

[0145] To express a therapeutic agent in accordance with the present invention one may prepare a tyrosine-modified rAAV expression vector that comprises a therapeutic agent-encoding nucleic acid segment under the control of one or more promoters. To bring a sequence "under the control of" a promoter, one positions the 5' end of the transcription initiation site of the transcriptional reading frame generally between about 1 and about 50 nucleotides "downstream" of (*i.e.*, 3' of) the chosen promoter. The "upstream" promoter stimulates transcription of the DNA and promotes expression of the encoded polypeptide. This is the meaning of "recombinant expression" in this context.

Particularly preferred recombinant vector constructs are those that comprise an rAAV vector. Such vectors are described in detail herein.

[0146] When the use of such vectors is contemplated for introduction of one or more exogenous proteins, polypeptides, peptides, ribozymes, and/or antisense oligonucleotides, to a particular cell transfected with the vector, one may employ the capsid-modified rAAV vectors disclosed herein to deliver one or more exogenous polynucleotides to a selected host cell.

PHARMACEUTICAL COMPOSITIONS

[0147] The genetic constructs of the present invention may be prepared in a variety of compositions, and may also be formulated in appropriate pharmaceutical vehicles for administration to human or animal subjects. The rAAV molecules of the present invention and compositions comprising them provide new and useful therapeutics for the treatment, control, and amelioration of symptoms of a variety of disorders, and in particular, articular diseases, disorders, and dysfunctions, including for example osteoarthritis, rheumatoid arthritis, and related disorders.

[0148] The invention also provides compositions comprising one or more of the disclosed capsid-modified rAAV vectors, expression systems, virions, viral particles, mammalian cells, or combinations thereof. In certain embodiments, the present invention provides pharmaceutical formulations of one or more capsid-modified rAAV vectors disclosed herein for administration to a cell or an animal, either alone or in combination with one or more other modalities of therapy, and in particular, for therapy of human cells, tissues, and diseases affecting man. Formulation of pharmaceutically-acceptable excipients and carrier solutions is well-known to those of skill in the art, as is the development of suitable dosing and treatment regimens for using the particular compositions described herein in a variety of treatment regimens, including *e.g.*, oral, parenteral, intravenous, intranasal, intra-articular, intramuscular administration and formulation.

EXEMPLARY DEFINITIONS

[0149] In accordance with the present invention, polynucleotides, nucleic acid segments, nucleic acid sequences, and the like, include, but are not limited to, DNAs (including and not limited to genomic or extragenomic DNAs), genes, peptide nucleic acids (PNAs) RNAs (including, but not limited to, rRNAs, mRNAs and tRNAs), nucleosides, and suitable nucleic acid segments either obtained from natural sources, chemically synthesized, modified, or otherwise prepared or synthesized in whole or in part by the hand of man.

[0150] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and compositions similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and compositions are described herein. For purposes of the present invention, the following terms are defined below:

[0151] In accordance with long standing patent law convention, the words "a" and "an" when used in this application, including the claims, denote "one or more."

[0152] The terms "about" and "approximately" as used herein, are interchangeable, and should generally be understood to refer to a range of numbers around a given number, as well as to all numbers in a recited range of numbers (e.g., "about 5 to 15" means "about 5 to about 15" unless otherwise stated). Moreover, all numerical ranges herein should be understood to include each whole integer within the range.

[0153] As used herein, the term "carrier" is intended to include any solvent(s), dispersion medium, coating(s), diluent(s), buffer(s), isotonic agent(s), solution(s), suspension(s), colloid(s), inert(s) or such like, or a combination thereof, that is pharmaceutically acceptable for administration to the relevant animal. The use of one or more delivery vehicles for chemical compounds in general, and chemotherapeutics in particular, is well known to those of ordinary skill in the pharmaceutical arts. Except insofar as any conventional media or agent is incompatible with the active ingredient, its use in the diagnostic, prophylactic, and therapeutic compositions is contemplated. One or more supplementary active ingredient(s) may also be incorporated into, or administered in association with, one or more of the disclosed chemotherapeutic compositions.

[0154] As used herein, the term "DNA segment" refers to a DNA molecule that has been isolated free of total genomic DNA of a particular species. Therefore, a DNA segment obtained from a biological sample using one of the compositions disclosed herein refers to one or more DNA segments that have been isolated away from, or purified free from, total genomic DNA of the particular species from which they are obtained. Included within the term "DNA segment," are DNA segments and smaller fragments of such segments, as well as recombinant vectors, including, for example, plasmids, cosmids, phage, viruses, and the like.

[0155] The term "effective amount," as used herein, refers to an amount that is capable of treating or ameliorating a disease or condition or otherwise capable of producing an intended therapeutic effect. The term "for example" or "e.g.," as used herein, is used merely by way of example, without limitation intended, and should not be construed as referring only those items explicitly enumerated in the specification.

[0156] As used herein, a "heterologous" is defined in relation to a predetermined referenced gene sequence. For example, with respect to a structural gene sequence, a heterologous promoter is defined as a promoter which does not naturally occur adjacent to the referenced structural gene, but which is positioned by laboratory manipulation. Likewise, a heterologous gene or nucleic acid segment is defined as a gene or segment that does not naturally occur adjacent to the referenced promoter and/or enhancer elements.

[0157] As used herein, the term "homology" refers to a degree of complementarity between two or more polynucleotide or polypeptide sequences. The word "identity" may substitute for the word "homology" when a first nucleic acid or amino acid sequence has the exact same primary sequence as a second nucleic acid or amino acid sequence. Sequence homology and sequence identity can be determined by analyzing two or more sequences using algorithms and computer programs known in

the art. Such methods may be used to assess whether a given sequence is identical or homologous to another selected sequence.

[0158] As used herein, "homologous" means, when referring to polynucleotides, sequences that have the same essential nucleotide sequence, despite arising from different origins. Typically, homologous nucleic acid sequences are derived from closely related genes or organisms possessing one or more substantially similar genomic sequences. By contrast, an "analogous" polynucleotide is one that shares the same function with a polynucleotide from a different species or organism, but may have a significantly different primary nucleotide sequence that encodes one or more proteins or polypeptides that accomplish similar functions or possess similar biological activity. Analogous polynucleotides may often be derived from two or more organisms that are not closely related (*e.g.*, either genetically or phylogenetically).

[0159] The terms "identical" or percent "identity," in the context of two or more nucleic acid or polypeptide sequences, refer to two or more sequences or subsequences that are the same or have a specified percentage of amino acid residues or nucleotides that are the same, when compared and aligned for maximum correspondence, as measured using one of the sequence comparison algorithms described below (or other algorithms available to persons of ordinary skill) or by visual inspection.

[0160] As used herein, the phrase "in need of treatment" refers to a judgment made by a caregiver such as a physician or veterinarian that a patient requires (or will benefit in one or more ways) from treatment. Such judgment may made based on a variety of factors that are in the realm of a caregiver's expertise, and may include the knowledge that the patient is ill as the result of a disease state that is treatable by one or more compound or pharmaceutical compositions such as those set forth herein.

[0161] As used herein, the term "nucleic acid" includes one or more types of: polydeoxyribonucleotides (containing 2-deoxy-D-ribose), polyribonucleotides (containing D-ribose), and any other type of polynucleotide that is an N-glycoside of a purine or pyrimidine base, or modified purine or pyrimidine bases (including abasic sites). The term "nucleic acid," as used herein, also includes polymers of ribonucleosides or deoxyribonucleosides that are covalently bonded, typically by phosphodiester linkages between subunits, but in some cases by phosphorothioates, methylphosphonates, and the like. "Nucleic acids" include single- and double-stranded DNA, as well as single- and double-stranded RNA. Exemplary nucleic acids include, without limitation, gDNA; hnRNA; mRNA; rRNA, tRNA, micro RNA (miRNA), small interfering RNA (siRNA), small nucleolar RNA (snORNA), small nuclear RNA (snRNA), and small temporal RNA (stRNA), and the like, and any combination thereof.

[0162] The term "naturally occurring" as used herein as applied to an object refers to the fact that an object can be found in nature. For example, a polypeptide or polynucleotide sequence that is present in an organism (including viruses) that can be isolated from a source in nature and which has not been intentionally modified by the hand of man in a laboratory is naturally-occurring. As used

herein, laboratory strains of rodents that may have been selectively bred according to classical genetics are considered naturally occurring animals.

[0163] The term "operably linked," as used herein, refers to that the nucleic acid sequences being linked are typically contiguous, or substantially contiguous, and, where necessary to join two protein coding regions, contiguous and in reading frame. However, since enhancers generally function when separated from the promoter by several kilobases and intronic sequences may be of variable lengths, some polynucleotide elements may be operably linked but not contiguous.

[0164] As used herein, the term "patient" (also interchangeably referred to as "host" or "subject") refers to any host that can receive one or more of the pharmaceutical compositions disclosed herein. Preferably, the subject is a vertebrate animal, which is intended to denote any animal species (and preferably, a mammalian species such as a human being). In certain embodiments, a "patient" refers to any animal host including without limitation any mammalian host. Preferably, the term refers to any mammalian host, the latter including but not limited to, human and non-human primates, bovines, canines, caprines, cavines, corvines, epines, equines, felines, hircines, lapines, leporines, lupines, murines, ovines, porcines, ranines, racines, vulpines, and the like, including livestock, zoological specimens, exotics, as well as companion animals, pets, and any animal under the care of a veterinary practitioner. A patient can be of any age at which the patient is able to respond to inoculation with the present vaccine by generating an immune response. In particular embodiments, the mammalian patient is preferably human.

[0165] The phrase "pharmaceutically-acceptable" refers to molecular entities and compositions that preferably do not produce an allergic or similar untoward reaction when administered to a mammal, and in particular, when administered to a human. As used herein, "pharmaceutically acceptable salt" refers to a salt that preferably retains the desired biological activity of the parent compound and does not impart any undesired toxicological effects. Examples of such salts include, without limitation, acid addition salts formed with inorganic acids (e.g., hydrochloric acid, hydrobromic acid, sulfuric acid, phosphoric acid, nitric acid, and the like); and salts formed with organic acids including, without limitation, acetic acid, oxalic acid, tartaric acid, succinic acid, maleic acid, fumaric acid, gluconic acid, citric acid, malic acid, ascorbic acid, benzoic acid, tannic acid, pamoic (embonic) acid, alginic acid, naphthoic acid, polyglutamic acid, naphthalenesulfonic acids, naphthalenedisulfonic acids, polygalacturonic acid; salts with polyvalent metal cations such as zinc, calcium, bismuth, barium, magnesium, aluminum, copper, cobalt, nickel, cadmium, and the like; salts formed with an organic cation formed from N,N'-dibenzylethylenediamine or ethylenediamine; and combinations thereof.

[0166] The term "pharmaceutically acceptable salt" as used herein refers to a compound of the present disclosure derived from pharmaceutically acceptable bases, inorganic or organic acids. Examples of suitable acids include, but are not limited to, hydrochloric, hydrobromic, sulfuric, nitric, perchloric, fumaric, maleic, phosphoric, glycollic, lactic, salicyclic, succinic, toluene-p-sulfonic,

tartaric, acetic, citric, methanesulfonic, formic, benzoic, malonic, naphthalene-2-sulfonic, trifluoroacetic and benzenesulfonic acids. Salts derived from appropriate bases include, but are not limited to, alkali such as sodium and ammonia.

[0167] As used herein, the terms "prevent," "preventing," "prevention," "suppress," "suppressing," and "suppression" as used herein refer to administering a compound either alone or as contained in a pharmaceutical composition prior to the onset of clinical symptoms of a disease state so as to prevent any symptom, aspect or characteristic of the disease state. Such preventing and suppressing need not be absolute to be deemed medically useful.

[0168] The term "promoter," as used herein refers to a region or regions of a nucleic acid sequence that regulates transcription.

[0169] As used herein, the term "polypeptide" is intended to encompass a singular "polypeptide" as well as plural "polypeptides," and includes any chain or chains of two or more amino acids. Thus, as used herein, terms including, but not limited to "peptide," "dipeptide," "tripeptide," "protein," "enzyme," "amino acid chain," and "contiguous amino acid sequence" are all encompassed within the definition of a "polypeptide," and the term "polypeptide" can be used instead of, or interchangeably with, any of these terms. The term further includes polypeptides that have undergone one or more post-translational modification(s), including for example, but not limited to, glycosylation, acetylation, phosphorylation, amidation, derivatization, proteolytic cleavage, post-translation processing, or modification by inclusion of one or more non-naturally occurring amino acids. Conventional nomenclature exists in the art for polynucleotide and polypeptide structures. For example, one-letter and three-letter abbreviations are widely employed to describe amino acids: Alanine (A; Ala), Arginine (R; Arg), Asparagine (N; Asn), Aspartic Acid (D; Asp), Cysteine (C; Cys), Glutamine (Q; Gln), Glutamic Acid (E; Glu), Glycine (G; Gly), Histidine (H; His), Isoleucine (I; Ile), Leucine (L; Leu), Methionine (M; Met), Phenylalanine (F; Phe), Proline (P; Pro), Serine (S; Ser), Threonine (T; Thr), Tryptophan (W; Trp), Tyrosine (Y; Tyr), Valine (V; Val), and Lysine (K; Lys). Amino acid residues described herein are preferred to be in the "**l**" isomeric form. However, residues in the "**d**" isomeric form may be substituted for any **l**-amino acid residue provided the desired properties of the polypeptide are retained.

[0170] "Protein" is used herein interchangeably with "peptide" and "polypeptide," and includes both peptides and polypeptides produced synthetically, recombinantly, or in vitro and peptides and polypeptides expressed in vivo after nucleic acid sequences are administered into a host animal or human subject. The term "polypeptide" is preferably intended to refer to any amino acid chain length, including those of short peptides from about 2 to about 20 amino acid residues in length, oligopeptides from about 10 to about 100 amino acid residues in length, and longer polypeptides including from about 100 amino acid residues or more in length. Furthermore, the term is also intended to include enzymes, *i.e.*, functional biomolecules including at least one amino acid polymer. Polypeptides and proteins of the present invention also include polypeptides and proteins that are or

have been post-translationally modified, and include any sugar or other derivative(s) or conjugate(s) added to the backbone amino acid chain.

[0171] The term "recombinant" indicates that the material (*e.g.*, a polynucleotide or a polypeptide) has been artificially or synthetically (non-naturally) altered by human intervention. The alteration can be performed on the material within or removed from, its natural environment or state. Specifically, *e.g.*, a promoter sequence is "recombinant" when it is produced by the expression of a nucleic acid segment engineered by the hand of man. For example, a "recombinant nucleic acid" is one that is made by recombining nucleic acids, *e.g.*, during cloning, DNA shuffling or other procedures, or by chemical or other mutagenesis; a "recombinant polypeptide" or "recombinant protein" is a polypeptide or protein which is produced by expression of a recombinant nucleic acid; and a "recombinant virus," *e.g.*, a recombinant AAV virus, is produced by the expression of a recombinant nucleic acid.

[0172] The term "regulatory element," as used herein, refers to a region or regions of a nucleic acid sequence that regulates transcription. Exemplary regulatory elements include, but are not limited to, enhancers, post-transcriptional elements, transcriptional control sequences, and such like.

[0173] The term "RNA segment" refers to an RNA molecule that has been isolated free of total cellular RNA of a particular species. Therefore, RNA segments can refer to one or more RNA segments (either of native or synthetic origin) that have been isolated away from, or purified free from, other RNAs. Included within the term "RNA segment," are RNA segments and smaller fragments of such segments.

[0174] The term "substantially corresponds to," "substantially homologous," or "substantial identity," as used herein, denote a characteristic of a nucleic acid or an amino acid sequence, wherein a selected nucleic acid or amino acid sequence has at least about 70 or about 75 percent sequence identity as compared to a selected reference nucleic acid or amino acid sequence. More typically, the selected sequence and the reference sequence will have at least about 76, 77, 78, 79, 80, 81, 82, 83, 84 or even 85 percent sequence identity, and more preferably, at least about 86, 87, 88, 89, 90, 91, 92, 93, 94, or 95 percent sequence identity. More preferably still, highly homologous sequences often share greater than at least about 96, 97, 98, or 99 percent sequence identity between the selected sequence and the reference sequence to which it was compared.

[0175] The percentage of sequence identity may be calculated over the entire length of the sequences to be compared, or may be calculated by excluding small deletions or additions which total less than about 25 percent or so of the chosen reference sequence. The reference sequence may be a subset of a larger sequence, such as a portion of a gene or flanking sequence, or a repetitive portion of a chromosome. However, in the case of sequence homology of two or more polynucleotide sequences, the reference sequence will typically comprise at least about 18-25 nucleotides, more typically at least about 26 to 35 nucleotides, and even more typically at least about 40, 50, 60, 70, 80, 90, or even 100 or so nucleotides.

[0176] When highly-homologous fragments are desired, the extent of percent identity between the two sequences will be at least about 80%, preferably at least about 85%, and more preferably about 90% or 95% or higher, as readily determined by one or more of the sequence comparison algorithms well-known to those of skill in the art, such as *e.g.,* the FASTA program analysis described by Pearson and Lipman (1988).The term "subject," as used herein, describes an organism, including mammals such as primates, to which treatment with the compositions according to the present invention can be provided. Mammalian species that can benefit from the disclosed methods of treatment include, but are not limited to, apes; chimpanzees; orangutans; humans; monkeys; domesticated animals such as dogs and cats; livestock such as horses, cattle, pigs, sheep, goats, and chickens; and other animals such as mice, rats, guinea pigs, and hamsters.

[0177] As used herein, the term "structural gene" is intended to generally describe a polynucleotide, such as a gene, that is expressed to produce an encoded peptide, polypeptide, protein, ribozyme, catalytic RNA molecule, or antisense molecule.

[0178] The term "subject," as used herein, describes an organism, including mammals such as primates, to which treatment with the compositions according to the present invention can be provided. Mammalian species that can benefit from the disclosed methods of treatment include, but are not limited to, humans, non-human primates such as apes; chimpanzees; monkeys, and orangutans, domesticated animals, including dogs and cats, as well as livestock such as horses, cattle, pigs, sheep, and goats, or other mammalian species including, without limitation, mice, rats, guinea pigs, rabbits, hamsters, and the like.

[0179] "Transcriptional regulatory element" refers to a polynucleotide sequence that activates transcription alone or in combination with one or more other nucleic acid sequences. A transcriptional regulatory element can, for example, comprise one or more promoters, one or more response elements, one or more negative regulatory elements, and/or one or more enhancers.

[0180] As used herein, a "transcription factor recognition site" and a "transcription factor binding site" refer to a polynucleotide sequence(s) or sequence motif(s) which are identified as being sites for the sequence-specific interaction of one or more transcription factors, frequently taking the form of direct protein-DNA binding. Typically, transcription factor binding sites can be identified by DNA footprinting, gel mobility shift assays, and the like, and/or can be predicted on the basis of known consensus sequence motifs, or by other methods known to those of skill in the art.

[0181] "Transcriptional unit" refers to a polynucleotide sequence that comprises at least a first structural gene operably linked to at least a first cis-acting promoter sequence and optionally linked operably to one or more other cis-acting nucleic acid sequences necessary for efficient transcription of the structural gene sequences, and at least a first distal regulatory element as may be required for the appropriate tissue-specific and developmental transcription of the structural gene sequence operably positioned under the control of the promoter and/or enhancer elements, as well as any additional cis

sequences that are necessary for efficient transcription and translation (e.g., polyadenylation site(s), mRNA stability controlling sequence(s), etc.

[0182] As used herein, the term "transformed cell" is intended to mean a host cell whose nucleic acid complement has been altered by the introduction of one or more exogenous polynucleotides into that cell.

[0183] As used herein, the term "transformation" is intended to generally describe a process of introducing an exogenous polynucleotide sequence (e.g., a viral vector, a plasmid, or a recombinant DNA or RNA molecule) into a host cell or protoplast in which the exogenous polynucleotide is incorporated into at least a first chromosome or is capable of autonomous replication within the transformed host cell. Transfection, electroporation, and "naked" nucleic acid uptake all represent examples of techniques used to transform a host cell with one or more polynucleotides.

[0184] As used herein, the terms "treat," "treating," and "treatment" refer to the administration of one or more compounds (either alone or as contained in one or more pharmaceutical compositions) after the onset of clinical symptoms of a disease state so as to reduce, or eliminate any symptom, aspect or characteristic of the disease state. Such treating need not be absolute to be deemed medically useful. As such, the terms "treatment," "treat," "treated," or "treating" may refer to therapy, or to the amelioration or the reduction, in the extent or severity of disease, of one or more symptom thereof, whether before or after its development afflicts a patient.

[0185] The phrases "isolated" or "biologically pure" refer to material that is substantially, or essentially, free from components that normally accompany the material as it is found in its native state. Thus, isolated polynucleotides in accordance with the invention preferably do not contain materials normally associated with those polynucleotides in their natural, or *in situ,* environment.

[0186] "Link" or "join" refers to any method known in the art for functionally connecting one or more proteins, peptides, nucleic acids, or polynucleotides, including, without limitation, recombinant fusion, covalent bonding, disulfide bonding, ionic bonding, hydrogen bonding, electrostatic bonding, and the like.

[0187] As used herein, the term "plasmid" or "vector" refers to a genetic construct that is composed of genetic material (*i.e.,* nucleic acids). Typically, a plasmid or a vector contains an origin of replication that is functional in bacterial host cells, *e.g., Escherichia coli*, and selectable markers for detecting bacterial host cells including the plasmid. Plasmids and vectors of the present invention may include one or more genetic elements as described herein arranged such that an inserted coding sequence can be transcribed and translated in a suitable expression cells. In addition, the plasmid or vector may include one or more nucleic acid segments, genes, promoters, enhancers, activators, multiple cloning regions, or any combination thereof, including segments that are obtained from or derived from one or more natural and/or artificial sources.

[0188] The term "a sequence essentially as set forth in SEQ ID NO:X" means that the sequence substantially corresponds to a portion of SEQ ID NO:X and has relatively few nucleotides (or amino

acids in the case of polypeptide sequences) that are not identical to, or a biologically functional equivalent of, the nucleotides (or amino acids) of SEQ ID NO:X. The term "biologically functional equivalent" is well understood in the art, and is further defined in detail herein. Accordingly, sequences that have about 85% to about 90%; or more preferably, about 91% to about 95%; or even more preferably, about 96% to about 99%; of nucleotides that are identical or functionally equivalent to one or more of the nucleotide sequences provided herein are particularly contemplated to be useful in the practice of the invention.

[0189] Suitable standard hybridization conditions for the present invention include, for example, hybridization in 50% formamide, 5× Denhardt's solution, 5× SSC, 25 mM sodium phosphate, 0.1% SDS and 100 μg/ml of denatured salmon sperm DNA at 42°C for 16 h followed by 1 hr sequential washes with 0.1× SSC, 0.1% SDS solution at 60°C to remove the desired amount of background signal. Lower stringency hybridization conditions for the present invention include, for example, hybridization in 35% formamide, 5× Denhardt's solution, 5× SSC, 25 mM sodium phosphate, 0.1% SDS and 100 μg/ml denatured salmon sperm DNA or *E. coli* DNA at 42°C for 16 h followed by sequential washes with 0.8× SSC, 0.1% SDS at 55°C. Those of skill in the art will recognize that conditions can be readily adjusted to obtain the desired level of stringency.

[0190] Naturally, the present invention also encompasses nucleic acid segments that are complementary, essentially complementary, and/or substantially complementary to at least one or more of the specific nucleotide sequences specifically set forth herein. Nucleic acid sequences that are "complementary" are those that are capable of base-pairing according to the standard Watson-Crick complementarity rules. As used herein, the term "complementary sequences" means nucleic acid sequences that are substantially complementary, as may be assessed by the same nucleotide comparison set forth above, or as defined as being capable of hybridizing to one or more of the specific nucleic acid segments disclosed herein under relatively stringent conditions such as those described immediately above.

[0191] As described above, the probes and primers of the present invention may be of any length. By assigning numeric values to a sequence, for example, the first residue is 1, the second residue is 2, *etc.*, an algorithm defining all probes or primers contained within a given sequence can be proposed:

[0192] n to n + y, where n is an integer from 1 to the last number of the sequence and y is the length of the probe or primer minus one, where n + y does not exceed the last number of the sequence. Thus, for a 25-basepair probe or primer (*i.e.*, a "25-mer"), the collection of probes or primers correspond to bases 1 to 25, bases 2 to 26, bases 3 to 27, bases 4 to 28, and so on over the entire length of the sequence. Similarly, for a 35-basepair probe or primer (*i.e.*, a "35-mer), exemplary primer or probe sequence include, without limitation, sequences corresponding to bases 1 to 35, bases 2 to 36, bases 3 to 37, bases 4 to 38, and so on over the entire length of the sequence. Likewise, for 40-mers, such probes or primers may correspond to the nucleotides from the first basepair to bp 40, from the second bp of the sequence to bp 41, from the third bp to bp 42, and so forth, while for 50-

mers, such probes or primers may correspond to a nucleotide sequence extending from bp 1 to bp 50, from bp 2 to bp 51, from bp 3 to bp 52, from bp 4 to bp 53, and so forth.

[0193] In certain embodiments, it will be advantageous to employ one or more nucleic acid segments of the present invention in combination with an appropriate detectable marker (*i.e.*, a "label,"), such as in the case of employing labeled polynucleotide probes in determining the presence of a given target sequence in a hybridization assay. A wide variety of appropriate indicator compounds and compositions are known in the art for labeling oligonucleotide probes, including, without limitation, fluorescent, radioactive, enzymatic or other ligands, such as avidin/biotin, *etc.*, which are capable of being detected in a suitable assay. In particular embodiments, one may also employ one or more fluorescent labels or an enzyme tag such as urease, alkaline phosphatase or peroxidase, instead of radioactive or other environmentally less-desirable reagents. In the case of enzyme tags, colorimetric, chromogenic, or fluorigenic indicator substrates are known that can be employed to provide a method for detecting the sample that is visible to the human eye, or by analytical methods such as scintigraphy, fluorimetry, spectrophotometry, and the like, to identify specific hybridization with samples containing one or more complementary or substantially complementary nucleic acid sequences. In the case of so-called "multiplexing" assays, where two or more labeled probes are detected either simultaneously or sequentially, it may be desirable to label a first oligonucleotide probe with a first label having a first detection property or parameter (for example, an emission and/or excitation spectral maximum), which also labeled a second oligonucleotide probe with a second label having a second detection property or parameter that is different (*i.e.*, discreet or discernable from the first label. The use of multiplexing assays, particularly in the context of genetic amplification/detection protocols are well-known to those of ordinary skill in the molecular genetic arts.

[0194] The tern "vector," as used herein, refers to a nucleic acid molecule (typically comprised of DNA) capable of replication in a host cell and/or to which another nucleic acid segment can be operatively linked so as to bring about replication of the attached segment. A plasmid, cosmid, or a virus is an exemplary vector.

EXAMPLES

[0195] The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples that follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

EXAMPLE 1 -- NEXT GENERATION rAAV2 VECTORS: POINT MUTATIONS IN TYROSINES LEAD TO HIGH-EFFICIENCY TRANSDUCTION AT LOWER DOSES

[0196] The present example demonstrates that mutations of surface-exposed tyrosine residues on AAV2 capsids circumvents the ubiquitination step, thereby avoiding proteasome-mediated degradation, and resulting in high-efficiency transduction by these vectors in human cells *in vitro* and murine hepatocytes *in vivo*, leading to the production of therapeutic levels of human coagulation factor at reduced vector doses. The increased transduction efficiency observed for tyrosine-mutant vectors is due to lack of ubiquitination, and improved intracellular trafficking to the nucleus. In addition to yielding insights into the role of tyrosine phosphorylation of AAV2 capsid in various steps in the life cycle of AAV2, these studies have resulted in the development of novel AAV2 vectors that are capable of high-efficiency transduction at lower doses.

MATERIALS AND METHODS

[0197] ***Recombinant AAV2 Vectors.*** Highly purified stocks of scAAV2 vectors containing the enhanced green fluorescence protein (EGFP) gene driven by the chicken β-actin (CBA) promoter (scAAV2-EGFP), and ssAAV2 vectors containing the factor IX (F.IX) gene under the control of the apolipoprotein enhancer/human α-1 antitrypsin (ApoE/hAAT) promoter (ssAAV2-F.IX) were generated using published methods.

[0198] ***Localization of Surface-Tyrosines on the AAV2 Capsid.*** The crystal structure of AAV2 (PDB accession number 1lp3) was used to localize the tyrosine residues on the AAV2 capsid surface. The icosahedral two-, three- and five-fold related VP3 monomers were generated by applying icosahedral symmetry operators to a reference monomer using Program O on a Silicon graphics Octane workstation. The position of the tyrosine residues were then visualized and analyzed in the context of a viral asymmetric unit using the program COOT, and graphically presented using the program PyMOL Molecular Graphics System (DeLano Scientific, San Carlos, CA, USA).

[0199] ***Construction of Surface-Exposed Tyrosine Residue Mutant AAV2 Capsid Plasmids.*** A two-stage procedure, based on QuikChange II® site-directed mutagenesis (Stratagene, La Jolla, CA, USA) was performed using plasmid pACG-2. Briefly, in stage one, two PCR extension reactions were performed in separate tubes for each mutant. One tube contained the forward PCR primer and the other contained the reverse primer. In stage two, the two reactions were mixed and a standard PCR mutagenesis assay was carried out as per the manufacturer's instructions. PCR primers were designed to introduce changes from tyrosine to phenylalanine residues as well as a silent change to create a new restriction endonuclease site for screening purposes. All mutants were screened with the appropriate restriction enzyme and were sequenced prior to use.

[0200] ***Preparation of Whole Cell Lysates (WCL) and Co-Immunoprecipitations.*** Approximately 2×10^6 HeLa cells, mock-treated or treated with MG132, were also subjected to mock-infection or infection with the WT scAAV2-EGFP or Y730F mutant vectors at 5×10^3 particles/cell for 2 hr at 37°C. For immunoprecipitations, cells were treated with 0.01% trypsin and

washed extensively with PBS. WCL were cleared of non-specific binding by incubation with 0.25 mg of normal mouse IgG together with 20 µl of protein G-agarose beads. After preclearing, 2 µg of capsid antibody against intact AAV2 particles (mouse monoclonal IgG_3, clone A20; Research Diagnostics, Inc. (Flanders, NJ, USA), or 2 µg of normal mouse IgG (as a negative control) were added and incubated at 4°C for 1 hr, followed by precipitation with protein G-agarose beads. For immunoprecipitations, resuspended pellet solutions were used for SDS-PAGE. Membranes were treated with monoclonal HRP–conjugated anti-Ub antibody (1:2,000 dilution) specific for ubiquitin (Ub) (mouse monoclonal immunoglobulin G_1 γIgG_1], clone P4D1; Santa Cruz, CA, USA). Immunoreactive bands were visualized using chemiluminescence (ECL–plus, Amersham Pharmacia Biotech, Piscataway, NJ, USA).

[0201] ***Isolation of Nuclear and Cytoplasmic Fractions from HeLa Cells.*** Nuclear and cytoplasmic fractions from HeLa cells were isolated and mock-infected or recombinant wt scAAV2-EGFP or Y700F vector-infected cells were used to isolate the cytoplasmic and nuclear fractions. The purity of each fraction was determined to be >95%.

[0202] ***Southern Blot Analysis for AAV2 Trafficking.*** Low-M_r DNA samples from nuclear and cytoplasmic fractions were isolated and electrophoresed on 1% agarose gels or 1% alkaline-agarose gels followed by Southern blot hybridization using a ^{32}P-labeled EGFP-specific DNA probe.

[0203] ***Recombinant AAV2 Vector Transduction Assays* In Vitro.** Approximately 1×10^5 HeLa cells were used for transductions with recombinant AAV2 vectors. The transduction efficiency was measured 48-hr post-transduction by EGFP imaging using fluorescence microscopy. Images from three to five visual fields were analyzed quantitatively by ImageJ analysis software (NIH, Bethesda, MD, USA). Transgene expression was assessed as total area of green fluorescence (pixel^2) per visual field (mean ± SD). Analysis of variance (ANOVA) was used to compare between test results and the control and they were determined to be statistically significant.

[0204] ***Recombinant AAV2 Vector Transduction Studies* In Vivo.** scAAV2-EGFP vectors were injected intravenously *via* the tail vein into C57BL/6 mice at 1×10^{10} virus particles per animal. Liver sections from three hepatic lobes of the mock-injected and injected mice 2 weeks after injection were mounted on slides. The transduction efficiency was measured by EGFP imaging as described. ssAAV2-FI.X vectors were injected intravenously (*via* the tail vein) or into the portal vein of C57BL/6, BALB/c, and C3H/HeJ mice at 1×10^{10} or 1×10^{11} virus particles per animal. Plasma samples were obtained by retro-orbital bleed and analyzed for hF.IX expression by ELISA.

RESULTS

[0205] ***Mutations in Surface-Exposed Tyrosine Residues Significantly Improve Transduction Efficiency of AAV2 Vectors.*** To demonstrate that tyrosine-phosphorylation of AAV2 capsids leads to increased ubiquitination and results in impaired intracellular trafficking, and is therefore unfavorable to viral transduction, surface-exposed tyrosine residues were modified on AAV2 capsids. Inspection of the capsid surface of the AAV2 structure revealed seven surface-exposed tyrosine residues (Y252,

Y272, Y444, Y500, Y700, Y704, and Y730). Site-directed mutagenesis was performed for each of the seven tyrosine residues, which were conservatively substituted with phenylalanine residues (tyrosine-phenylalanine, Y-F) (Table 1). scAAV2-EGFP genomes encapsidated in each of the tyrosine-mutant capsids were successfully packaged, and mutations of the surface-exposed tyrosine residues did not lead to reduced vector stability.

TABLE 1

TITERS OF WILDTYPE (WT) AND TYROSINE-MODIFIED Y→F MUTANT AAV2 VECTORS

AAV Vectors	1st packaging titers (vgs/mL)	2nd packaging titers (vgs/mL)	3rd packaging titers (vgs/mL)	4th packaging titers (vgs/mL)
WT scAAV2-EGFP	3.4×10^{11}	1.0×10^{12}	3.2×10^{11}	3.0×10^{11}
Y252F scAAV2-EGFP	3.8×10^{11}	4.0×10^{11}	ND	ND
Y272 scAAV2-EGFP	7.7×10^{11}	1.0×10^{11}	ND	ND
Y444F scAAV2-EGFP	9.7×10^{10}	4.0×10^{10}	6.0×10^{9}	5.0×10^{10}
Y500F scAAV2-EGFP	8.8×10^{10}	2.0×10^{9}	4.0×10^{10}	6.0×10^{10}
Y700F scAAV2-EGFP	1.0×10^{11}	4.0×10^{11}	ND	ND
Y704F scAAV2-EGFP	6.0×10^{11}	2.0×10^{11}	ND	ND
Y730F scAAV2-EGFP	1.2×10^{11}	5.0×10^{11}	1.2×10^{11}	4.0×10^{11}

ND = Not done.

[0206] The transduction efficiency of each of the tyrosine-mutant vectors was analyzed and compared with the WT scAAV2-EGFP vector in HeLa cells *in vitro* under identical conditions. From the results, it was evident that whereas mock-infected cells showed no green fluorescence, the transduction efficiency of each of the tyrosine-mutant vectors was significantly higher compared with the WT scAAV2-EGFP vector at 2,000 viral particles/cell. Specifically, the transduction efficiency of Y444F, Y500F, Y730F vectors was ~8- to 11-fold higher than the WT vector.

[0207] ***Mutations in Surface-Exposed Tyrosine Residues Dramatically Improve Transduction Efficiency of AAV2 Vectors in Murine Hepatocytes in Vivo.*** The efficacy of WT and tyrosine-mutant scAAV2-EGFP vectors was also evaluated in a mouse model in vivo. The transduction efficiency of tyrosine-mutant vectors was significantly higher, and ranged between 4-29-fold, compared with the WT vector. When other tissues, such as heart, lung, kidney, spleen, pancreas, GI tract (jejunum, colon), testis, skeletal muscle, and brain were harvested from mice injected with 1×10^{10} particles of the tyrosine-mutant vectors and analyzed, no evidence of EGFP gene expression was seen. Thus, mutations in the surface-exposed tyrosine residues did not appear to alter the liver-tropism following tail vein injection of these vectors in vivo.

[0208] ***Increased Transduction Efficiency of Tyrosine-Mutant Vectors is Due to Lack of Uubiquitination, and Improved Intracellular Trafficking to the Nucleus.*** To further confirm the hypothesis that EGFR-PTK-mediated phosphorylation of capsid proteins at tyrosine residues is a pre-requisite for ubiquitination of AAV2 capsids, and that ubiquitinated virions are

recognized and degraded by cytoplasmic proteasome on their way to the nucleus, leading to inefficient nuclear transport, a series of experiments were performed as follows.

[0209] In the first study, HeLa C12 cells, carrying adenovirus-inducible AAV2 *rep* and *cap* genes, were mock infected, or infected with WT, Y444F or Y730F scAAV2-EGFP vectors. Whereas mock-infected cells showed no green fluorescence, and ~15% of cells were transduced with the WT scAAV2-EGFP vectors in the absence of co-infection with adenovirus, the transduction efficiency of Y444F and Y730F scAAV2-EGFP vectors was increased by ~9 and ~18-fold, respectively, compared with the WT vector. Interestingly, whereas co-infection with adenovirus led to ~11-fold increase, the transduction efficiency of Y444F and Y730F scAAV2-EGFP vectors was not further enhanced by co-infection with adenovirus. Since adenovirus can improve AAV2 vector nuclear transport in HeLa cells, these data suggested that the surface-exposed tyrosine residues play a role in intracellular trafficking of AAV2, and that their removal leads to efficient nuclear transport of AAV2 vectors.

[0210] In a second study, HeLa cells, either mock-treated or treated with Tyr23, a specific inhibitor of EGFR-PTK, or MG132, a proteasome inhibitor, both known to increase the transduction efficiency of AAV vectors, were mock-infected or infected with the WT or Y730F scAAV2-EGFP vectors. Whereas mock-infected cells showed no green fluorescence, and ~5% of cells were transduced with the WT scAAV2-EGFP vectors in mock-treated cells, pretreatment with Tyr23 or MG132 led to an ~9-fold and ~6-fold increase in the transduction efficiency, respectively. Although the transduction efficiency of Y730F scAAV2-EGFP vectors was increased by ~14-fold compared with the WT vectors, it was not further enhanced by pretreatment with either Tyr23 or MG132. These data strongly suggest that the absence of surface-exposed tyrosine residues, which prevented phosphorylation of the mutant vectors, likely prevented ubiquitination of the capsid proteins, and these vectors could not be recognized on their way to the nucleus and degraded by the proteasome, which led to their efficient nuclear translocation.

[0211] In a third study, HeLa cells, either mock-treated or treated with MG132, were mock-infected or infected with the WT, Y730F, or Y444F scAAV2-EGFP vectors. WCL were prepared 4 hrs post-infection and equivalent amounts of proteins were immunoprecipitated first with anti-AAV2 capsid antibody (A20) followed by Western blot analyses with anti-Ub monoclonal antibody. Whereas ubiquitinated AAV2 capsid proteins (Ub-AAV2 Cap) were undetectable in mock-infected cells, the signal of ubiquitinated AAV2 capsid proteins was weaker in untreated cells, and a significant accumulation of ubiquitinated AAV2 capsid proteins occurred following treatment with MG132. Interestingly, infections with Y730F or Y444F vectors dramatically decreased the extent of accumulation of MG132-induced ubiquitinated AAV2 capsid proteins. These results substantiate that mutation in tyrosine residues circumvents proteasome-mediated degradation of the vectors.

[0212] In a fourth study, the fate of the input WT, Y444F, and Y730F vector viral DNA was determined in HeLa cells. Southern blot analysis of low-M_r DNA samples isolated from cytoplasmic [C] and nuclear [N] fractions and densitometric scanning of autoradiographs, revealed that ~36% of

the input scAAV2 DNA was present in the nuclear fraction in cells infected with the WT vector . Interestingly, however, the amount of input Y730F and Y444F scAAV2 vector DNA in the nuclear fraction was increased to ~72% and ~70%, respectively. These results further documented that mutations in the surface-exposed tyrosine residues prevent ubiquitination of AAV2 capsids, resulting in a decrease of proteasome-mediated degradation, and in turn, facilitate nuclear transport of AAV2 vectors.

[0213] ***Tyrosine-Mutant Vectors Express Therapeutic Levels of Human Factor IX Protein at ~10-Fold Reduced Vector Dose in Mice.*** It was important to examine whether tyrosine-mutant AAV2 vectors were capable of delivering a therapeutic gene efficiently at a reduced vector dose *in vivo*. To this end, a single-stranded, hepatocyte-specific human Factor IX (h.FIX) expression cassette was encapsidated in the Y730F vector, and the efficacy of this vector was tested in three different strains of mice (BALB/c, C3H/HeJ, and C57BL/6). Consistently in all three strains, Y730F vector achieved ~10-fold higher circulating hF.IX levels compared with the WT vector following tail vein or portal vein administration, with the latter being the more effective route. These results clearly indicated that the Y730F vectors expressed therapeutic levels of human F.IX protein (~50 ng/mL) at ~10-fold reduced vector dose (10^{10} particles/mouse) in C57BL/6 mice by port vein injection. It should be noted that hepatic viral gene transfer in C57BL/6 mice is generally more efficient than in the other two strains.

[0214] These results demonstrated here are consistent with the interpretation that EGFR-PTK-induced tyrosine phosphorylation of AAV2 capsid proteins promotes ubiquitination and degradation of AAV2, thus leading to impairment of viral nuclear transport and decrease in transduction efficiency. Mutational analyses of each of the seven surface-exposed tyrosine residues yield AAV2 vectors with significantly increased transduction efficiency *in vitro* as well as *in vivo*. Specifically, Y444F and Y730F mutant vectors bypass the ubiquitination step, which results in a significantly improved intracellular trafficking and delivery of the viral genome to the nucleus.

[0215] Despite long-term therapeutic expression achieved in preclinical animal models by AAV2 vectors composed of the WT capsid proteins, in a recent gene therapy trial, two patients with severe hemophilia B developed vector dose-dependent transaminitis that limited duration of hepatocyte-derived hF.IX expression to <8 weeks. Subsequent analyses demonstrated presence of memory $CD8^+$ T cells to AAV capsids in humans and an MHC I-restricted, capsid-specific cytotoxic T lymphocyte (CTL) response in one of the hemophilia B patients, which mirrored the time course of the transaminitis. It was concluded that this $CD8^+$ T cell response to input capsid eliminated AAV2-transduced hepatocytes. These data demonstrated that a lower capsid antigen dose is sufficient for efficient gene transfer with the Y730F vector, and show much-reduced ubiquitination of AAV-Y730F compared to WT capsid, a prerequisite for MHC I presentation. Thus, the T-cell response to AAV2 capsid (a serious hurdle for therapeutic gene transfer in the liver), may be avoided by using the surface-exposed tyrosine-mutant AAV2 vectors.

[0216] Dramatically increased transduction efficiency of tyrosine-mutant vectors have also been observed in primary human neuronal and hematopoietic stem cells *in vitro* and in various tissues and organs in mice *in vivo*. Double, triple, and quadruple tyrosine-mutants have also been constructed to examine whether such multiple mutants are viable, and whether the transduction efficiency of these vectors can be augmented further. It is noteworthy that with a few exceptions (Y444 positioned equivalent to a glycine in AAV4 and arginine in AAV5; Y700 positioned equivalent to phenylalanine in AAV4 and AAV5; and Y704 positioned equivalent to a phenylalanine in AAV7), these tyrosine residues are highly conserved in AAV serotypes 1 through 10.

EXAMPLE 2 - ACTIVATION OF THE NF-κB PATHWAY BY RAAV VECTORS

[0217] Since the *in silico* analysis with human transcription factor database demonstrated the presence of several binding sites for NF-κB, a central regulator of cellular immune and inflammatory responses, in the adeno-associated virus (AAV) genome, the present example investigates whether AAV utilizes NF-κB during its life cycle. Small molecule modulators of NF-κB were used in HeLa cells transduced with recombinant AAV vectors. VP16, an NF-κB activator, augmented AAV vector-mediated transgene expression up to 25-fold. Of the two NF-κB inhibitors (Bay11), which blocks both the canonical and the non-canonical NF-κB pathways, totally ablated the transgene expression, whereas pyrrolidone dithiocarbamate (PDTC), which interferes with the classical NF-κB pathway, had no effect. Western blot analyses confirmed the abundance of the nuclear p52 protein component of the non-canonical NF-κB pathway in the presence of VP16, which was ablated by Bay11, suggesting that the non-canonical NF-κB pathway is triggered during AAV infection. Similar results were obtained with primary human dendritic cells (DCs) *in vitro*, in which cytokines-induced expression of DC maturation markers, CD83 and CD86, was also inhibited by Bay11. Administration of Bay11 prior to gene transfer in normal C57BL/6 mice *in vivo* resulted in up to 7-fold decrease in AAV vector-induced production of pro-inflammatory cytokines and chemokines such as, IL-1β, IL-6, TNFα, IL-12β, KC, and RANTES. These studies suggested that transient immuno-suppression with NF-κB inhibitors prior to transduction with AAV vectors leads to a dampened immune response, which has significant implications in the optimal use of AAV vectors in human gene therapy.

[0218] Recent studies have begun to define the initial activation signals that result from AAV gene transfer. One study found AAV-induced signaling through the Toll-like receptor 9 (TLR9)-myeloid differentiation factor 88 (MyD88) pathway to induce a type I interferon response in plasmacytoid dendritic cells (pDCs), thereby driving subsequent adaptive immune responses to the vector and transgene product upon gene transfer to murine skeletal muscle (Zhu *et al.*, 2009). These data indicate sensing of the DNA genome by the endosomal TLR9 receptor in pDCs. No evidence for induction of pro-inflammatory cytokines following *in vitro* pulsing of DCs or macrophages with AAV was found. Still, earlier reports demonstrated a rapid, albeit highly transient, Kupffer cell-dependent innate response to AAV vectors in the liver, which included expression of several inflammatory cytokines (Zaiss and Muruve, 2008; Zaiss *et al.*, 2008; Zaiss and Muruve, 2005; Zaiss *et al.*, 2002).

[0219] Interestingly, the role of NF-κB, a key cellular responder to many stress- and pathogen-derived signals and regulator of pro-inflammatory cytokine expression (Hayden and Ghosh, 2004; Hiscott *et al.*, 2006; Li and Verma, 2002), has not been previously studied in the AAV life cycle. In this example, it is shown that infection of human cells with AAV can lead to activation of the non-canonical NF-κB pathway. In addition, activation of NF-κB substantially increases transgene expression (including in DCs), while inhibition of NF-κB blunts expression. Prevention of inflammatory cytokine induction by transient inhibition of NF-κB reveals a role for NF-κB in the innate response to AAV *in vivo*, and importantly, does not interfere with long-term transgene expression.

RESULTS

[0220] ***AAV-ITRs Contain Binding sites for NF-κB-Responsive Transcription Factors.*** The existence of a cellular protein which interacts specifically with the single-stranded D[-]-sequence in the left inverted terminal repeat (ITR) of the AAV2 genome has been previously described (Qing *et al.*, 1997). Since the ssD[+]-sequence in the right ITR is complementary to the ssD[-]-sequence in the left ITR, it was reasoned that a putative cellular protein might also exist, and interact with the ssD[+]-sequence in the right ITR. In electrophoretic mobility-shift assays, using the ssD[+]-sequence probe, a distinct cellular protein was indeed detected, which was designated as ssD[+]-sequence binding protein (ssD[+]-BP) (Qing *et al.*, 1997). Following purification and mass spectrometry, ssD[+]-BP was found to have partial amino acid homology to a cellular NF-κB repressing factor, a negative regulator of transcription. Additional *in silico* analysis with human transcription factor database [TRANSFAC] demonstrated the presence of several binding sites for NF-κB binding co-factors, such as p300, TFIIB, and SpII. One of these is the p300/CREB transcription factor that has been recently shown to be associated with the AAV genome (Dean *et al.*, 2009). Although it is not known whether the NF-κB signaling is activated by AAV binding to the cell surface receptors/co-receptors, recent studies have demonstrated that the innate immune response could be triggered either a) through the Toll like receptor 9 (TLR9)-myeloid differentiation factor 88 (MYD88) pathway, or b) through the activation of the CD40 ligand on the cell surface in mouse models *in vivo* (Zhu *et al.*, 2009; Mays *et al.*, 2009). Both of these ligands are known to interact down-stream with NF-κB transcription factors during their biological activation (Mineva *et al.*, 2007; Loiarro *et al.*, 2005). The following data demonstrated that the NF-κB is involved in the AAV life cycle.

[0221] ***AAV Infection Activates Non-Canonical NF-κB Pathway in Human Cells.*** Small molecule activators and inhibitors of NF-κB signaling were used in HeLa cells transduced with a self-complementary serotype 2 vector expressing EGFP (scAAV-EGFP). VP16, an NF-κB activator (Wu and Miyamoto, 2008), augmented EGFP expression by ~25-fold (FIG. 1A and FIG. 1B). Between the two inhibitors tested, Bay11, that blocks the activity of both IKKκ and IKKκ, totally ablated EGFP expression, whereas PDTC, which inhibits IKB degradation by blocking IKB ubiquitin ligase in the

classical pathway (Cuzzocrea *et al.,* 2002), had no noticeable effect on EGFP expression (FIG. 1A and FIG. 1B). Furthermore, VP16-mediated augmented transgene expression was completely ablated by Bay11, but not by PDTC (FIG. 6A). Similar results were obtained with both ssAAV vectors (FIG. 6B) and with the tyrosine triple-mutant scAAV vector (Y730+500+444F; TM-AAV), which were described in the previous examples (Markusic *et al.,* 2010) (FIG. 6C). It was concluded, therefore, that transgene expression from the AAV vector was regulated by the alternative (non-canonical) pathway of NF-κB. This conclusion was confirmed by Western blot analysis (FIG. 6D, and FIG. 6E), which revealed an increase in the cytosolic p100 and the nuclear p52 protein components of the non-canonical NF-κB pathway by ~3- to 6-fold in the presence of VP16. Moreover, transduction with AAV vector by itself (*i.e.,* in the absence of activator) increased p100 and p52 (FIG. 1C), indicating that infection of the cell activated the alternative NF-κB pathway. This increase was ablated by Bay11 treatment, while p65, the marker used for the classical NF-κB pathway, was unaffected (FIG. 1C).

[0222] NF-κB Pathway is Operational in Primary Human Antigen-Presenting Cells.

[0223] ***Following AAV Infection.*** In primary human dendritic cells (DCs), on the other hand, while transgene expression was again substantially increased with the NF-κB activator (FIG. 2A), AAV infection by itself did not activate NF-κB (FIG. 2B). In the presence of VP16, ~20-fold increase in EGFP expression was observed compared with scAAV vector-transduced DCs. Treatment with cytokines (TNF-α, IL-6, IL-1β, PGE2), known to activate the NF-κB pathway, led to a further increase in transgene expression to ~26%, which was reduced to ~12% following treatment with Bay11 (FIG. 2A). Western blot analyses of nuclear fractions further corroborated that the alternative pathway of NF-κB activation (accumulation of p52 proteins) was operational (FIG. 2B). Similar results were obtained following scAAV vector-mediated gene delivery to murine livers *in vivo* (FIG. 7). The inventors also tested the capability of NF-κB modulators to induce phenotypic changes in DCs. Flow cytometric analyses of two DC maturation markers, CD83 and CD86 indicated that VP16 alone was not able to induce maturation or enhance the expression of co-stimulatory molecules when used together with the cytokines cocktail. However, treatment with Bay11 led to inhibition of cytokine-mediated maturation of APCs, further implicating the involvement of NF-κB (Table 2). This reduction of maturation markers expression diminishes the main function of DCs to process antigenic material and reduces T-cell activation and proliferation. Thus, it was hypothesized that suppression of NF-κB activation prior to vector administration might lead to a dampened innate immune response against AAV.

TABLE 2

FACS ANALYSES OF MARKERS OF MATURATION OF PRIMARY HUMAN DENDRITIC CELLS

Group	Geometric means of levels of expression in cells expressing	
	CD83	CD86
Immature DCs	10.38	7.04
DCs - No maturation supplement	18.08	13.63
Mature DCs + Cytokines	20.60	26.80
DCs + AAV	18.29	12.65
DCs + VP16	16.48	13.70
Mature DCs + AAV + Cytokines	24.25	23.75
Mature DCs + AAV + Cytokines + VP16	19.92	21.92
Mature DCs + AAV + Cytokines + Bay11	16.88	10.11

Data from a representative experiment are shown (n = 3).

[0224] Inhibition of NF-κB Activation Leads to Suppression of Pro-Inflammatory Cytokine Production Prior to AAV Vector-Mediated Gene Transfer in Mice in Vivo. In in vivo studies, a single dose of Bay11 at 20 mg/kg body weight was administered intra- peritoneally (i.p.) 12 hrs prior to vector administration in C57BL/6 mice. Transcript levels from liver homogenates of innate immune mediators (FIG. 3A) or for activation of NF-κB (FIG. 3B) genes were measured from Bay11- and vector-injected groups and compared with sham-injected mice. These data revealed that 2 hrs post-vector administration, mice injected with Bay11+AAV vector had significantly reduced levels of pro-inflammatory cytokines or chemokines including IL-1α, IL-6, TNFα, IL-12α, KC, and RANTES, compared with sham- and AAV vector-injected animals (FIG. 3A), and additionally, the up-regulation of the NF-κB gene expression profile was prevented (FIG. 3B). A similar down-regulation trend of these innate immune response markers was seen in mice injected with the more efficacious tyrosine triple-mutant AAV vector (Y730+500+444F; TM-AAV). The up-regulation of type I interferon expression by both wild-type (WT-AAV) and TM-AAV vectors was unaffected by Bay11 (FIG. 8A, FIG. 8B, FIG. 8C, FIG. 8D, FIG. 8E, and FIG. 8F). Administration of Bay11 also significantly reduced the anti-AAV2 antibody response in these mice (FIG. 9). The sum of these results implies that the transient inflammatory cytokine response, typically seen during in vivo hepatic AAV gene transfer, is mediated by NF-κB activation.

[0225] AAV Vector-Mediated Transgene Expression in Murine Hepatocytes. In view of the observation that Bay11 strongly inhibits AAV-mediated transgene expression in HeLa cells in vitro 48 hrs post-transduction (FIG. 1A and FIG. 1B), which would be counter-productive to achieve long-term transgene expression in vivo, it was important to examine the effect of Bay11 in mice. As can be seen in FIG. 4A, animals injected with or without Bay11 had similar levels of EGFP expression from either vector when analyzed 2 weeks after gene transfer. Transduction efficiency of the TM-AAV

vector was ~12-fold higher than that of the WT-AAV vector (FIG. 4B), consistent with recently published studies (Markusic et al., 2010). These data suggested that Bay11 administration could safely and effectively down-regulate mediators of innate immune response without compromising long-term transgene expression.

MATERIALS AND METHODS

[0226] ***Recombinant AAV Vectors.*** Highly purified stocks of self-complementary (sc) AAV2 vectors were generated containing either the wild-type (WT) plasmid or the triple tyrosine-mutant (TM; Y730+500+444F) plasmid and the enhanced green fluorescence protein (EGFP) gene driven by the chicken β-actin (CBA) promoter (WT-scAAV2-EGFP, TM-scAAV2-EGFP) by triple transfection of HEK-293 cells. The vectors were then purified by CsCl gradient centrifugation, filter sterilized, and quantified by slot-blot hybridization as described (Liu *et al.,* 2003; Kube and Srivastava, 1997). The tyrosine-mutant pACG2-Y730+500+444F-Rep/Cap plasmid has been described recently (Markusic *et al.,* 2010).

[0227] ***Recombinant AAV Vector Transduction Assays*** **in Vitro.** Optimal concentration of NF-κB-modulating compounds was determined by a cell viability assay with tenfold-dilutions from the IC_{50} or were used as described previously (Wu and Miyamoto, 2008; Kumar *et al.,* 2008). VP16 or Bay11 (10 or 5 μM, final concentration), and PDTC (50 or 25 μM final concentration) were used either alone or in activator/inhibitor combinations. For transduction experiments, approximately 1×10^5 HeLa cells were either pre-treated with these compounds 24 hrs prior to vector infection. Cells were transduced with 500 or 2,000 vector genomes (vgs) per cell of recombinant WT-AAV or TM-AAV vectors encoding the EGFP transgene as described previously (Markusic *et al.,* 2010). After 7 days of culture, primary human dendritic cells were transduced with AAV vectors at 2000 vgs/cell and incubated for 48 hrs. Transgene expression was assessed as total area of green fluorescence ($pixel^2$) per visual field (mean ± SD), or by flow cytometry. Analysis of variance (ANOVA) was used to compare between test results and the control and they were determined to be statistically significant.

[0228] ***Recombinant AAV Vector Transduction Studies*** **in Vivo.** Groups of 6-weeks old normal C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME, USA) were administered intra-peritoneally, with a single dose (20 mg/kg) of NF-κB inhibitor Bay11, in a 200-μL volume diluted in DMSO (day 0). Animals injected with only the DMSO carrier solvent were considered as baseline (mock) group (n = 75) and animals injected with Bay11 were the test group (n = 75). At this point, the animals from mock and Bay11 groups were randomized to receive either phosphate buffered saline (PBS, pH 7.4) or WT-AAV or TM-AAV vectors (n = 25 mice each group). On day 1, $\sim 1 \times 10^{11}$ viral genome (vg) particles of WT-AAV2-EGFP or TM-AAV2-EGFP vectors or PBS were administered intravenously *via* the tail vein. To measure the modulation of immune response to AAV, 5 animals each from PBS-, WT-AAV-, or TM-AAV vector-injected groups were sacrificed by carbon-dioxide inhalation at different time points post-vector administration (2, 6, 10, 24 hrs and day 10). Hepatic lobes were

collected, cross-sectioned and mounted on slides to study the effect of Bay11 on AAV-mediated EGFP expression (from day 10 mice). All animal studies were conducted in accordance with institutional animal care and use committee guidelines.

[0229] ***Gene-Expression Analysis of Innate Immune Response by RT-PCR Assay.*** Groups of 6-weeks old normal C57BL/6J mice were administered intra-peritoneally, with a single dose (20 mg/kg) of NF-κB inhibitor, Bay11, in a 200-μL volume diluted in DMSO (day 0). On day 1, mice were injected with either phosphate-buffered saline (PBS, pH 7.4), or with ~1×10^{11} vgs of the wild-type (WT) AAV-EGFP vectors, or the tyrosine triple-mutant (TM) AAV-EGFP vectors intravenously *via* the tail-vein ($n = 5$ mice each group). At 2 hr post-vector administration, gene expression profiling of the innate immune response was performed that included Toll-like receptors 1-9, MyD88, MIP-1, IL-1α, IL-1β, IL-12α, IL6, KC, TNFα, RANTES, MCP-1, IFNα, IFNβ, and IP-10. Data were captured and analyzed using an ABI Prism 7500 Sequence Detection System with v 1.1 Software (Applied Biosystems). The baseline was determined automatically for the 18S rRNA and for other genes. Thresholds were determined manually for all genes. Gene expression was measured by the comparative threshold cycle (Ct) method. The parameter threshold cycle (Ct) was defined as the cycle number at which the reporter fluorescence generated by the cleavage of the probe passed a fixed threshold above baseline. Cytokine gene expression was normalized using the endogenous reference 18S rRNA gene and mock-infected murine mRNA were used as reference sample. Relative gene expression was determined for each group of treated and untreated animals and values > 2.6 and < 0.38 were considered as significant up-regulations and down-regulations between the groups and was calculated by assessing the variability in the 96-well plates used to measure specific gene expression.

[0230] ***Cells, Antibodies and Chemicals.*** HeLa cells were obtained from the American Type Culture Collection (Rockville, MD, USA) and maintained as monolayer cultures in Iscove's-modified Dulbecco's medium (IMDM, Invitrogen Carlsbad, CA, USA) supplemented with 10% newborn calf serum (NCS) (Lonza, Inc., Basel, Switzerland) and antibiotics. Leukapheresis-derived PBMCs were resuspended in serum-free AIM-V medium (Lonza) and semi-adherent cell fractions were incubated in serum-free AIM-V medium supplemented with recombinant human IL-4 (500 U/mL) and GM-CSF (800 U/mL) (R&D Systems, MN, USA). Cells were treated with NF-κB modulators (10 mM VP16 or 10 mM Bay11), and cytokines cocktail including 10 ng/mL TNF-α, 10 ng/mL IL-1, 10 ng/mL IL-6, 1 mg/mL PGE2 (R&D Systems) for 20 hr. Cells were harvested, characterized to ensure they met the typical phenotype of mature DCs (CD83, RPE, murine IgG1, CD86, FITC, murine IgG1; Invitrogen). All primary and secondary antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA) or Santa Cruz Biotechnology, Inc (Santa Cruz, CA, USA). NF-kB activators [Etoposide (VP16), Aphidicolin, Hydroxyurea (HU)] and NF-kB inhibitors [Bay11-7082 (Bay11), Pyrrolidine dithiocarbamate (PDTC)] were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). These

compounds were re-suspended in either DMSO (Sigma-Aldrich) or in sterile, DNAase-, RNAase-free water (Invitrogen) as per the manufacturer's instructions.

[0231] ***Western Blot Analyses.*** Homogenized lysates of the cell pellets from ~2×10^6 HeLa cells or DCs, mock or pre-treated with the optimal concentration of NF-κB activators or inhibitors were used for sample preparation. Whole cell proteins were isolated using the RIPA lysis buffer (Sigma-Aldrich) and cytoplasmic and nuclear proteins were extracted using a commercial kit (NE-PER Extraction Reagent Kit, Pierce Biotech, Rockford, IL, USA) as per the manufacturer's protocol in the presence of a protease inhibitor cocktail (Halt™ Protease Inhibitor Cocktail Kit, Pierce Biotech). The protein extracts were boiled for 5 min under reducing conditions [SDS-sample buffer containing 62.5 mM Tris-HCl (pH 6.8 at 25°C), 2% wt./vol. SDS, 10% glycerol, 50 mM DTT, 0.01% (wt./vol.) bromo-phenol blue (Cell Signaling Technology, Inc.)] and stored at -86°C until further analysis. Equal volumes of samples were run on 4–15% SDS-PAGE (Bio-Rad, Hercules, CA, USA). Gels were transferred onto a 0.2-μm nitrocellulose membrane (Bio-Rad) and typically incubated overnight with 1:1000 dilution of primary antibodies [p100/52, p65, inhibitory kinase-IκBκ, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), Lamin B (Cell Signaling Technology, Inc.), β-actin (Santa Cruz Biotechnology)]. The next day, blots were incubated with 1:2,000-1:5,000 of the appropriate anti-idiotypic HRP labeled IgG secondary antibody (Santa Cruz Biotechnology). Immunoblot detection was performed using the ECL plus Western blotting detection kit (Amersham Biosciences, Piscataway, NJ, USA). The intensity of the protein bands was measured with Adobe Photoshop CS3 software® and normalized to proteins levels from the housekeeping gene products used as loading controls.

[0232] The basis for the present study was the finding that the host cellular NF-κB can bind to the 20-bp D-sequence present in the AAV inverted terminal repeats (ITRs) (Qing *et al.*, 1997), which was identified by electrophoretic mobility-shift assays followed by mass-spectrometry (FIG. 10A and FIG. 10B). The data presented in this example provide the first evidence of the involvement of NF-κB in AAV infection. Using a variety of pharmacological modulators, which have been extensively used by other investigators (Wu and Miyamoto, 2008; Kumar *et al.*, 2008) to study the NF-κB signaling pathway, it was shown that the non-canonical NF-κB pathway is up-regulated following AAV infection. This is significant considering that activation of the NF-κB transcriptional program is a fundamental immediate early step of inflammatory and immune activation (Li and Verma, 2002), and NF-κB signaling represents a prime candidate for viral susceptibility or interference (Hiscott *et al.*, 2006). Viruses which activate NF-κB have been shown to be susceptible to innate immune response through an interferon response (Vesicular stomatitis virus, Measles virus) (Hiscott *et al.*, 2003), toll-like receptor (TLR) dependent (Ebola virus, Respiratory syncytial virus) (Okumura *et al.*, 2010; Lizundia *et al.*, 2008), and TLR-independent signaling pathway (Cytomegalovirus, Hepatitis C virus) (Castanier *et al.*, 2010; Gourzi *et al.*, 2007). On the other hand, many viruses disrupt the innate

immune responses and NF-κB using multifunctional viral decoy proteins that target specific aspects of the NF-κB pathway. Viruses, including human immunodeficiency virus type I (HIV-I), human T-cell leukemia virus type 1 (HTLV-1), Human herpesvirus 8 (HHV8) and Epstein-Barr virus (EBV), have incorporated aspects of NF-κB signaling into their life cycle and pathogenicity, and thus utilize NF-κB activation to promote their survival (Hiscott *et al.*, 2006).

[0233] In contrast, it stands to reason that the non-canonical pathway of NF-κB is activated following AAV infection both because the non-canonical NF-κB activation is known to be important for innate and adaptive immune response (Gilmore, 2006), and AAV vectors lack complex structural gene elements necessary to develop any NF-κB-like decoy proteins. The exacerbated activation of the non-canonical pathway has been associated to a wide range of inflammatory disorders like rheumatoid arthritis, ulcerative colitis or B cell lymphomas (Dejardin, 2006). Monarch-1, a pyrin-containing protein expressed exclusively in cells of myeloid lineage suppresses pro-inflammatory cytokines and chemokines through inhibition of NF-κB inducing kinase (NIK) necessary to activate non-canonical NF-κB pathway (Lich *et al.*, 2007). The activation of non-canonical pathway of NF-κB activation has been shown to result in maturation and T- cell priming activity of DCs over-expressing a mutated IκBκ which blocks activation of the classical pathway (Lind *et al.*, 2008). In alymphoplasia (Aly) mouse deficient in NIK, the cross-priming of CD8+ T cells to exogenous antigens in DCs is affected suggesting the importance of this pathway in adaptive immunity (Lind *et al.*, 2008). Mice deficient in non-canonical pathway components are also deficient in secondary lymphoid organ development and homeostasis (Guo *et al.*, 2008). It is not known whether AAV-binding activates the NF-κB signaling to a cell surface receptor. Recent studies have demonstrated that the innate immune response to AAV could be triggered through the TLR9-MYD88 pathway or through activation of the CD40 ligand on cell surface in murine models *in vivo* (Zhu *et al.*, 2009; Mays *et al.*, 2009). It is interesting to note that while both rely on NF-κB signaling down-stream for mounting an innate immune response (Mineva *et al.*, 2007; Loiarro *et al.*, 2005), activation of TNF super family receptors such as CD40L can activate the non-canonical NF-κB pathway (Qing *et al.*, 2005).

[0234] Based on the evidence that the first "danger-signal" or "trigger" to immune surveillance directed against AAV vectors may be the activation of alternative NF-κB signaling pathway, it was reasoned that transient blocking of NF-κB during AAV vector administration could dampen the host immune response. One possible strategy to negate the NF-κB-priming by AAV is to generate targeted mutations against the NF-κB responsive transcription factor binding sites in the AAV-ITRs. However, given the pleiotropic functions of NF-κB proteins in cellular physiology (Hayden and Ghosh, 2004), it is possible that different NF-κB-responsive cytokine promoter-binding transcription factors might be operational in different cell types. Alternatively, a protocol for transient immuno-suppression by targeting the NF-κB pathway might be universally applicable. The selective NF-κB

inhibitor, Bay11, can markedly reduce markers of inflammation and innate immune response to AAV vectors yet does not affect its transgene expression *in vivo*. Bay11 was able to down-regulate the activity of several key regulators namely, IL-1α, IL-6, TNFα, IL-12α, KC and RANTES, suggesting the benefit of using this pharmacologic modulator to selectively down-regulate the inflammatory and innate immune response against AAV vectors. Interestingly, NIK that is critical for activation of the non-canonical NF-κB pathway, is also known induce activation of IL-1α, IL-6, IL-12α, TNFα and RANTES in response to a variety of viral infections (DiPaolo *et al.*, 2009; Yanagawa and Onoe, 2006; Andreakos *et al.*, 2006; Habib *et al.*, 2001). In addition, it is well recognized that NIK is pivotal to the activation and function of the quiescent professional antigen presenting cells, the DCs, whose activity is critical for priming of the antigen specific CD4+ helper T cells, leading to immune responses to relevant targets such as the delivery vector (Andreakos *et al.*, 2006; Habib *et al.*, 2001; Martin *et al.*, 2003; Brown and Lillicrap, 2002). *In vitro*, NIK increases DC antigen presentation by potently activating NF-κB and consequently up-regulating the expression of cytokines (TNFα, IL-6, IL-12, IL-15, and IL-18), chemokines {IL-8, RANTES, macrophage inflammatory protein-1α, monocyte chemo-attractant protein-1, and monocyte chemo-attractant protein-3}, MHC antigen-presenting molecules (class I and II), and co-stimulatory molecules (CD80 and CD86) (Andreakos *et al.*, 2006). *In vivo*, NIK enhances immune responses against a vector-encoded antigen and shifts them toward a T helper 1 immune response with increased IgG2a levels, T-cell proliferation, IFN-γ production, and cytotoxic T lymphocyte responses more potently than complete Freund's adjuvant (Andreakos *et al.*, 2006). Bay11, used in this study, prevents the activity of IKKα and β, which are the substrates for NIK in the non-canonical pathway (Pierce *et al.*, 1997). These data indicate the high specificity of Bay11 in targeting the non-canonical NF-κB pathway as well as its ability to prevent the activation of major modulators of immune response.

[0235] A protocol for transient immuno-suppression by targeting the NF-κB pathway might be universally applicable to limit immuno-toxicities. Indeed, a recent report showed decreased AAV capsid antigen presentation by the use of a proteasomal inhibitor, Bortezomib [Velcade®] (Finn *et al.*, 2010). Bortezomib has a considerable anti-myeloma efficacy (Kube and Srivastava, 1997), which is likely in large part due to repression of NF-κB signaling. It may therefore be possible to simultaneously block MHC I presentation of capsid and inflammatory signals or use more selective NF-κB-targeted therapies, such as Bay11 in this study, or the newer IKK inhibitors in order to further enhance the safety and therapeutic efficacy of AAV vectors.

EXAMPLE 3 - DEVELOPMENT OF OPTIMIZED AAV3 SEROTYPE VECTORS

[0236] Adeno-associated virus 2 (AAV2), a non-pathogenic human parvovirus, contains a single-stranded DNA genome, and possesses a wide tissue-tropism that transcends the species barrier (Muzyczka, 1992). Recombinant AAV2 vectors have gained attention as a promising vector system for the potential gene therapy of a variety of human diseases, and are currently in use in a number of gene therapy clinical trials (Daya and Berns, 2008). More recently, several additional AAV serotypes

have been isolated, and have been shown to transduce specific cell types efficiently (Muramatsu *et al.*, 1996; Chiorini *et al.*, 1997; Chiorini *et al.*, 1999; Rutledge *et al.*, 1998; Gao GP *et al.*, 2002; Vandenberghe *et al.*, 2004). Whereas various steps in the life cycle of AAV2 are reasonably well understood (Summerford and Samulski 1998; Qing *et al.*, 1999; Summerford *et al.* 1999; Hansen *et al.*, 2000; Hansen *et al.*, 2001; Sanlioglu *et al., 2000; Douar et al.*, 2001; Zhao *et al.*, 2006; Thomas *et al.* 2004; Zhong *et al.* 2004; Ferrari *et al.*, 1996; Fisher *et al.* 1996; Qing *et al.*, 2004; Zhong *et al.*, 2004; Zhong *et al.*, 2004; Zhong *et al.*, 2008; McCarty *et al.*, 2004; Bainbridge *et al.*, 2008), less is known about the other serotypes.

[0237] Of the 10 commonly used AAV serotypes, AAV3 has been reported to transduce cells and tissues poorly (Zincarelli *et al.*; Zincarelli *et al.*, 2008). However, recent studies revealed that AAV3 vectors transduce established human hepatoblastoma (HB) and human hepatocellular carcinoma (HCC) cell lines as well as primary human hepatocytes extremely efficiently (Glushakova *et al.*, 2009). Subsequently, it was documented that AAV3 infection was strongly inhibited by hepatocyte growth factor (HGF), HGF receptor (HGFR) specific siRNA, and anti-HGFR antibody, which suggested that AAV3 utilizes HGFR as a cellular receptor/co-receptor for viral entry (Ling *et al.*, 2010).

[0238] The ubiquitin-proteasome pathway plays a crucial role in intracellular trafficking of AAV vectors (Douar *et al.*, 2001; Zhong *et al.*, 2007; Duan *et al.*, 2000). Intact AAV2 capsids can be phosphorylated at tyrosine residues by epidermal growth factor receptor protein tyrosine kinase (EGFR-PTK), and that tyrosine-phosphorylation of AAV capsids negatively affects viral intracellular trafficking and transgene expression. These observations led to the suggestion that tyrosine-phosphorylation is a signal for ubiquitination of AAV capsids followed by proteasome-mediated degradation (Duan *et al.*, 2000; Zhong *et al.*, 2008). This led to the hypothesis that mutations of the surface-exposed tyrosine residues (Y) to phenylalanine (F) might allow the vectors to evade phosphorylation, ubiquitination and proteasome-mediated degradation. Indeed, mutations of the surface-exposed tyrosine residues in AAV2 vectors led to high-efficiency transduction at lower doses both in HeLa cells *in vitro* and murine hepatocytes *in vivo* (Zhong *et al.*, 2008). Therapeutic levels of expression of human factor IX have been obtained in several different strains of mice using the single and multiple tyrosine-mutant AAV2 vectors (Zhong *et al.*, 2008; Markusic *et al.*, 2010). Additional studies have corroborated that similar Y-to- F mutations in AAV serotypes 6, 8 and 9 also lead to augmented transgene expression (Petrs-Silva *et al.*, 2009; Qiao *et al.*, 2010; Taylor and Ussher, 2010). Six of seven surface-exposed tyrosine residues in AAV2 are also conserved in AAV3, but their involvement in AAV3-mediated transduction has not been evaluated.

[0239] This example demonstrates that: (i) AAV3 vector-mediated transduction is dramatically increased in T47D cells, a human breast cancer cell line that expresses undetectable levels of the endogenous hHGFR (Abella *et al.*, 2005), following stable transfection and over-expression of hHGFR; (ii) the tyrosine kinase activity associated with hHGFR negatively affects the transduction

efficiency of AAV3 vectors; (iii) the use of proteasome inhibitors significantly improves AAV3 vector-mediated transduction; (iv) site-directed mutagenesis of three surface-exposed tyrosine residues on the AAV3 capsid leads to improved transduction efficiency; (v) a specific combination of two tyrosine-mutations further improves the extent of transgene expression; and (vi) AAV3 vectors efficiently transduce human HB and HCC tumors in a murine xenograft model *in vivo*, following both intratumoral or systemic administration. These optimized AAV3 vectors provide improved tools for gene therapy, and particularly for the therapy of liver cancer in humans.

MATERIALS AND METHODS

[0240] ***Cell Lines and Cultures.*** Human cervical cancer (HeLa) and hepatocellular carcinoma (Huh7) cell lines were purchased from American Type Culture Collection (Manassas, VA, USA), and maintained in complete DMEM medium (Mediatech, Inc., Manassas, VA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Sigma-Aldrich, St. Louis, MO, USA), 1% penicillin and streptomycin (P/S, Lonza, Walkersville, MD, USA). A newly established human hepatoblastoma (Hep293TT) cell line (Chen *et al.*, 2009) was maintained in complete RPMI medium 1640 (Invitrogen, Camarillo, CA, USA) supplemented with 15% heat-inactivated FBS (Sigma-Aldrich), 1% penicillin and streptomycin (P/S, Lonza, Walkersville, MD). Cells were grown as adherent cultures in a humidified atmosphere at 37°C in 5% CO_2 and were sub-cultured after treatment with trypsin-versene mixture (Lonza) for 2-5 min at room temperature, washed and re-suspended in complete medium. A human breast cancer cell line, T47D, and T47D cells stably transfected with a hHGFR expression plasmid (T47D+hHGFR), were maintained in complete DMEM medium (Mediatech, Inc.) with or without 600 μg/mL of G418, supplemented with 10% heat-inactivated fetal bovine serum (FBS, Sigma-Aldrich, St. Louis, MO, USA), 1% penicillin and streptomycin (Lonza).

[0241] ***Recombinant AAV Plasmids and Vectors.*** Recombinant AAV3 packaging plasmid and recombinant AAV2-CBAp-EGFP vector plasmid were generously provided respectively by Drs. R. Jude Samulski and Xiao Xiao, University of North Carolina at Chapel Hill, Chapel Hill, NC. Highly purified stocks of scAAV2 and scAAV3 vectors containing the enhanced green fluorescence protein (EGFP) gene driven by the chicken β-actin promoter (CBAp) were packaged by the calcium phosphate triple-plasmid transfection protocol described previously (Wu *et al.*, 2007; Kube and Srivastava, 1997). The physical particle titers of recombinant vector stocks were determined by quantitative DNA slot-blot analyses (Kube and Srivastava, 1997).

[0242] ***Construction of Surface-Exposed Tyrosine Residue Mutant AAV3 Capsid Plasmids.*** A two-stage procedure, based on QuikChange II® site-directed mutagenesis (Stratagene) was performed by using plasmid pAAV3 as described previously (Glushakova *et al.*, 2009; Ling *et al.*, 2010). Briefly, in stage one, two PCR extension reactions were performed in separate tubes for each mutant. One tube contained the forward PCR primer and the other contained the reverse primer (Table 3).

[0243] In stage two, the two reactions were mixed and a standard PCR mutagenesis assay was carried out as the manufacturer's instructions. PCR primers were designed to introduce changes from tyrosine to phenylalanine residues and a silent change to create a new restriction endonuclease site for screening purposes (Table 3). All mutants were screened with the appropriate restriction enzyme and were sequenced before use.

TABLE 3

NUCLEOTIDE SEQUENCES OF PRIMERS USED FOR SITE-DIRECTED MUTAGENESIS

Mutants	Primer Sequences (5′ to 3′)
Y252F	ACCAGAACCT**GGGC**CTGCCCACT**TT**CAACAACCATCTCTACAAG (SEQ ID NO:11)
	*Apa*I Tyr→Phe
Y272F	CAATCAGGAGC**TTC**GAACGACAACCAC**TT**CTTTGGCTACAGCACC (SEQ ID NO:12)
	+*Bst*BI Tyr→Phe
Y444F	CTT**AT**CGATCAGTATCTGTAC**TT**CCTGAACAGAACGCAAGGAACA (SEQ ID NO:13)
	+*Cla*I Tyr→Phe
F501Y	GCTAACGACAACAACAACAGTAACTA**TCC**TGGACAGCGGCCAGCAAA (SEQ ID NO:14)
	Phe→Tyr +*Nco*I
Y701F	TGGAATCCAGAGATTCAGT**TT**CAC**TC**CAACTACAACAAGTCTGTT (SEQ ID NO:15)
	Tyr→Phe +*Bmg*BI
Y705F	GAGATTCAGTACAC**TC**CAACT**TT**CAACAAGTCTGTTAATGTGGAC (SEQ ID NO:16)
	+*Afl*III Tyr→Phe
Y731F	GTGAACCTCGCCCTATTGGAACCCGGT**TT**CTCACACGAAACTTG (SEQ ID NO:17)
	Tyr→Phe

The codon triplets are shown in bold; red fonts denote the mutations from tyrosine to phenylalanine residues, and green fonts indicate the silent mutations to eliminate/create the restriction enzyme sites (underlined), which were used to identify the desired clones.

[0244] ***AAV Vector Transduction Assays.*** Huh7 or HeLa cells were seeded in 96-well plates at a concentration of 5,000 cells per well in complete DMEM medium. AAV infections were performed in serum- and antibiotic-free DMEM medium. Hep293TT cells were seeded in 96-well plates at a concentration of 10,000 cells per well in complete RPMI medium. The infections were performed in serum- and antibiotic-free RPMI medium. The expression of EGFP was analyzed by direct fluorescence imaging 72-hrs' post-transduction.

[0245] ***Western Blot Analyses.*** Cells were harvested and disrupted in a radio-immunoprecipitation assay (RIPA) lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% SDS, 1% NP-40, 0.25% sodium deoxycholate and 1 mM EDTA with protease inhibitor cocktail, 1 mM NaF and 1 mM Na_3VO_4). Total protein concentration was measured using a Bradford reagent (Bio-Rad) and equal amounts (50 μg) of whole cell lysates were resolved by SDS-PAGE. After electrophoresis, samples were electro-transferred to a nitrocellulose membrane (Bio-Rad), probed with relevant primary antibodies at 4°C overnight, incubated with horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA, USA), and detected with an enhanced chemi-

luminescence substrate (Amersham). Antibodies against phospho-c-Met (Y1234/1235), total c-Met, phospho-Akt (S473) and phospho-ERK (T202/Y204) were purchased from Cell Signaling, and anti-β-actin (AC-74) antibody was obtained from Sigma-Aldrich.

[0246] ***Recombinant AAV3 Vector Transduction Studies in Mouse Xenograft Models.*** Groups of 6-weeks old NSG mice (Jackson Laboratories) were injected subcutaneously with 5×10^6 Hep293TT or Huh7 cells. Four-week post-injection, indicated numbers of AAV3 vector genomes (vgs) were administered either intratumorally or through tail-vein. Four days post-vector administration, tumors were resected, cross-sectioned and evaluated for EGFP expression using a fluorescent microscope. Sections were also stained with DAPI to visualize the cell nucleus. All animal studies were conducted in accordance with approved institutional guidelines.

[0247] ***Statistical Analysis.*** Results are presented as mean ± standard deviation (SD). Differences between groups were identified using a grouped-unpaired two-tailed distribution of Student's T test. *P* values < 0.05 were considered statistically significant.

RESULTS

[0248] ***Human HGFR is Required for AAV3 Infectivity.*** AAV3 utilizes human hepatocyte growth factor receptor (HGFR) as a cellular co-receptor (Ling *et al.*, 2010). To unequivocally corroborate this finding, a human breast cancer cell line, T47D, was used that expresses undetectable levels of hHGFR (Abella *et al.*, 2005), as well as T47D cells stably transfected with hHGFR expression plasmids (T47D+hHGFR) (Abella *et al.*, 2005). The expression of hHGFR protein in the established cell line T47D+hHGFR was confirmed by Western blot analysis (see FIG. 12C). Equivalent numbers of T47D and T47D+hHGFR cells were transduced with various multiplicities-of-infection (MOI) of self-complementary (sc) AAV3-CBAp-EGFP vectors under identical conditions and transgene expression was determined 72 hr post-transduction. These results, shown in FIG. 11A, document that the transduction efficiency of AAV3 vectors is ~8-13-fold higher in cells that express hHGFR than those that do not. AAV3 vector-mediated transduction of T47D+hHGFR cells could be completely blocked in the presence of 5 μg/mL of hHGF (FIG. 11B). Taken together, these data provide conclusive evidence that cell surface expression of hHGFR is required for successful transduction by AAV3 vectors.

[0249] ***Inhibition of HGFR Protein Tyrosine Kinase Activity Enhances Transduction Efficiency of AAV3 Vectors.*** To examine whether in addition to the extracellular domain, the intracellular domain of HGFR, which contains protein tyrosine kinase activity, is also involved in AAV3 infection, a further study was performed. Binding of its ligand, HGF, results in dimerization of the receptor and intermolecular trans-phosphorylation of multiple tyrosine residues in the intracellular domain (Nguyen *et al.*, 1997). T47D+hHGFR cells were treated for two hrs with increasing concentrations of a specific HGFR kinase inhibitor, BMS-77760707 (BMS) (Schroeder *et al.*, 2009; Dai and Siemann, 2010). Cells were subsequently infected with scAAV3 vectors at 2,000 vgs/cell. These results are shown in FIG. 12A. It is evident that BMS-777607-treatment led to

~2-fold increase in AAV3 transduction efficiency. Although the p-value is higher when BMS-777607 was used at the highest concentration of 10 μM, compared with the lower concentration of 1 μM, this change is most likely due to drug toxicity. In previous studies, it was reported that BMS-777607 treatment had no significant effect on cell growth at doses $\leq$1 μM. However, doses of 10 μM did result in significant reduction in cell proliferation, which suggests that this concentration is toxic to cells (Dai and Siemann, 2010). In the next experiment, to rule out any possible non-specific nature of this drug, the parental T47D cells were included as a control. Both cell types were treated with 1 μM BMS-777607 for 2 hr and then infected with scAAV3 vectors at 10,000 vg/cell. The results, shown in FIG. 12B, indicated that whereas BMS-777607-treatment significantly enhanced AAV3 infectivity in T47D+hHGFR cells, it had no effect in T47D cells that lack expression of hHGFR.

[0250] To examine whether inhibition of the HGFR kinase led to alterations in the phosphorylation status of specific cellular proteins involved in the downstream signaling pathway total and phosphorylation levels of the HGFR protein in both T47D and T47D+hHGFR lysates were determined following a 2-hr drug-incubation period. Activation of signaling pathways downstream from HGFR kinase, ERK1/2 and Akt, were analyzed using phosphorylation-specific antibodies. These results, shown in FIG. 12C, confirmed that whereas little expression of hHGFR occurs in T47D cells, the level of expression is significantly higher in T47D+hHGFR cells for both total HGFR and phosphorylated HGFR, which is consistent with previously published reports (Abella *et al.*, 2005). Treatment of T47D+hHGFR cells with BMS-777607 completely blocked the phosphorylation of HGFR, but not total HGFR. In addition, BMS-777607-treatment had no effect on the expression of phosphorylated AKT and ERK1/2. These results suggest that the enhancement of AAV3 vector infectivity by the BMS-777607-treatment is due to inhibition of HGFR kinase.

[0251] To date, only AAV2 has been reported to use hHGFR as a co-receptor (Yan *et al.*, 2002). The roles of hHGFR and hHGFR kinase inhibitor on other AAV serotypes are not known. To rule out any non-specific enhancement of transduction by BMS-777607, other serotypes of AAV, which are not dependent on HGFR, as well as AAV2 vectors, were compared for transduction efficiency following treatment of cells with BMS-777607. These results, shown in FIG. 13, indicate that whereas AAV2 and AAV3 vectors can efficiently transduce T47D+hHGFR cells, other serotypes (AAV4-AAV9) can only transduce these cells at a very low efficiency. This result suggests that hHGFR is not involved in the life cycle of these AAV serotypes. Treatment of cells with BMS-777607 significantly increased the transduction efficiency of both AAV2 and AAV3 vectors, but not the other AAV serotypes, which suggested that the effect of the BMS-777607-treatment is AAV serotype-specific.

[0252] ***Proteasome Inhibitors Increase the Transduction Efficiency of AAV3 Vectors.*** Previous studies have shown that proteasome inhibitors, such as MG132, can significantly enhance the transduction efficiency of AAV2 vectors by facilitating intracellular trafficking (Zhong *et al.*, 2007; Yan *et al.*, 2002). To evaluate whether MG132 can also improve AAV3 trafficking in target

cells, Huh7, a well-established human hepatocellular carcinoma cell line (Nakabayashi *et al.*, 1982), and Hep293TT, a recently established human hepatoblastoma cell line (Chen *et al.*, 2009), were either mock-treated or treated with increasing concentrations of MG132. Following a two-hour treatment, cells were infected with scAAV3-EGFP vectors. HeLa cells, treated with 5 μM MG132 and transduced with scAAV2 vectors, were included as a positive control. Transgene expression was determined by fluorescence microscopy 72 hrs post-transduction. These data are shown in FIG. 14A and FIG. 14B. As can be seen, pretreatment with MG132 significantly increased the transduction efficiency of scAAV2 vectors in HeLa cells, which is consistent with previously results (Zhong *et al.*, 2008). Interestingly, a dose-dependent increase in the transduction efficiency of scAAV3 vectors in both Huh7 and Hep293TT cells occurred following MG132-treatment, suggesting that AAV3 vectors also undergo ubiquitination followed by proteasome-mediated degradation.

[0253] Previous studies have also shown that inhibition of EGFR-PTK signaling by Tyrphostin 23 (Tyr23), a specific inhibitor of EGFR-PTK (May *et al.*, 1998), modulates the Ub/proteasome pathway, which in turn, facilitates intracellular trafficking and transgene expression mediated by AAV2 vectors (Zhong *et al.*, 2007). Hep293TT cells were mock-treated or treated with Tyr23 for 2 hr and transduced with scAAV3 vectors. HeLa cells, pretreated with Tyr23 and transduced with scAAV2 vectors, were included as appropriate controls. Transgene expression was determined 72 hr post-transduction. These results, shown in FIG. 14C and FIG. 14D, indicate that Tyr23-treatment led to a significant increase in the transduction efficiency of both scAAV2 and scAAV3 vectors. The increased transgene expression was independent of vector entry, since there was no significant difference in the amounts of internalized viral DNA in the presence or absence of either MG132 or Tyr23. These results further corroborate the involvement of the host cell Ub/proteasome machinery in the life cycle of AAV3 vectors as well.

[0254] ***Site-directed Mutagenesis of Surface-Exposed Tyr Residues Significantly Improves Transduction Efficiency of scAAV3 Vectors.*** In the preceding examples, the inventors have demonstrated that there are seven surface-exposed tyrosine residues (Y252, Y272, Y444, Y500, Y700, Y704 and Y730) on AAV2 capsids that are phosphorylated by EGFR-PTK and negatively affect the transduction efficiency of AAV2 vectors (Zhong *et al.*, 2008). Alignment of amino acid sequences from AAV2 and AAV3 capsids indicated that six of seven tyrosine residues (Y252, Y272, Y444, Y701, Y705 and Y731) are conserved in AAV3 capsid (Table 4).

TABLE 4

SURFACE-EXPOSED TYR RESIDUES ON AAV CAPSIDS, AND SITE-DIRECTED MUTAGENESIS TO CONVERT THEM TO PHENYLALANINE RESIDUES

AAV2	AAV3
Y252	Y252→F
Y272	Y272→F
Y444	Y444→F
Y500	F501
Y700	Y701→F
Y704	Y705→F
Y730	Y731→F

The surface-exposed tyrosine (Y) residues on AAV2 and AAV3 capsids are shown; arrows denote the site-directed mutations from Y to phenylalanine (F) residues on AAV3 capsids.

[0255] One tyrosine residue, Y500 in AAV2, is present as F501 in AAV3. Since it has been shown that Y to F mutations in several AAV serotypes enhance transgene expression by circumventing ubiquitination and proteasome-mediated degradation (Zhong *et al.*, 2008; Petrs-Silva *et al.*, 2009; Qiao *et al.*, 2010; Taylor and Ussher *et al.*, 2010), it was reasoned that mutation of F501 back to a tyrosine residue would reduce the transduction efficiency of AAV3 vectors. This hypothesis was tested by generating a mutant AAV3 vector in which the phenylalanine residue was substituted with a tyrosine residue (F501Y). The transduction efficiency of the mutant vector was compared with its wild-type (WT) AAV3 counterpart using Huh7 cells under identical conditions. As can be seen in FIG. 15A, the extent of the transgene expression mediated by the F501Y mutant vector was reduced by ~50% compared with the WT AAV3 vector.

[0256] To further test the hypothesis that tyrosine-mutations on AAV3 capsids would lead to decreased EGFR-PTK-mediated phosphorylation followed by reduced ubiquitination and impaired proteasome-mediated degradation resulting in increased transgene expression, all six surface-exposed tyrosine residues on AAV3 capsids were modified and substituted with phenylalanine residues (tyrosine-phenylalanine, Y-F). Each of the single tyrosine-mutant vectors encapsidating scAAV2-CBAp-EGFP genomes could be successfully packaged. Vector titers for each of the mutants were determined by both quantitative DNA slot blots and qPCR, and no significant differences in the packaging efficiency were observed. The transduction efficiency of each of the tyrosine-mutant vectors was analyzed and compared with the WT scAAV3-CBAp-EGFP vector in both Huh7 (FIG. 15B) and Hep293TT (FIG. 15C) cells under identical conditions. From these results, it is evident that, the transduction efficiency of three of the tyrosine-mutant vectors (Y701F, Y705F and Y731F) is significantly higher compared with the WT scAAV3 vector. Specifically, the transduction efficiency of Y731F vector was ~8-fold higher than the WT vector, followed by Y705F (~3-fold) and Y701F (~2-fold) vectors.

[0257] ***Multiple-Mutations in Surface-Exposed Tyrosine Residues Further Improve the Transduction Efficiency of AAV3 Vectors.*** In the prior examples involving Y-F mutant AAV2 vectors, it was observed that specific combinations of the most efficient single-mutations of surface-exposed tyrosine residues further augmented the transduction efficiency of AAV2 vectors (Markusic *et al.*, 2010). To examine whether a similar enhancement could be achieved with AAV3 vectors, the following double- and triple-mutant AAV3 vectors were constructed: Y701+731F, Y705+731F, and Y701+705+731F. Each of these mutant vectors was packaged to similar titers, as determined by both quantitative DNA slot blots and qPCR. The transduction efficiency of these multiple-mutants was compared with the WT and the Y731F single-mutant AAV3 vectors in Huh7 cells under identical conditions. These results are shown in FIG. 16A. As can be seen, whereas the Y731F mutation significantly increased the transduction efficiency of AAV3 vectors, as observed before, only one of the double-mutations (Y705+731F) led to an additional significant increase in transgene expression. Interestingly, the transduction efficiency of both the double mutant (Y701+731F) and the triple mutant (Y701+705+731F) vectors was reduced to levels similar to the WT AAV3 vector. The best-performing single and multiple tyrosine-mutants on human liver cancer cells were then evaluated for transduction of T47D and T74D+hHGFR cells (FIG. 16B). Similar to human liver cancer cells, the tyrosine-mutant rAAV3 vectors led to high-efficiency transduction of both cell types, with or without hHGFR expression.

[0258] To examine the possibility whether the observed enhanced transduction efficiency of the Y-F mutant vectors was due to the involvement of one or more additional putative cellular receptor/co-receptor functions, the WT, Y731F, and Y705+731F mutant scAAV3-CBAp-EGFP vectors were used to transduce Huh7 cells in the absence or the presence of 5 μg/ml hHGF under identical conditions. These results are shown in FIG. 16C. As is evident, the presence of hHGF dramatically inhibited the transduction efficiency and transgene expression of all three AAV3 vectors, which is consistent with the interpretation that the tyrosine-mutant vectors also utilize hHGFR as a cellular receptor/co-receptor for viral entry.

[0259] ***AAV3 Vectors Transduce Human Liver Tumors in Murine Xenograft Models.*** To demonstrate AAV3 vectors could also transduce human HB and HCC tumors in a xenograft mouse model *in vivo*, ~5×10^6 HCC (Huh7) or HB (Hep293TT) cells were injected sub-cutaneously in NOD/Scid gamma (NSG) mice. Four-weeks later, when tumors were clearly visible and palpable in both groups of animals, ~2×10^{10} vgs of scAAV3-CBAp-EGFP vectors were injected directly into tumors. Four-days post-vector injections, tumors were excised and thin sections were examined under a fluorescence microscope. These results indicated that AAV3 vectors were effective to transduce both human HCC (FIG. 17A) and HB (FIG. 17B) tumors *in vivo*. Consistent with the *in vitro* data, the transduction efficiency of AAV3 vectors was higher in Hep293TT cell-derived tumors than that in Huh7 cell-derived tumors.

[0260] ***Optimized Tyrosine-Mutant AAV3 Vectors are Highly Efficient in Transducing Human Liver Tumors in Murine Xenografts.*** Next, the best performing double tyrosine-mutant AAV3 vectors were further evaluated *in vivo* for xenograft human liver tumors gene transfer. In the first set of studies, ~5×10^{10} vgs of either the wild-type (WT) scAAV3- or Y705+731F-AAV3-CBAp-EGFP vectors were intratumorally injected in NSG mice bearing human HB (Hep293TT) tumors. Four-days post-vector injections, tumors were excised, and thin sections were examined under a fluorescence microscope (FIG. 17C). As can be seen, tumors injected with the WT-AAV3 vectors exhibited detectable levels expression of EGFP. The transduction efficiency of the double tyrosine-mutant AAV3 vectors was significantly higher compared with the WT AAV3 vectors, which is consistent with the *in vitro* data.

[0261] In the second set of studies, ~5×10^{11} vgs of either the WT-scAAV3-CBAp-EGFP vector or the Y705+731F-scAAV3-CBAp-EGFP vector were injected *via* the tail-vein in NSG mice bearing human HB (Hep293TT) tumors. Phosphate-buffered saline (PBS) injections were used as an appropriate control. Whereas little trangene expression occurred in tumors from mice injected with pBS (FIG. 18A), direct tumor-targeting could be achieved following systemic administration of AAV3 vectors. The transduction efficiency of the optimized tyrosine-mutant AAV3 vectors (FIG. 18C), once again, was significantly higher than that of the WT AAV3 vectors (FIG. 18B). These data suggest that the observed increased transduction efficiency of tyrosine-mutant AAV3 vectors was independent of viral administration route.

[0262] HGFR is a trans-membrane receptor tyrosine kinase, and binding of its ligand, HGF, results in dimerization of the receptor and intermolecular trans-phosphorylation of multiple tyrosine residues in the intracellular domain. (Liu *et al.*, 2008) Whereas it is clear that AAV3 capsid interacts with the extracellular domain of hHGFR, it is less clear, whether AAV3-binding to hHGFR also triggers its activation and phosphorylation of the downstream target proteins. The data does indeed demonstrate that suppression of the hHGFR-PTK activity leads to a modest increase in AAV3 vector-mediated transgene expression. In this context, it is of interest to note that the transduction efficiency of AAV3 vectors is significantly higher in a more recently established human hepatoblastoma (HB) cell line, Hep293TT, compared with that in a HB cell line, Huh6, which was established nearly three decades ago. Although subtle differences might exist between the two cell lines, specific mutations have been identified in the tyrosine kinase domain of hHGFR in Hep293TT cells, which render it inactive, and that the hHGFR-specific kinase inhibitor, BMS-777607, which augments the transduction efficiency in Huh6 cells, has little effect on AAV3 transduction efficiency in Hep293TT cells.

[0263] Despite the utilization of two distinct cellular growth factor receptors as co-receptors by AAV2 (hFGFR1) and AAV3 (hHGFR), the two serotypes appear to share certain post-receptor entry and intracellular trafficking pathways. For example, both capsids become phosphorylated at tyrosine residues by EGFR-PTK, presumably in the late endosomes, followed by ubiquitination, which leads

to proteasome-mediated degradation. (Zhong *et al.*, 2008) However, although 6 of 7 surface-exposed tyrosines in AAV2 are conserved in AAV3, the patterns of behavior of the corresponding Y-F mutants are somewhat divergent. For example, Y730F (for AAV2) and Y731F (for AAV3) are the most efficient single-mutants, followed by Y444F (for AAV2), and Y705F (for AAV3), the transduction efficiency of Y444F (for AAV3) remains unaltered. Similarly, whereas the transduction efficiency of the Y730+444F double-mutant (for AAV2) is not significantly different from that of Y730F, the transduction efficiency of the Y705+731F double-mutant (for AAV3) is significantly higher than Y731F. Furthermore, the Y730+500+444F triple-mutant (for AAV2) is the most efficient, the Y731+501+705F triple-mutant (for AAV3) is the most efficient, the Y501 residue having already been mutated in the WT AAV3 capsid. Interestingly, even the WT AAV3 vectors were able to transduce human liver tumors reasonably well in a mouse xenograft model *in vivo* following intratumor injection. However, evidence that the tyrosine-mutant vector resulted in higher gene transfer efficiency *in vivo* has been demonstrated.

[0264] Human liver cancer, especially hepatocellular carcinoma (HCC), is one of the most aggressive malignant tumors. The major obstacle to survival with HCC is recurrence after HCC resection (Tang, 2005). Thus, transduction of 100% of target cells is desirable in order to completely eliminate the tumor. In previous studies, it was observed that melittin, a toxic peptide derived from bee venom, inhibits the viability and motility of HCC cells both *in vitro* and *in vivo via* the suppression of Rac1-dependent pathway (Liu *et al.*, 2008) and up-regulation of mitochondria membrane protein 7A6 (Zhang *et al.*, 2007). Melittin has been shown to induce apoptosis of HCC cells potentially by activating CaMKII/TAK1/JNK/p38 signaling pathway (Wang *et al.*, 2009).

[0265] Based on previous studies with recombinant adenovirus vectors containing the melittin gene driven by a liver cancer cell-specific promoter to achieve specific killing of liver cancer cells both *in vitro* and *in vivo* (Ling *et al.*, 2005), this example provides optimized tyrosine-mutant AAV3-melittin vectors under the control of a liver cancer cell-specific promoter that can be used to selectively target both primary and metastatic liver cancer.

EXAMPLE 4 - HIGH-EFFICIENCY TRANSDUCTION OF HUMAN MONOCYTE-DERIVED DENDRITIC CELLS BY CAPSID-MODIFIED RECOMBINANT AAV2 VECTORS

[0266] Dendritic cells (DCs) are antigen-presenting cells (APCs), which play a critical role in the regulation of the adaptive immune response. DCs are unique APCs and have been referred to as "professional" APCs, since the principal function of DCs is to present antigens, and because only DCs have the ability to induce a primary immune response in resting naïve T lymphocytes.(Banchereau and Steinman, 1998) Although a naturally occurring anti-tumor immune response is detectable in patients, this response fails to control tumor growth. On the other hand, monocyte-derived DCs (moDCs) generated *ex vivo* in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin 4 (IL-4) possess the capacity to stimulate antigen-specific T-cells after endogenous expression of antigens. (Chapuis *et al.*, 1997; den Brok *et al.*, 2005) For this reason,

genetically-modified DCs have been extensively studied and numerous Phase I and II clinical trials evaluating the efficacy of DCs in patients with cancer have been initiated. (Figdor *et al.*, 2004; Palucka *et al.*, 2011) However, current methods for DC loading are inadequate in terms of cell viability, uncertainty regarding the longevity of antigen presentation, and the restriction by the patient's haplotype. (Palucka *et al.*, 2011)

[0267] The possibility of manipulating viral genomes by biotechnological techniques, together with the recent identification of many tumor-associated antigens (TAAs), has sparked an interest in using recombinant viruses to express TAAs in the hope of inducing a protective antitumor immune response in patients. (Liu, 2010; Robert-Guroff, 2007) Among different methods for gene delivery, vectors based on a human parvovirus, the adeno-associated virus serotype 2 (AAV2), have attracted much attention mainly because of the non-pathogenic nature of this virus, and its ability to mediate long-term, sustained therapeutic gene expression. (Daya and Berns, 2008; Mueller and Flotte, 2008; Srivastava, 2008) Successful transduction of different subsets of DCs by different commonly used serotypes of AAV vectors has been demonstrated and the potential advantage of an AAV-based antitumor vaccine discussed. (Pannazhagan *et al.*, 2001; Veron *et al.*, 2007; Mahadevan *et al.*, 2007; Shin *et al.*, 2008; Taylor and Ussher, 2010) However, further improvements in gene transfer by recombinant AAV vectors to DCs in terms of specificity and transduction efficiency are warranted to achieve a significant impact when used as an anti-tumor vaccine.

[0268] Cellular epidermal growth factor receptor protein tyrosine kinase (EGFR-PTK) negatively impacts nuclear transport and subsequent transgene expression by recombinant AAV2 vectors primarily due to phosphorylation of capsids at surface tyrosine residues. (Zhong *et al.*, 2007) These studies resulted in the development of next generation recombinant AAV2 vectors containing point mutations in surface exposed tyrosine residues that transduce various cells and tissues with high-efficiency at lower doses compared to the wild-type (WT) vector. (Zhong *et al.*, 2008) However, such single or multiple tyrosine-mutant AAV vectors failed to increase the transduction efficiency of monocyte-derived DCs (moDCs) more than 2-fold, most likely due to lower levels of expression and/or activity of EGFR-PTK compared with that in HeLa cells or hepatocytes. (Taylor and Ussher, 2010)

[0269] Serine/threonine protein kinases are involved in a wide variety of cellular processes such as differentiation, transcription regulation, and development of many cell types including immune cells. Such kinases can also negatively regulate the efficiency of recombinant AAV vector-mediated gene transfer by phosphorylating the surface-exposed serine and/or threonine residues on the viral capsid and target the vectors for proteasome-mediated degradation. In the present example, the following were documented: (i) Site-directed mutagenesis of the 15 surface-exposed serine (S) residues on the AAV2 capsid to valine (V) residues leads to improved transduction efficiency of S458V, S492V, and S662V mutant vectors compared with the WT AAV2 vector; (ii) The S662V mutant vector efficiently transduces human monocyte-derived dendritic cells (moDCs), a cell type not

readily amenable to transduction by the conventional AAV vectors; (iii) High-efficiency transduction of moDCs by S662V mutant does not induce any phenotypic changes in these cells; and (iv) Recombinant S662V- vectors encoding a truncated human telomerase (hTERT) gene, used to transduced DCs result in rapid, specific T-cell clone proliferation and generation of robust CTLs, which leads to specific cell lysis of K562 cells.

MATERIALS AND METHODS

[0270] ***Cells and Antibodies.*** HEK293, HeLa and NIH3T3 cells were obtained from the American Type Culture Collection and maintained as monolayer cultures in DMEM (Invitrogen) supplemented with 10% FBS (Sigma) and antibiotics (Lonza). Leukapheresis-derived peripheral blood mononuclear cells (PBMCs) (AllCells) were purified on Ficoll-Paque (GEHeathCare), resuspended in serum-free AIM-V medium (Lonza), and semi-adherent cell fractions were incubated for 7 days with recombinant human IL-4 (500 U/mL) and GM-CSF (800 U/mL) (R&D Systems). Cell maturation was initiated with a cytokine mixture including 10 ng/mL TNF-α, 10 ng/mL IL-1, 10 ng/mL IL-6, and 1 mg/mL PGE2 (R&D Systems) for 48 hrs. Prior to EGFP expression cells were characterized for co-stimulatory molecules expression to ensure that they met the typical phenotype of mature dendritic cells (mDC) (CD80, RPE, murine IgG1; CD83, RPE, murine IgG1; CD86, FITC, murine IgG1; Invitrogen). (Jayandharan *et al.*, 2011)

[0271] ***Site-Directed Mutagenesis.*** A two-stage PCR was performed with plasmid pACG2 as described previously (Wang and Malcolm, 1999) using Turbo *Pfu* Polymerase (Stratagene). Briefly, in stage one, two PCR extension reactions were performed in separate tubes for the forward and reverse PCR primer for 3 cycles. In stage two, the two reactions were mixed and a PCR reaction was performed for an additional 15 cycles, followed by *Dpn*I digestion for 1 hr. Primers were designed to introduce changes from serine (TCA or AGC) to valine (GTA or GTC) for each of the residues mutated.

[0272] ***Production of Recombinant AAV Vectors.*** Recombinant AAV2 vectors containing the EGFP gene driven by the chicken β-actin promoter were generated as described previously (Zologukhin *et al.*, 2002). Briefly, HEK293 cells were transfected using polyethelenimine (PEI, linear, MW 25,000, Polyscinces, Inc.). Seventy-two hrs post transfection, cells were harvested and vectors were purified by iodixanol (Sigma) gradient centrifugation and ion exchange column chromatography (HiTrap Sp Hp 5 mL, GE Healthcare). Virus was then concentrated and the buffer exchanged in three cycles to lactated Ringer's using centrifugal spin concentrators (Apollo, 150-kDa cut-off, 20-mL capacity, CLP) (Cheng *et al.*, 2011). Ten μL of purified virus was treated with DNAse I (Invitrogen) for 2 hr at 37°C, then an additional 2 hr with proteinase K (Invitrogen) at 56°C. The reaction mixture was purified by phenol/chloroform, followed by chloroform treatment. Packaged DNA was precipitated with ethanol in the presence of 20 μg glycogen (Invitrogen). DNAse I-resistant AAV particle titers were determined by RT-PCR with the following primer-pair, specific for the CBA promoter:

Forward 5′-TCCCATAGTAACGCCAATAGG-3′ (SEQ ID NO:18),
Reverse 5′-CTTGGCATATGATACACTTGATG-3′ (SEQ ID NO:19)
and SYBR Green PCR Master Mix (Invitrogen) (Aslanidi *et al.*, 2009).

[0273] ***Recombinant AAV Vector Transduction Assays* In Vitro.** HEK293 or monocyte-derived dendritic cells (moDCs), were transduced with AAV2 vectors with 1,000 vgs/cell or 2,000 vgs/cell respectively, and incubated for 48 hrs. Alternatively, cells were pretreated with 50 μM of selective serine/threonine kinase inhibitors 2-(2-hydroxyethylamino)-6-aminohexylcarbamic acid tert-butyl ester-9-isopropylpurine (for CaMK-II), anthra[1,9-cd]pyrazol-6(2H)-one, 1,9-pyrazoloanthrone (for JNK), and 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole (for MAPK) (CK59, JNK inhibitor 2, PD 98059, Calbiochem), 1 hr before transduction. Transgene expression was assessed as the total area of green fluorescence (pixel2) per visual field (mean ± SD) as described previously (Markusic *et al.*, 2011; Jayandharan *et al.*, 2011). Analysis of variance was used to compare test results and the control, which were determined to be statistically significant.

[0274] ***Western Blot Analysis.*** Western blot analysis was performed as described previously. (Akache *et al.*, 2006) Cells were harvested by centrifugation, washed with PBS, and resuspended in lysis buffer containing 50 mM TrisHCl, pH 7.5, 120 mM NaCl, 1% Nonidet P-40, 10% glycerol, 10 mM Na4P2O7, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM EDTA, and 1 mM EGTA supplemented with protease and phosphotase inhibitors mixture (Set 2 and 3, Calbiochem). The suspension was incubated on ice for 1 hr and clarified by centrifugation for 30 min at 14,000 rpm at 4°C. Following normalization for protein concentration, samples were separated using 12% polyacrylamide/SDS electrophoresis, transferred to a nitrocellulose membrane, and probed with primary antibodies, anti p-p38 MAPK (Thr180/Tyr182) rabbit mAb, total p38 MAPK rabbit mAb and GAPDH rabbit mAb (1:1000, CellSignaling), followed by secondary horseradish peroxidase-linked linked antibodies (1:1000, CellSignaling).

[0275] ***Specific Cytotoxic T-Lymphocytes Generation and Cytotoxicity Assay.*** Monocyte-derived dendritic cells (moDCs) were generated as described above. Immature DCs were infected with AAV2-S662V vectors encoding human telomerase cDNA, separated into two overlapping ORF – hTERT838-2229 and hTERT2042-3454 at MOI 2,000 vgs/cell of each. Cells were then allowed to undergo stimulation with supplements to induce maturation. After 48 hr, the mature DCs expressing hTERT were harvested and mixed with the PBMCs at a ratio of 20:1. CTLs were cultured in AIM-V medium containing recombinant human IL-15 (20 IU/mL) and IL-7 (20 ng/mL) at 20×10^6 cells in 25 cm^2 flasks. Fresh cytokines were added every 2 days. After 7 days post-priming, the cells were harvested and used for killing assays (Heiser *et al.*, 2002). A killing curve was generated and specific cell lysis was determined by FACS analysis of live/dead cell ratios as described previously (Mattis *et al.*, 1997). Human immortalized myelogenous leukemia cell line, K562, was used as a target.

[0276] ***Statistical Analysis.*** Results are presented as mean ± S.D. Differences between groups were identified using a grouped-unpaired two-tailed distribution of Student's T-test. P-values <0.05 were considered statistically significant.

RESULTS

[0277] ***Inhibition of Specific Cellular Serine/Threonine Kinase Increases Transduction Efficiency of rAAV2 Vectors.*** In previous studies, inhibition of cellular epidermal growth factor receptor protein tyrosine kinase (EGFR-PTK) activity and site-directed mutagenesis of the 7 surface-exposed tyrosine residues was shown to significantly increase to the transduction efficiency of AAV2 vectors by preventing phosphorylation of these residues, thereby circumventing ubiquitination and subsequent proteasome-mediated degradation of the vectors (Zhong *et al.*, 2008). However, AAV2 capsids also contain 15 surface-exposed serine residues, which can potentially be phosphorylated by cellular serine/threonine kinases widely expressed in various cell types and tissues. To test the hypothesis that inhibition of such kinase activity can prevent phosphorylation of surface-exposed serine residues and thus improve intracellular trafficking and nuclear transport of AAV2 vectors, several commercially available specific inhibitors of cellular serine/threonine kinases were used, including calmodulin-dependent protein kinase II (CamK-II), c-Jun N-terminal kinase (JNK); and mitogen-activated protein kinase (p38 MAPK). HEK293 cells were pre-treated with specific inhibitors, such as 2-(2-hydroxyethylamino)-6-aminohexylcarbamic acid tert-butyl ester-9-isopropylpurine (for CaMK-II), anthra[1,9-cd]pyrazol-6(2H)-one, 1,9-pyrazoloanthrone (for JNK), and 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole (for p38 MAPK) for 1 hr at various concentrations. Cells were subsequently transduced with either single-stranded (ss) or self-complementary (sc) AAV2 vectors at 1,000 vector genomes (vgs) per cell. These results indicated that all inhibitors at an optimal concentration of 50 μM significantly increased the transduction efficiency of ssAAV2 and scAAV2 vectors, the p38 MAPK inhibitor being the most effective (FIG. 19A and FIG. 19B). This observation suggests, albeit does not prove, that the increase in the transduction efficiency was most likely due to prevention of phosphorylation of vector capsids rather than improved viral second-strand DNA synthesis.

[0278] Site-Directed Mutagenesis of Surface-Exposed Serine Residues on AAV2 Capsid Improves AAV2 Vector-Mediated Transgene Expression. The AAV2 capsid contains 50 serine (S) residues in the viral protein 3 (VP3) common region of the three capsid VPs, of which 15 (S261, S264, S267, S276, S384, S458, S468, S492, S498, S578, S658, S662, S668, S707, S721) are surface-exposed. (Xie et al., 2002) Each of the 15 S residues was substituted with valine (V) by site-directed mutagenesis as described (Zhong et al., 2008). Most mutants could be generated at titers similar to the WT AAV2 vectors, with the exception of S261V, S276V, and S658V, which were produced at ~10 times lower titers, and S267V and S668V, which produced no detectable levels of DNAse I-resistant vector particles. The titers of S468V and S384V mutants were ~3-5 times higher than the WT AAV2 vectors. Each of the S-V mutant vectors was evaluated for transduction efficiency in

HEK293 cells. These results, shown in FIG. 20, indicate that of the 15 mutants, the S662V mutant transduced HEK293 cells ~20-fold more efficiently than its WT counterpart did. The transduction efficiency of the S458V and the S492V mutant vectors was increased by ~4- and 2-fold, respectively. The positions of these three critical surface exposed serine residues on the AAV2 capsid are shown in FIG. 21A and FIG. 21B. No further increase in transduction efficiency was observed with the double-mutants (S458+662V and S492+662V), or the triple-mutant (S458+492+662V), indicating that unlike some of the tyrosine-mutants, combining multiple mutations in the serine residues was neither additive nor synergistic. Interestingly, the transduction efficiency of the S468V and the S384V mutants, which were produced at titers higher than the WT AAV2 vectors, remained unchanged (S468V) or were reduced ~10-fold (S384V) at the same multiplicity of infection (MOI). These data are summarized in FIG. 34.

[0279] Substitution of S662 with Different Amino Acids has Diverse Effects on AAV2 Capsid Assembly and AAV2 Vector-Mediated Transgene Expression. In addition to S-to-V substitution at position 662, the following 7 mutants with different amino acids were also generated: S662→Alanine (A), S662→Asparagine (N), S662→Aspartic acid (D), S662→Histidine (H), S662→Isoleucine (I), S662→Leucine (L), and S662→Phenylalanine (F), and evaluated their transduction efficiency in 293 cells. These results, shown in FIG. 22 and summarized in FIG. 35, demonstrate that the substitution of S with V led to the production of the most efficient mutant without any change in vector titers. Replacement of S with N, I, L, or F decreased the packaging efficiency ~10-fold with no significant effect on the transduction efficiency, whereas substitution with D or H increased the transduction efficiency ~8-fold and ~4-fold, respectively, with no effect on vector titers. Interestingly, substitution of S to A increased the viral titer up to ~5-fold, and enhanced the transgene expression ~3-fold compared with the WT AAV2 vector. The observed variability in titers and infectivity of the serine-mutants at position 662 suggests the critical role each of the amino acids plays in modulating both AAV2 packaging efficiency and biological activity.

[0280] ***Transduction Efficiency of S662V Vectors Correlate with p38 MAPK Activity.*** Since all of the S662V vector-mediated transgene expression data thus far were derived using 293 cells, these studies were extended to include the following cells types: (i) NIH3T3 (mouse embryonic fibroblasts), (ii) H2.35 (mouse fetal hepatocytes), (iii) HeLa (human cervical cancer cells), and (iv) primary human monocyte-derived dendritic cells (moDCs). These cell types were transduced with WT scAAV2-EGFP or S662V scAAV2-EGFP vectors at an MOI of 2,000 vgs per cell under identical conditions. EGFP gene expression was evaluated 48 hrs post-infection (p.i.) for HeLa, 293 and moDCs, and 5 days p.i. for H2.35 and NIH3T3 cells. These results are shown in FIG. 23A. As can be seen, although the absolute differences in the transduction efficiency between WT and S662V mutant vectors ranged from ~3-fold (in H2.35 cells) to ~20-fold (in 293 cells) the mutant vector was consistently more efficient in each cell type tested. Since pre-treatment of cells with an inhibitor of cellular p38 MAPK was the most effective in increasing the transduction efficiency (FIG. 19A and

FIG. 19B), the inventors examined whether or not the observed differences in the transduction efficiency of the WT and the mutant vectors was due to variations in the levels of expression and/or activity of the cellular p38 MAPK. Cell lysates prepared from each cell type were analyzed on Western blots probed with specific antibodies to detect both total p38 MAPK and phospho-p38 MAPK levels. GAPDH was used as a loading control. These results, shown in FIG. 23B, indicate that whereas the p38 MAPK protein levels were similar, the kinase activity, as determined by the level of phosphorylation, varied significantly among different cell types, and the transduction efficiency of the S662V mutant vector correlated roughly with the p38 MAPK activity. These approximate correlations between p38 MAPK activity and the efficiency of the S662V mutant vector can probably be explained by different cell susceptibilites for AAV infection, the overall number of viral particles entered cell after primary infection. It remains unclear as to which precise steps in the life cycle of AAV are modulated by p38 MAPK-mediated phosphorylation. It is also possible that other serine/threonine kinases contributing to the difference in efficiency of transduction by S662V and WT vectors. Interestingly, however, transduction by the WT-AAV2 vectors did not lead to up regulation of phosphorylation of p38 MAPK in 293 cells or in moDC, further supporting a previous report that AAV does not induce robust phenotypic changes in moDCs (Markusic *et al.*, 2011).

[0281] ***S662V Vector-Mediated Transduction of Primary Human moDCs Does Not Lead to Phenotypic Alterations.*** MAPK family members play important roles in the development and maturation of APCs. moDCs, isolated from healthy donor leukapheresis, were treated with 50 μM selective kinase inhibitors as described above and then transduced with WT scAAV2-EGFP vectors. Two hrs p.i., cells were treated with supplements (TNF-α, IL-1β, Il-6, PGE2) to induce maturation. EGFP transgene expression was evaluated 48 hrs p.i. by fluorescence microscopy. Pre-treatment of moDCs with specific inhibitors of JNK and p38 MAPK increased EGFP expression levels ~2-fold and ~3-fold, respectively, and the transduction efficiency was enhanced by ~5-fold with the S662V mutant vectors (FIG. 24). Since inhibition of these kinases has previously been reported to prevent maturation of dendritic cells (Beisleve *et al.*, 2005; Nakahara *et al.*, 2006; Nakahara *et al.*, 2004; Harley, 2008), the capability of S662V mutant to induce phenotypic changes in DCs also was evaluated. moDC were infected with an increasingly higher MOI up to 50,000 vgs per cell, harvested at 48 hrs p.i., and analyzed by fluorescence-activated cell sorting (FACS) for up regulation of surface co-stimulatory molecules. Flow cytometric analyses of DC maturation markers such as CD80, CD83 and CD86 indicated that, similar to WT AAV2 vectors, the S662V mutant vectors also did not induce the maturation of moDCs (FIG. 24C). This observation supports the previously described low immunogenicity of AAV vectors. (Shin *et al.*, 2008; Jayandharan *et al.*, 2011)

[0282] ***hTERT-Specific CTL Generation by moDC Transduced with AAV2-S662V Vectors.*** Since the serine-mutant AAV2 vector-mediated transgene expression in moDC was significantly improved compared with the WT-AAV2 vectors, the ability of S662V-loaded moDCs to stimulate the generation of cytotoxic T-lymphocytes and effect specific killing of the target cell was examined.

Given that human telomerase is recognized as a unique anti-cancer target (Harley, 2008; Beatty and Vonderheide, 2008) commonly expressed in most cancer cells, a truncated human telomerase (hTERT) gene was cloned under the control of the chicken β-actin promoter and packaged the DNA into the AAV2 S662V mutant. Non-adherent peripheral blood mononuclear cells (PBMC) containing up to 25% of CD8 positive cells were stimulated once with moDC/hTERT delivered by the S662V vector. An immortalized myelogenous leukemia cell line, K562, was used for a two-color fluorescence assay of cell-mediated cytotoxicity to generate a killing curve with subsequently reduced effector to target cell ratio. Result of these experiments, shown in FIG. 25, suggest that moDC loaded with hTERT can effectively stimulate specific T cell clone proliferation and killing activity compared with moDC expressing GFP. Thus, since immunization strategies that generate rapid and potent effector responses are essential for effective immunotherapy, these results support the efficacy of AAV-based delivery methods for vaccination studies.

DISCUSSION

[0283] Although the possibility of genetically-modified dendritic cells stimulating a specific anti-tumor cytotoxic T cell response has been proven in a number of clinical trials, a reliable method for therapeutic antigen loading, control of expression, and antigen presentation has not yet been previously developed (O'Neill and Bhardwaj, 2007; Tacken *et al.*, 2007). Since the first attempts to transduce dendritic cells with conventional ssAAV vectors nearly a decade ago (Pannazhagan *et al.*, 2001), significant progress has been made in increasing the transduction efficiency of these vectors. For example, the development of self-complementary AAV (scAAV) vectors has circumvented a major rate-limiting step of viral second-strand DNA synthesis, which dramatically increases transgene expression levels in different subsets of dendritic cells. (Shin *et al.*, 2008; Aldrich *et al.*, 2006; Wang *et al.*, 2003) AAV vector-based antigen delivery to dendritic cells has successfully been utilized for several cancer models. (Mahadevan *et al.*, 2007; Eisold *et al.*, 2007; Yu *et al.*, 2008)

[0284] The natural flexibility of AAV structural and regulatory viral components promotes rapid molecular evolution and formation of numerous serologically distinct serotypes (Gao *et al.*, 2003; Vandenberghe *et al.*, 2009; Wu *et al.*, 2006). Several studies have shown that one can take advantage of such plasticity of AAV to generate new vectors with different cell and tissue tropism (Wu *et al.*, 2000; Girod *et al.*, 1999). Other studies revealed that substitution of a single amino acid on the viral capsid can strongly affect viral titer, interaction with cellular receptor, tissue-tropism and trafficking from endosome to the nucleolus (Zhong *et al.*, 2008; Wu *et al.*, 2006). Wu *et al.* (2006) have reported that replacement of lysine to glutamine at position 531 (K531E) on AAV6 capsid reduces gene transfer to mouse hepatocytes *in vivo* and affinity for heparin. The reverse mutation (E531K) on AAV1 capsid increased liver transduction and imparted heparin binding.

[0285] Data with AAV2 serotype vectors indicate that a single substitution of tyrosine to phenylalanine (Y→F) dramatically improves viral trafficking from endosome to the nucleolus by preventing capsid phosphorylation, subsequent ubiquitination and degradation via proteasome (Zhong

et al., 2008). These studies have led to the generation of a number of vectors with increased transduction efficiency in different cell types and tissues. Such vectors were used to improve F.IX gene transfer to murine hepatocytes for the phenotypic correction of hemophilia B (Markusic *et al.*, 2011). These tyrosine-mutant AAV vectors also led to high efficiency transduction of mouse retina for the potential treatment of ocular diseases (Petrs-Zilva *et al.*, 2009). Although AAV6 serotype has shown higher transduction efficiency than AAV2 in dendritic cells (Veron *et al.*, 2007; Taylor and Ussher, 2010), these studies have focused on AAV2 because these vectors have been studied more extensively in both basic research and clinical settings, however AAV6 vectors may be developed with a similar strategy as described herein.

[0286] It has become abundantly clear that phosphorylation of surface-exposed tyrosine-residues on AAV2 capsids negatively impacts the transduction efficiency of these vectors, which can be dramatically augmented by the use of specific inhibitors of cellular EGFR-PTK, known to phosphorylate these residues (Zhong *et al.*, 2008). In the present example, the role of phosphorylation of serine residues in the life cycle of AAV2 vectors was more fully delineated.

[0287] Indeed, the transduction efficiency of both ssAAV and scAAV vectors could be augmented by pre-treatment of cells with specific inhibitors of JNK and p38 MAPK, implying that one or more surface-exposed serine and/threonine residues on the AAV2 capsid becomes phosphorylated inside the host cell and that this modification is detrimental to capsid trafficking to the nucleus.

[0288] Next, each of 15 surface-exposed serine residues was mutated individually, but only three of these mutations led to an increase in transduction efficiency in different cell types, which ranged from ~2-fold to ~20-fold. However, unlike the tyrosine-mutants (Markusic *et al.*, 2011), combining multiple mutations did not augment the transduction efficiency of either the double-mutants (S458+662V and S492+662V), or the triple-mutant (S458+492+662V) AAV2 vectors *in vitro*. In this context, it is noteworthy that in a report by DiPrimio *et al.*, (DiPrimio *et al.*, 2008), in which the HI loop located between the H and I strands of the conserved core β-barrel and contains residue S662 was characterized, both site-directed mutagenesis and peptide substitutions showed that this capsid region plays a crucial role in AAV capsid assembly and viral genome packaging (FIG. 22A and FIG. 22B) (Xie *et al.*, 2002). Although the S662 residue was not specifically targeted in those studies, the transduction efficiency of most of these mutants was either unchanged, or was reduced by up to 27-fold. The HI loop, which forms interactions between icosahedral five-fold symmetry related VPs and lies on the floor of the depression surrounding this axis, was also proposed to undergo a conformational re-arrangement that opens up the channel located at the icosahedral fivefold axis following heparin binding by AAV2 (Levy *et al.*, 2009). Residues S458 and 492 are located adjacent to each other (contributed from symmetry related VPs) on the outer surface of the protrusions (surrounding the icosahedral three-fold axes) facing the depression at the two-fold axes. Previous mutation of residues adjacent to S458A, S492A and S492T had no effect on capsid assembly and

resulted in no effect on transduction efficiency (Lochrie *et al.*, 2006), which confirms the critical role that particular amino acids plays in packaging efficiency and biological activity of AAV. Additional structural analyses of these data revealed the following: For the three mutants with low yields, the side-chain of the residues interact with main-chain atoms from the same VP monomer, and S267V with a low titer, interacts with D269 from the same monomer. For another capsid mutant, S668V, which is located in the HI loop and shown to play a role in capsid assembly (DiPrimio *et al.*, 2008), no obvious disruption of interaction was observed with the substitution. Interestingly, all of these residues, regardless of assembly phenotype, are at interface positions but only 458 and 492 involved in inter-VP interactions. The other residues are only involved in intra-VP interactions, if any. Thus, it is possible that the changes in the no capsid or low capsid yield mutants result in misfolding for their VPs or the abrogation of formation of multimers formation required for assembly when changed to alanine.

[0289] In the setting of tumor immunotherapy, the time of T cell activation and the potency and longevity of CD8 T cell responses are crucial factors in determining therapeutic outcome. Thus, the investors further evaluated whether increased transduction efficiency of moDC by the serine-mutant AAV2 vectors correlated with superior priming of T cells. Human telomerase was used as a specific target since it has been shown in numerous studies and clinical trials to be an attractive candidate for a broadly expressed rejection antigen for many cancer patients (Harley, 2008; Beatty and Vonderheide, 2008). These results suggest that modification of the AAV2 capsid might be beneficial in terms of producing more specific and effective vectors for gene delivery.

[0290] It is also important that one of the main obstacles, the induction of immuno-competition in cellular immune responses against vector-derived and transgene-derived epitopes, can probably be overcome not only by the replication-deficiency and lack of viral proteins expressed by recombinant AAV2, but also the fact that less capsid of modified viral particles will be degraded by host proteosomes and thus, provide less material for presentation.

EXAMPLE 5 -- OPTIMIZATION OF THE CAPSID OF RAAV2 VECTORS

[0291] Adeno-associated virus (AAV) vectors are currently in use in a number of Phase I/II clinical trials as delivery vehicles to target a variety of tissues to achieve sustained expression of therapeutic genes (Daya and Berns 2008; Mueller and Flotte 2008; Srivastava 2008; Asokan *et al.*, 2012; Flotte *et al.*, 2012). However, large vector doses are needed to achieve therapeutic benefits. The requirements for sufficient amounts of the vector pose a production challenge, as well as the risk of initiating the host immune response to the vector (High and Aubourg, 2011; Mendell *et al.*, 2012, Mingozzi and High, 2011). More specifically, recombinant vectors based on AAV2 serotype were initially used in a clinical trial for the potential gene therapy of hemophilia B, but in this trial, therapeutic level of expression of human Factor IX (hF.IX) was not achieved at lower vector doses, and at higher vector doses, the therapeutic level of expression of hF.IX was short-lived due to a

cytotoxic T cell (CTL) response against AAV2 capsids (Manno *et al.*, 2006; Mingozzi and High, 2007; Mingozzi *et al.*, 2007).

[0292] In a more recent trial with recombinant vectors based on AAV8 serotype, therapeutic levels of expression of hF.IX were been achieved, but an immune response to AAV8 capsid proteins was observed (Aslanidi *et al.*, 2012). Thus, it is critical to develop novel AAV vectors with high transduction efficiency that can be used at lower doses. Cellular epidermal growth factor receptor protein tyrosine kinase (EGFR-PTK) negatively affects transgene expression from recombinant AAV2 vectors primarily due to phosphorylation of AAV2 capsids at tyrosine residues, and tyrosine-phosphorylated capsids are subsequently degraded by the host proteasome machinery (Zhong *et al.*, 2008; Markusic *et al.*, 2010). Selective inhibitors of JNK and p38 MAPK serine/threonine kinases also improved the transduction efficiency of AAV2 vectors, suggesting that phosphorylation of certain surface-exposed serine and/or threonine residues might also decrease the transduction efficiency of these vectors. These studies led to the development of tyrosine- and serine-mutant AAV2 vectors, which has been shown to transduce various cell types with significantly higher efficiency than the WT vectors. (Aslanidi *et al.*, 2012; Zhong *et al.*, 2008; Markusic *et al.*, 2010; Petrs-Silva *et al.*, 2009) In addition to the tyrosine and serine residues, the elimination of surface-exposed threonine residues by site-directed mutagenesis also led to an increase in the transduction efficiency at lower vector doses. In this example, each of the 17 surface-exposed threonine residues was substituted with valine (V) residues by site-directed mutagenesis, and four of these mutants, T455V, T491V, T550V, T659V, were shown to increase the transduction efficiency between ~2-4-fold in human HEK293 cells. Because the tyrosine triple-mutant (Y730F+500+444F) vector transduced murine hepatocytes most efficiently than WT (Aslanidi *et al.*, 2012; Zhong *et al.*, 2008; Markusic *et al.*, 2010; Petrs-Silva *et al.*, 2009), these mutations were subsequently combined with the best-performing single serine-mutant (S662V) and single threonine-mutant (T491V) to generate the following vectors: two quadruple (Y444+500+730F+S662V; Y730+500+44F+T491V) and one quintuple (Y444+500+730F+S662V+T491V). The quadruple-mutant (Y444+500+730F+T491V) vector efficiently transduced a murine hepatocyte cell line *in vitro* as well as primary murine hepatocytes *in vivo* at reduced doses, which implicated the use of these vectors in human gene therapy in general, and hemophilia in particular.

MATERIALS AND METHODS

[0293] ***Cells.*** Human embryonic kidney cell line, HEK293, and murine hepatocyte cell line, H2.35, cells were obtained from the American Type Culture Collection (Manassas, VA, USA), and maintained as monolayer cultures in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (FBS; Sigma) and antibiotics (Lonza).

[0294] ***Production of Recombinant Vectors.*** Recombinant AAV2 vectors containing either EGFP (scAAV2-GFP) or firefly luciferase gene (Fluc) (ssAAV2-Fluc) driven by the chicken β-actin promoter (CBA) were generated as described previously (Aslanidi *et al.*, 2012; Aslanidi *et al.*, 2009;

Zolotukhin *et al.*, 2002; Kohlbrenner *et al.*, 2005). Briefly, HEK293 cells were transfected using polyethylenimine (PEI, linear, MW 25,000, Polysciences, Inc.). Seventy-two hrs' post-transfection, cells were harvested and vectors were purified by iodixanol (Sigma) gradient centrifugation and ion exchange column chromatography (HiTrap Sp Hp 5 mL, GE Healthcare). Virus was then concentrated and buffer exchanged into Lactated Ringer's solution in three cycles using centrifugal spin concentrators (Apollo, 150-kDa cut-off, 20-mL capacity, CLP). To determine genome titers, ten µl of purified virus were incubated with DNase I (Invitrogen) at 37°C for 2 hr, then with Proteinase K (Invitrogen) at 55°C for an additional 2 hr. The reaction mixture was purified by phenol/chloroform, followed by chloroform extraction. Packaged DNA was precipitated O/N with ethanol in the presence of 20 µg glycogen (Invitrogen). DNase I-resistant AAV2 particle titers were determined by qPCR with the following primer-pairs specific for the CBA promoter:

Forward: 5′-TCCCATAGTAACGCCAATAGG-3′ (SEQ ID NO:20),

Reverse: 5′-CTTGGCATATGATACACTTGATG-3′ (SEQ ID NO: 21),

and SYBR GreenER PCR Master Mix (Invitrogen) (Aslanidi *et al.*, 2012; Aslanidi *et al.*, 2009).

[0295] ***Site-Directed Mutagenesis.*** A two-stage PCR was performed with plasmid pACG2 as described previously (Aslanidi *et al.*, 2012; Wang and Malcolm, 1999) using Turbo *Pfu* Polymerase (Stratagene). Briefly, in stage one, two PCR extension reactions were performed in separate tubes for the forward and reverse PCR primers for 3 cycles. In stage two, the two reactions were mixed and a PCR reaction was performed for an additional 15 cycles, followed by *Dpn*I digestion for 1 hr. Primers were designed to introduce changes from threonine (ACA) to valine (GTA) for each of the residues mutated.

[0296] ***Recombinant AAV Vector Transduction Assays*** **In Vitro.** Human HEK293 were transduced with 1×10^3 vgs/cell, and murine hepatocytes H2.35 cells were transduced with 2×10^3 vgs/cell with WT and mutant scAAV2-GFP vectors, respectively, and incubated for 48 hr. Transgene expression was assessed as the total area of green fluorescence (pixel2) per visual field (mean ± SD) as described previously (Aslanidi *et al.*, 2012; Zhong *et al.*, 2008; Markusic *et al.*, 2010). Analysis of variance was used to compare test results and the control, which were determined to be statistically significant.

[0297] ***Analysis of Vector Genome Distribution in Cytoplasm and Nuclear Fractions.*** Approximately 1×10^6 H2.35 cells were infected by either WT or mutant scAAV2-GFP vectors with MOI 1×10^4 vgs/cell. Cells were collected at various time points by trypsin treatment to remove any adsorbed and un-adsorbed viral particles and then washed extensively with PBS. Nuclear and cytoplasmic fractions were separated with Nuclear and Cytoplasmic Extraction Reagents kit (Thermo Scientific) according to manufacturer instruction. Viral genome was extracted and detected by qPCR analysis with the CBA specific primers described above. The difference in amount of viral genome between cytoplasmic and nuclear fractions was determined by the following rule: C_T values for each

sample from cells treated with virus were normalized to corresponding C_T from mock treated cells (ΔC_T). For each pairwise set of samples, fold change in packaged genome presence was calculated as fold change = $2^{-(\Delta CT\text{-cytoplasm} - \Delta CT\text{-nucleus})}$. Data from three independent experiments were presented as a percentage of the total amount of packaged genome in the nuclear and cytoplasmic fractions.

[0298] **In Vivo *Bioluminescence Imaging.*** All animal experiments were performed per institutional policies, and all procedures were done in accordance with the principles of the National Research Council's Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize suffering. Ten-week-old C57BL/6 male mice (Jackson Laboratory, Bar Harbor, ME) were injected intravenously with 1×10^{10} vgs/animal of WT and mutant ssAAV2-Fluc vectors (n = 3). Luciferase activity was analyzed two weeks post injection using a Xenogen IVIS Lumina System (Caliper Life Sciences). Briefly, mice were anesthetized with 2% isofluorane and injected intraperitoneally with luciferin substrate (Beetle luciferin, Caliper Life Sciences) at a dose of 150 μg/g of body weight. Mice were placed in a light-tight chamber and images were collected at 5 min after the substrate injection. Images were analyzed by the Living Image 3.2 software (Caliper Life Sciences) to determine relative signal intensity.

[0299] ***Visualization of the Position of the Mutant Residues on the AAV2 Capsid.*** The atomic coordinates for the AAV2 VP3 crystal structure (residues 217 to 735, VP1 numbering) (Protein Data Bank (PDB) accession no. 1lp3; [Xie *et al.*, 2002]) was downloaded and used to generate a complete capsid model using the Oligomer generator application in VIPERdb (Carrillo-Trip *et al.*, 2009). This generates 60 VP3 copies for creating the T = 1 icosahedral capsid via matrix multiplication. The structure was viewed with the program COOT (Xie *et al.*, 2002) and figures were generated using either of the computer programs, PyMOL (Schrodinger, LLC) and RIVEM (Xiao and Rossman, 2007).

[0300] ***Statistical Analysis.*** Results are presented as mean ± S.D. Differences between groups were identified using a grouped-unpaired two-tailed distribution of Student's *t*-test. *P*-values < 0.05 were considered statistically significant.

RESULTS

[0301] ***Site-Directed Mutagenesis of Surface-Exposed Threonine Residues on AAV2 Capsid.*** The AAV2 capsid contains 45 threonine (T) residues in the capsid viral protein 3 (VP3) common region of the three capsid VPs, VP1, VP2, and VP3. Seventeen of these (251, 329, 330, 454, 455, 503, 550, 592, 581, 597, 491, 671, 659, 660, 701, 713, 716) are surface-exposed. (Xie *et al.*, 2002) Each of the 17 T residues was substituted with valine (V) by site-directed mutagenesis as described previously (Aslanidi *et al.*, 2012; Zhong *et al.*, 2008). Most mutants could be generated at titers similar to the WT AAV2 vectors, with the exception of T329V and T330V that were produced at ~10-fold lower titers, and T713V and T716V, which produced no detectable levels of DNase I-resistant vector particles. Each of the T-V mutant vectors was evaluated for transduction efficiency in HEK293 cells. These results, shown in FIG. 26A and FIG. 26B, indicate that of the 17 mutants, the T491V mutant

transduced HEK293 cells ~4-fold more efficiently than its WT counterpart did. The transduction efficiency of the T455V, T550V, T659V mutant vectors were increased by ~2-fold. These data indicated that phosphorylation of specific tyrosine, serine, and threonine residues on AAV2 capsid by cellular kinases is a critical determinant of the transduction efficiency of these vectors.

[0302] ***Multiple Mutations of Surface-Exposed Threonine Residues Further Improve Transduction Efficiency of AAV2 Vectors.*** To evaluate whether the transduction efficiency of the threonine-mutant AAV2 vectors could be enhanced further, the following multiple-mutant vectors were generated: three double-mutants (T455+491V; T550+491V; T659+491V), two triple-mutants (T455+491+550V; T491+550+659V), and one quadruple-mutant (T455+491+550+659V). Each of the multiple-mutant vectors packaged genome titers similar to the WT AAV2 vectors. In side-by-side comparisons, each of the multiple-mutant vectors was shown to transduce HEK293 more efficiently than the WT and the single-threonine mutant AAV2 vectors (FIG. 27A and FIG. 27B). The best performing vector was identified to be the triple-mutant (T491+550+659V), with the transduction efficiency ~10-fold higher than the WT vector, and ~3-fold higher than the best single-mutant (T491V) vector. These data confirmed that combining several threonine-mutations on a single viral capsid led to a synergetic effect in augmenting the transduction efficiency.

[0303] ***Optimized Threonine-Mutant AAV2 Vectors Efficiently Transduce Murine Hepatocytes* in Vitro.** The tyrosine triple-mutant (Y444+550+730F) vector described in previous examples has been shown to be efficient in transducing murine hepatocytes in a comparison of vectors containing up to 7 surface tyrosine to phenylalanine changes (Markusic *et al.* 2010; Jayandharan *et al.*, 2011). Thus, it was of interest to evaluate whether combining the best performing single-serine (S662V) and single-threonine (T491V) mutations with the triple-tyrosine mutant could further increase the transduction efficiency of these vectors to produce even further improved expression vectors in accordance with the methods described herein.

[0304] To that end, several multiple-mutants were generated as follows: two quadruple (Y444+500+730F+T491V; Y444+500+730F+S662V), and one quintuple (Y444+500+730F+T491V+S662V) mutant vectors. Comparison of the transduction efficiency of these mutants with the WT and the tyrosine triple-mutant AAV2 vectors in H2.35 cells showed that the expression level from the Y444+500+730F+T491V mutant was ~2-3-fold higher than for the tyrosine triple-mutant AAV2 vector, and ~24-fold higher than the WT AAV2 vector (FIG. 28A and FIG. 28B). Interestingly, combining the S662V mutation with the tyrosine triple-mutant vector, or with the tyrosine-threonine quadruple-mutant vector, negatively affected their transduction efficiency. Addition of several other threonine mutations, such as T550V and T659V, also did not augment the transduction efficiency of the Y444+500+730F+T491V quadruple-mutant AAV2 vector. Additional studies are warranted to gain a better understanding of the complex interactions among these surface-exposed Y, S, and T residues as well as their phosphorylation status.

[0305] ***Multiple-Mutations Enhance Intracellular Trafficking and Nuclear Translocation of AAV2 Vectors.*** Prevention of phosphorylation of surface-exposed tyrosine residues on the AAV2 capsid improved intracellular trafficking of tyrosine-mutant vectors and increases the number of the viral genomes translocated to the nucleus (Zhong *et al.*, 2008; Zhong *et al.*, 2008). In this example, the addition of the T491V mutant to the tyrosine triple-mutant vector was assigned for its ability to augment this transduction efficiency by further increasing nuclear transport of these vectors. To this end, the kinetics of transgene expression in H2.35 cells mediated by the Y444+500+730F+T491V quadruple-mutant were evaluated and compared to the Y444+500+730F triple-mutant and the WT AAV2 vectors. These results are shown in FIG. 29A and FIG. 29B. As can be seen, EGFP expression from the tyrosine-threonine quadruple-mutant vector was ~2-3fold higher at each tested time point, and could be detected as early as 16 hr post-infection. These results suggested that the early-onset of transgene expression from the quadruple-mutant vectors could be due to more efficient nuclear transport of these vectors. To test this possibility experimentally, qPCR analysis was used to quantitate the vector genomes in cytoplasmic and nuclear fractions of H2.35 cells infected with the WT and the two mutant AAV2 vectors at different time points. The vector genome ratios in the two cellular fractions are shown in FIG. 30A and FIG. 30B. Whereas ~20% of the genomes from the WT AAV2 vectors, and ~45% of the genomes from the triple-mutant vectors were detected in the nuclear fraction 16 hr post-infection, more than 70% of the vector genomes from the quadruple-mutant were detected at the same time-point. Similarly, only ~45% of the genomes from the WT AAV2 vectors were detected in the nuclear fraction 48 hr post-infection, ~80% of the genomes from the triple-mutant vectors, and ~90% of the vector genomes from the quadruple-mutant were detected in the nuclear fraction at the same time-point. Thus, these data corroborated the hypothesis that combining the threonine (T491V) mutation with the tyrosine triple-mutant (Y444+500+730F) vector leads to a modest improvement in the nuclear translocation of these vectors, which correlated with a faster onset of gene expression and the observed improvement in the transduction efficiency.

[0306] ***Optimized AAV2 Vectors are Highly Efficient in Transducing Murine Hepatocytes* in Vivo.** The transduction efficiency of the optimized AAV2 vectors was evaluated in a murine model *in vivo*. Each of multiple-mutant vectors was packaged with a single-stranded firefly luciferase (Fluc) AAV2 genome, and ~1×10^{10} vgs of each vectors were injected intravenously into C57BL/6 mice (n = 3 for each group). Levels of expression of Fluc gene, assessed two weeks post-injection by bioluminescence imaging, showed that expression from the Y444+500+730F+T491V quadruple-mutant vector was ~3-fold higher than that from the tyrosine triple-mutant vector. One representative animal from each group and the quantification of these data are presented in FIG. 31A and FIG. 31B. Consistent with the data obtained *in vitro*, the addition of S662V mutation had a negative effect on the transduction efficiency of both the tyrosine-triple-mutant and the tyrosine-threonine quadruple-mutant vectors. Exemplary single and multiple-mutation capsid proteins of the present invention include, but are not limited to, those illustrated in Table 5:

TABLE 5

SUMMARY OF EXEMPLARY MUTATIONS OF SURFACE-EXPOSED TYROSINE (Y), SERINE (S), AND THREONINE (T) RESIDUES ON THE AAV2 CAPSID

Single Mutations	Double Mutations	Triple Mutations	Multiple Mutations
Y252F	Y252F+Y730F	Y444+500+730F	Y272+444+500+730F
Y272F	Y272F+Y730F	Y730F+S662V+T491V	Y272+444+500+730F
Y444F	Y444F+Y730F	S458+492+662V	Y272+444+500+730F
Y500F	Y500F+Y730F	T455+550+491V	Y272+444+500+700+730F
Y700F	Y700F+Y730F	T550+659+491V	Y272+444+500+704+730F
Y704F	Y704F+Y730F		Y252+272+444+500+704+730F
Y730F	Y444F+T550F		Y272+444+500+700+704+730F
S261V	S458V+S492V		Y252+272+444+500+700+704+730F
S264V	S458V+S662V		Y444+500+730F+T491V
S267V	S492V+S662V		Y444+500+730F+S458V
S276V	T455+T491V		Y444+500+730F+S662V+T491V
S384V	T550+T491V		Y444+500+730F+T550+T491V
S458V	T659+T491V		Y444+500+730F+T659+T491V
S468V	T671+T491V		
S492V	Y730F+T491V		
S498V	S662V+T491V		
S578V	Y730F+S662V		
S658V			
S662V			
S662A			
S662D			
S662F			
S662H			
S662N			
S662L			
S662I			
S668V			
S707V			
S721V			
T251V			
T329V			
T330V			
T454V			
T455V			
T491V			
T503V			
T550V			

T592V
T597V
T581V
T671V
T659V
T660V
T701V
T713V
T716V

The first letter corresponds to the amino acid in the wild-type AAV2 capsid, the number is the VP3 amino acid position that was mutated, and the last letter is the mutant amino acid.

DISCUSSION

[0307] Recombinant AAV-based vectors are attractive delivery vehicles for gene replacement therapy as a potential treatment for a variety of genetic disorders. Although AAV vectors have been used successfully in many animal models, and recently shown efficacy in several clinical trials, a number of steps in the life cycle of AAV continue to appear to limit the effectiveness of these vectors in gene therapy. Some of these steps include intracellular trafficking, nuclear transport, uncoating, and viral second-strand DNA synthesis (Ding *et al.*, 2005; Harbison *et al.*, 2005; Nonnenmacher and Weber, 2012).

[0308] The simple organization and natural plasticity of AAV structural and regulatory components provide a unique opportunity to manipulate the viral capsid and the genome to develop customized recombinant vectors with distinctive features. Significant progress has been made in the past decade to improve the specificity and the transduction efficiency of recombinant AAV vectors. For example, specific mutations in the viral inverted terminal repeat (ITR) sequences have led to development of self-complementary AAV (scAAV) vectors, which overcome the rate-limiting step of viral second-strand DNA synthesis, and dramatically increase transgene expression levels in various types of the cells and tissues (McCarty *et al.*, 2003; Wang *et al.*, 2003). Additional studies on capsid structure analyses, combined with a wealth of information emanating from mutagenesis studies on the capsid genes, have led to the identification of specific regions which play a critical role in vector encapsidation, tissue-tropism, and intracellular trafficking of these vectors (Lochire *et al.*, 2006; Muzyczka and Warrington, 2005; Wu *et al.*, 2006; Gao *et al.*, 2003; Vandenberghe *et al.*, 2009; Wu *et al.*, 2006).

[0309] In the previous examples, it was shown that substitution of surface-exposed specific tyrosine (Y) and serine (S) residues on AAV2 capsids significantly increased the transduction efficiency of these vectors, both *in vitro* and *in vivo*, presumably by preventing phosphorylation, subsequent ubiquitination, and proteasome-mediated degradation. Since surface-exposed specific threonine (T) residues on AAV2 capsids would likewise be expected to undergo phosphorylation, in this example each of the 17 surface-exposed T residues were systematically mutagenized, and several

single-mutant vectors were identified that could increase the transduction efficiency up to 4-fold. Combinations of multiple T mutations on a single capsid identified modifications that further augmented the transduction efficiency up to ~10-fold, compared with that of the WT AAV2 vector in HEK293 cells.

[0310] Two independent groups have previously reported mutations of specific T residues on AAV2 capsids. For example, Lochrie *et al.*, 2006, targeted the T residues at positions 330, 454, 455, 491, 503, and 550 in a *tour de force* effort to identify surface regions that bind antibodies, and DiPrimio *et al.* (2008), targeted the T residue at position 659 in an effort to identify regions critical for capsid assembly and genome packaging. In both studies, the T residues were substituted with either alanine (A), serine (S), or lysine (K) residues, or by peptide substitution. However, no increase in the transduction efficiency of any of the mutant vectors was observed. In contrast, in the example, the surface-exposed T residues were substituted with valine residues. This further corroborates the recent observation for the critical role played by specific amino acid type in modulating the biological activity of AAV vectors (Aslanidi *et al.*, 2012; Li *et al.*, 2012).

[0311] When the most efficient threonine-mutation (T491V) was combined with a previously reported tyrosine triple-mutation (Y444+500+730F) (Markusic *et al.* 2010) to generate a Y-T quadruple-mutant (Y444+500+730F+T491V) vector, the transduction efficiency of this vector was ~2-3-fold higher than the tyrosine triple-mutant vector in murine hepatocytes, both *in vitro* and *in vivo*. However, combining the most efficient S-mutation (S662V) (Aslanidi *et al.*, 2012) with the tyrosine triple-mutation negatively affected the transduction efficiency of the Y-S quadruple mutant (Y444+500+730F+S662V) vector as well as the Y-S-T pentuple-mutant (Y444+500+730F+S662V+T491V) vector. Although several other combinations showed greater transduction efficiency compared with the WT AAV2 vector, neither combination of similar (quadruple, pentuple or sextuple-tyrosine; and triple and quadruple-threonine mutants), nor combination of the best performing YST mutations reached the level of expression from the triple-tyrosine mutant vector. In view of the large number of combinations of mutations tested, only the mutations that significantly increased the transduction efficiency over the triple-tyrosine mutant vector were characterized in detail here.

[0312] The 17 AAV2 surface-exposed threonine residues are scattered throughout the capsid. Four of the mutations (T329V, T330V, T713V, and T716V) resulted in significant defects in assembly and vector production, and they could not be further characterized. Residues 329 and 330 are located in the α-surface loop (DE loop) located between the βD and βE strands of the core β-barrel of the AAV2 VP3 structure (Xie *et al.*, 2002). Five of these loops, from icosahedral five-fold symmetry related VP3s assembly a channel at this axis which connects the interior and exterior surfaces of the capsid (FIG. 32A). As was observed in a previous study (Bleker *et al.*, 2006), titers for these mutants were significantly reduced consistent with a role for the channel in genome packaging. Residues 713 and 716 are located on the wall/raised capsid region between the deprssions at and

surrounding the icosahedral two- and five-fold axes, respectively (FIG. 32A and FIG. 32B). Their side-chains participate in polar interactions with symmetry related VP3 monomers and it is likely that mutation results in a defect in capsid assembly. A role in capsid assembly for residues located at the icosahedral two-fold axis is consistent with a recent report in which they observe that the AAV2 residues that mediated the interaction with the assembly-activating protein (AAP) were located at this capsid region (Naumer *et al.*, 2012).

[0313] Residues T455, T491, T550, and T659, showing an increased transduction phenotype when mutated to valine or alanine, are located on the protrusions which surround the icosahedral three-fold axis (T455, T491, and T550) or on the HI loop (between βH and βI of the core β-barrel) (T659) which is lies on the depression surrounding the channel at the icosahedral five-fold axis of the AAV2 capsid. The residues on the protrusion, a prominent feature on the capsid assembled from two VP3 monomers, are located close to the top (455), side facing the two-fold depression (491), and side facing the depression surrounding the five-fold (550), respectively, of the protrusions. This AAV region contains the most variability in sequence and structure, and with the exception of residue 659, the other three threonine residues are located to define VP3 variable regions (VRs) (Govindasamy *et al.*, 2006). Along with T659, these residues form a footprint on the capsid surface that extends over the top of the protrusion towards the depression surrounding the icosahedral five-fold axis (FIG. 32A and FIG. 32B). Their surface exposure is consistent with the potential to interact with host molecules, which could include kinases. Interestingly, this footprint is flanked by the residues in the triple-tyrosine mutant, Y444, Y500, and Y730, with T491 located proximal to tyrosine residue Y730 in a depiction of the capsid surface amino acids (FIG. 32B). This residue, which sits in the depression at the icosahedral axis of the capsid, showed the highest increase in transduction compared to WT AAV2 when of the seven surface-exposed tyrosines where mutated to phenylanine residues (Zhong *et al.* 2008). Significantly, the two-fold capsid region is observed to undergo pH-mediated structural transitions when the homologous AAV8 was examined at the conditions encountered during trafficking in the endocytic pathway (Nam *et al.*, 2011). It is possible that the mutations of the AAV2 improve transduction efficiency through altered receptor binding mechanisms. Residues mediating AAV2 and AAV6 interaction with heparan sulfate receptors, R585 and R588, and K531 (structurally equivalent to E530 in AAV2), respectively, are close to this foot (FIG. 26B), and residues 491 and 500, in VRV, are located in one of two large regions on the surface of the AAV2 capsid that has been implicated in binding to the LamR receptor in AAV8 (Akache *et al.*, 2006). Amino acids in VRV also play a role in the AAV9 capsid binding to its glycan receptor, galactose.

[0314] The decreased transduction efficiency phenotype of the mutants containing the S662V mutations is difficult to explain given the location of this residue within the footprint delineated by the residues which enhance transduction when mutated to eliminate potential phosphorylation (FIG. 32A and FIG. 32B). In addition, it has been shown that a mutation of this residue to valine improved transduction relative to WT AAV2 (Aslanidi *et al.*, 2012). Residue S662, like T659, is located in the

HI loop that extends over adjacent five-fold symmetry related VP3 monomers and likely plays a role in stabilizing the pentameric subunits. However, the serine side-chain is not engaged in any inter- or intra-subunit interactions, and while the HI loop has been reported to be a determinant of capsid assembly and genome packaging (DiPrimio *et al.*, 2008), it tolerated single amino acid substitution (Aslanidi *et al.*, 2012). Thus, its effect is likely due to the abrogation of a capsid interaction utilizing the footprint containing the triple-tyrosine mutant residues and T491. Significantly, the phenotypes for mutations in nearby amino acids that make up the HI loop, for example, amino acid residue 664, substituting either serine (mut45subSer14) or a FLAG epitope (mut45SubFLAG10), were non-infectious or not assembled into viral capsid (Wu *et al.*, 2000). However, an HA insertion at the same position produced capsids that were partially defective, yet still bound heparin (Wu *et al.*, 2000).

[0315] Whereas only ~45% of the vector genomes delivered by the WT AAV2 vectors were present in the nucleus at 48 h post infection, >90% of the vector genomes delivered by the Y-T quadruple-mutant vector were present at the same time point. This indicates improved trafficking kinetics for the mutant that would be consistent with reduced re-direction to the proteasome. The modest (~2-3-fold) increase in the transduction efficiency of these vectors compared to the tyrosine triple-mutant vectors is also consistent with the ~10% increase in nuclear vector genome delivery, *i.e.* ~90% compared to ~80%.

[0316] The various combinations of surface tyrosine, serine, and threonine modifications clearly showed that there is an optimal combination to achieve maximal augmentation. These studies also highlighted the requirement for specific residue types in AAV interactions during infection and for enhancing transduction. It is possible that the individual mutations, which did not show a significant increase in the transduction efficiency as single changes, can form superior vectors when combined in a single capsid.

TABLE 6

COMPARISON OF TYROSINE RESIDUES IN AAV SEROTYPES

(Surface exposed residues are shown with an "*" following their amino acid position)

AAV1	AAV2	AAV3	AAV4	AAV5	AAV6	AAV7	AAV8	AAV9	AAV10	AAV11	AAV12
Y6	Y6	Y6	Y5	NA	Y6	Y6	Y6	Y6	Y6	Y6	Y6
Y50	Y50	Y50	Y49	Y49	Y50	Y50	Y50	Y50	Y50	Y50	Y50
Y52	Y52	Y52	Y51	Y51	Y52	Y52	Y52	Y52	Y52	Y52	Y52
Y79	Y79	Y79	Y78	Y78	Y79	Y79	Y79	Y79	Y79	Y79	Y79
Y90	Y90	Y90	Y89	Y89	Y90	Y90	Y90	Y90	Y90	Y90	Y90
Y93	Y93	Y93	Y92	Y92	Y93	Y93	Y93	Y93	Y93	Y93	Y93
Y252	Y252*	Y252*	Y246*	Y242*	Y252*	Y253*	Y253*	Y252*	Y253	Y246*	Y255*
Y257	Y257	Y257	Y251	Y247	Y257	Y258	Y258	Y257	Y258	Y251	Y260
Y273*	Y272*	Y272*	Y263*	Y263*	Y273*	Y274*	Y275*	Y274*	Y275	Y263*	Y272*
Y276	Y275	Y275	NA	Y266	Y276	Y277	Y278	Y277	Y278	NA	NA
Y282	Y281	Y281	Y272	Y272	Y282	Y283	Y284	Y283	Y284	Y272	Y281
NA	NA	NA	NA	Y294	NA	NA	NA	NA	NA	NA	NA
Y349	Y348	Y348	Y339	Y339	Y349	Y350	Y351	Y350	Y351	Y339	Y348
Y353	Y352	Y352	Y343	Y343	Y353	Y354	Y355	Y354	Y355	Y343	Y352
Y376	Y375	Y375	Y366	Y366	Y376	Y377	Y378	Y377	Y378	Y366	Y375
Y378	Y377	Y377	Y368	Y368	Y378	Y379	Y380	Y379	Y380	Y368	Y377
Y394	Y393	Y393	Y387	NA	Y394	Y395	Y396	Y395	Y396	Y386	Y395
Y398	Y397	Y397	Y391	Y390	Y398	Y399	Y400	Y399	Y400	Y390	Y399
Y414	Y413	Y413	Y407	Y406	Y414	Y415	Y416	Y415	Y416	Y406	Y415
Y425	Y424	Y424	Y418	NA	Y425	Y426	Y427	Y426	Y427	Y417	Y426
Y442*	**Y441***	**Y441***	**Y435***	**Y434***	**Y442***	**Y443***	**Y444***	**Y443***	**Y444**	**Y434***	**Y443***
NA	NA	NA	NA	Y436	NA	NA	NA	NA	NA	NA	NA
Y444*	**Y443***	**Y443***	NA	NA	**Y444***	**Y445***	**Y446***	**Y445***	**Y446**	NA	NA
Y445*	**Y444***	**Y444***	NA	NA	**Y445***	**Y446***	**Y447***	**Y446***	**Y447**	NA	NA
NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	Y465
NA	NA	NA	NA	NA	NA	Y466	NA	NA	NA	NA	NA
NA	NA	NA	NA	Y457	NA	NA	NA	NA	NA	NA	NA
NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	Y475
NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	Y467	Y476
NA	NA	NA	NA	Y461	NA	NA	NA	NA	NA	NA	NA
NA	NA	NA	NA	NA	NA	NA	NA	Y478	NA	NA	NA
Y484	Y483	Y484	NA	NA	Y484	NA	Y486	Y484	Y486	NA	NA

NA	NA	NA	**Y491***	NA	NA	NA	NA	NA	NA	**Y490**	**Y499**
NA	**Y500**	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
NA	NA	NA	**Y504***	NA	NA	NA	NA	NA	NA	**Y503**	**Y512**
Y509	Y508	Y509	NA	NA	Y509	Y511	Y511	NA	Y511	Y507	NA
NA	NA	NA	NA	Y502	NA	NA	NA	NA	NA	NA	NA
NA	NA	NA	NA	**Y521***	NA	NA	NA	NA	NA	NA	NA
NA	NA	NA	NA	**Y542***	NA	NA	**Y557***	NA	**Y557**	NA	NA
NA	NA	NA	NA	**Y563***	NA	NA	NA	NA	NA	NA	NA
NA	Y576	Y577	NA	NA	NA	Y578	Y579	Y577	Y579	NA	NA
NA	NA	NA	NA	**Y585***	NA	NA	NA	NA	NA	NA	NA
Y613*	**Y612***	**Y613***	**Y611***	**Y602***	**Y613***	**Y614***	**Y615***	**Y613***	**Y615**	**Y610***	**Y619***
NA	NA	NA	Y612	NA	NA	NA	NA	NA	NA	Y611	Y620
Y674*	**Y673***	**Y674***	**Y672***	**Y662***	**Y674***	**Y675***	**Y676***	**Y674***	**Y676**	**Y671***	**Y680***
Y701	Y700	Y701	NA	Y689*	Y701*	Y702*	Y703*	Y701*	Y703	NA	NA
Y705*	**Y704***	**Y705***	**Y703***	**Y693***	**Y705***	**NA**	**Y707***	**Y705***	**Y707**	**Y702***	**Y711***
NA	NA	NA	NA	NA	NA	NA	Y708	Y706	Y708	NA	NA
Y721	Y720	Y721	Y719	Y709	Y721	Y722	Y723	Y721	Y723	Y717	Y727
Y731*	**Y730***	**Y731***	**Y729***	**Y719***	**Y731***	**Y732***	**Y733***	**Y731***	**Y733**	**Y728***	**NA**

TABLE 7

COMPARISON OF LYSINE RESIDUES IN AAV SEROTYPES

(Surface exposed residues are shown with an "*" following their amino acid position)

AAV 1	AAV 2	AAV 3	AAV 4	AAV 5	AAV 6	AAV 7	AAV 8	AAV 9	AAV 10	AAV 11	AAV 12
NA	K24	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
K26	K26	K26	NA	NA	K26	K26	K26	K26	K26	K26	K26
K31	NA	NA	K30	K30	K31	K31	K31	NA	K31	K31	NA
K33	K33	K33	K32	K32	K33	K33	K33	K33	K33	K33	K33
K38	NA	NA	NA	NA	K38	K38	K38	NA	K38	K38	NA
NA	K39	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
K51	K51	K51	K50	NA	K51	K51	K51	K51	K51	K51	K51
K61	K61	K61	K60	NA	K61	K61	K61	K61	K61	K61	K61
K77	K77	K77	K76	NA	K77	K77	K77	K77	K77	K77	K77
NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	K81
K84	NA	K84	K83	NA	K84	K84	NA	K84	K84	K84	NA
NA	K92	K92	K91	K91	NA	NA	NA	K92	NA	NA	K92
NA	NA	NA	NA	K102	NA	NA	NA	NA	NA	NA	NA
NA	K105	NA	NA	NA	NA	NA	NA	K105	NA	NA	NA
NA	NA	NA	NA	K115	NA	NA	NA	NA	NA	NA	NA
K122	K122	K122	K121	K121	K122	K122	K122	K122	K122	K122	K122
K123	K123	K123	K122	K122	K123	K123	K123	K123	K123	K123	K123
K137	K137	K137	NA	K136	K137	K137	K137	K137	K137	K137	K137
K142	K142	K142	K141	NA	K142	K142	K142	K142	K142	K142	K142
K143	K143	K143	K142	K142	K143	K143	K143	K143	K143	K143	K142
NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	K148
NA	NA	NA	NA	K150	NA	NA	NA	NA	NA	NA	NA
NA	NA	NA	NA	K152	NA	NA	NA	NA	NA	NA	NA
NA	NA	NA	NA	K153	NA	NA	NA	NA	NA	NA	K160
K161	K161	K161	K160	NA	K161	K162	K162	K161	K162	K160	K164
NA	NA	NA	K161	NA	NA	K163	K163	NA	K163	K161	K165
NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	K166
NA	NA	K164	K163	NA	NA	NA	NA	NA	NA	K163	NA
NA	NA	NA	NA	K161	NA	NA	NA	NA	NA	NA	K168
K168	NA	NA	K167	NA	K168	NA	NA	K168	K169	NA	NA
K169	K169	K169	K168	NA	K169	K170	K170	K169	K170	K168	NA

NA	NA	NA	K169	NA	NA	NA	NA	NA	NA	NA	NA
NA	NA	NA	NA	K232 I	NA	NA	NA	NA	NA	NA	NA
K258*	**K258**	**K258***	**K252***	**NA**	**K258***	**K259***	**K259***	**K258***	**K259***	**NA**	**NA**
NA	NA	NA	NA	K251*	NA	NA	NA	NA	NA	NA	NA
K310 I	K309	K309 I	K300 I	NA	K310 I	K311 I	K312 I	K311 I	K312 I	K300 I	K309I
NA	NA	K310 I	NA	NA	NA	K312 I	NA	NA	NA	NA	NA
K315 I	K314	K314 I	K305 I	K305 I	K315 I	K316 I	K317 I	K316 I	K317 I	K305 I	K314I
K322 I	K321	K321 I	K312 I	K312 I	K322 I	K323 I	K324 I	K323 I	K324 I	K312 I	K321I
NA	NA	NA	NA	NA	NA	NA	**K333***	**K332***	**K333***	NA	NA
NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	K384I
NA	NA	NA	NA	K394 I	NA	NA	NA	NA	NA	NA	NA
NA	NA	NA	K411 I	NA	NA	NA	NA	NA	NA	K410 I	K419I
NA	NA	NA	NA	K425 I	NA	NA	NA	NA	NA	NA	NA
NA	NA	NA	NA	NA	NA	NA	NA	**K449***	NA	NA	NA
K459 I	NA	NA	NA	NA	K459 I	NA	NA	NA	NA	NA	NA
NA	NA	NA	NA	NA	NA	NA	NA	K462*	NA	NA	NA
NA	NA	NA	K459 I	K451 I	NA	NA	NA	NA	NA	K458 I	K467I
NA	NA	NA	K469 I	NA	NA	NA	NA	NA	NA	NA	NA
K476 I	NA	NA	K470 I	K462 I	K476 I	K478 I	K478 I	NA	K478 I	K469 I	K478I
NA	NA	NA	K479 I	NA	NA	NA	NA	NA	NA	K478 I	K487I
NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	K490I
K491*	**K490**	**K491***	**K485***	NA	**K491***	**K493***	NA	NA	NA	**K484***	**K490**
K493*	NA	NA	NA	NA	**K493***	NA	NA	NA	NA	NA	NA
NA	NA	NA	**K492***	NA	NA	NA	NA	NA	NA	**K491***	**K493**
NA	NA	NA	**K503***	NA	NA	NA	NA	NA	NA	**K502***	**K511**
K508*	**K507**	**K508***	NA	NA	**K508***	**K510***	**K510***	NA	**K510***	NA	NA
K528*	**K527**	**K528***	NA	NA	**K528***	**K530***	**K530***	**K528***	**K530***	NA	NA
NA	NA	NA	NA	NA	**K531***	NA	NA	NA	NA	NA	NA
K533*	**K532**	**K533***	NA	NA	**K533***	NA	NA	NA	NA	NA	NA
NA	NA	NA	**K532***	NA	NA	NA	NA	NA	NA	NA	NA
K545*	**K544**	**K545***	NA	NA	**K545***	**K547***	**K547***	**K545***	**K547***	NA	NA
NA	NA	NA	**K544***	NA	NA	NA	NA	NA	NA	NA	NA
NA	**K549**	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
NA	NA	NA	NA	NA	NA	**K553***	NA	NA	NA	NA	NA
NA	**K556**	NA	NA	NA	NA	NA	NA	**K557***	NA	NA	NA
K567*-	NA	NA	NA	NA	**K567***	NA	**K569***	**K567***	**K569***	NA	NA

K621 I	K620	K621 I	K619 I	K610 I	K621 I	K622 I	K623 I	K621 I	K623 I	K618 I	K627I
K641 I	K640	K641 I	K639 I	K630 I	K641 I	K642 I	K643 I	K641 I	K643 I	K638 I	K647I
K650 I	K649	K650 I	K648 I	K639 I	K650 I	K651 I	K652 I	K650 I	K652 I	K647 I	K656I
NA	NA	NA	NA	NA	NA	NA	NA	K664 I	NA	NA	NA
K666*	**K665**	**K666***	NA	NA	**K666***	**K667***	**K668***	**K666***	**K668***	NA	NA
NA	NA	NA	NA	K676 I	NA	NA	NA	NA	NA	NA	NA
K689 I	K688	K689 I	K687 I	K677 I	K689 I	K690 I	K691 I	K689 I	K691 I	K686 I	K695I
K693 I	K692	K693 I	K691 I	K681 I	K693 I	K694 I	K695 I	K693 I	K695 I	K690 I	K699I
K707*	**K706**	**K707***	**NA**	**NA**	**K707***	**K708***	**K709***	**K707***	**K709***	NA	NA
NA	NA	NA	K718 I	NA	NA	NA	NA	NA	NA	K717 I	NA

Residues in bold are surface-associated lysines = *

Resides that are located on the interior of the capsid = I

No homologous lysine at that position for that serotype = NA

Residues not visible in the crystal structure of AAVs are shaded in grey; however, biochemical data suggests that these amino acids are located inside the AAV capsid until some point in the virus life cycle when they are then externalized.

EXAMPLE 6 - SUPPRESSION OF HUMAN LIVER TUMORIGENESIS BY AAV3 VECTORS

[0317] Hepatocellular carcinoma (HCC) ranks fifth among solid cancers with ~695,900 deaths worldwide each year (Thomas and Zhu, 2005; Jemal *et al.*, 2011). During the past two decades, the incidence of HCC in the USA has tripled, while the 5-year survival rate has remained below 12% (El-Serag, 2011). It is even worse in Asia and Africa, with an annual incidence of 1 per 3,000 in China (Chen, *et al.*, 2013). Currently, staging of HCC is considered crucial for planning of optimal therapy (Bruix *et al.*, 2005). Patients with early HCC may benefit from radical (curative) therapies and those with intermediate stage may benefit from palliative treatments. However, relapse is a frequent complication and treatment failure rates are high. For those with advanced HCC, unfortunately, best supportive care is the only option (Verslype *et al.*, 2009). Although there is one medicine, Sorafenib, approved by the U.S. Food and Drug Administration for advanced HCC, in a large Phase III clinical trial the median survival rate increased only from 7.9 to 10.7 months (Llovet *et al.*, 2008).

[0318] AAV vectors have shown remarkable efficacy in the treatment of Leber's congenital amaurosis (Bainbridge *et al.*, 2008; Maguire *et al.*, 2008; Cideciyan *et al.*, 2008; Cideciyan, 2010) hemophilia B (Nathwani *et al.*, 2011) and aromatic L-amino acid carboxylase deficiency (Hwu *et al.*, 2012). Glybera, a rAAV1 vector to treat lipoprotein lipase deficiency, is the first gene therapy product in the Western world (Melchiorri *et al.*, 2013). In the early 2000s, conventional, single-stranded (ss) rAAV2 vectors were used by investigators to target HCC *in vivo* (Su *et al.*, 2000). Unfortunately, since the transduction efficiency of ssAAV2 vectors is low, no transduction was observed in tumors larger than 2-mm *via* systemic administration (Peng *et al.*, 2000). More recently, delivery of specific miRNAs in a mouse endogenous HCC model using rAAV8 vectors was shown to

result in inhibition of cancer cell proliferation (Kota *et al.*, 2009; Hsu *et al.*, 2012). However, rAAV8 vectors have a broad tropism to normal tissues other than the liver in murine models (Zincarelli *et al.*, 2008; Gao *et al.*, 2002; Wang *et al.*, 2005) and in non-human primates (Nathwani, *et al.*, 2006; Nathwani *et al.*, 2007).

[0319] Interestingly, the inventors have demonstrated that rAAV3 vectors, which fail to efficiently transduce any normal murine tissue *in vivo*, (Zincarelli *et al.*, 2008; Palomeque *et al.*, 2007; Markakis *et al.*, 2010; Ling *et al.*, 2010) were shown to transduce human HCC cells highly efficiently both *in vitro* (Ling *et al.*, 2010; Glushakova *et al.*, 2009) and *in vivo* (Cheng *et al.*, 2012). Although rAAV3 vectors also transduce primary human hepatocytes, the transgene expression could be restricted to malignant cells by using a HCC-specific promoter, α-fetoprotein promoter (AFPp). In subsequent studies, the inventors observed that rAAV3 vectors utilize the human hepatocyte growth factor receptor (hHGFR, also named c-*Met*) as a cellular co-receptor, (Ling *et al.*, 2010) which indicates an opportunity to exploit rAAV3-based vectors in targeting human liver cancers, since hHGFR is over-expressed in most HCC cells (You *et al.*, 2011). Furthermore, since AAV3 has lower incidence of pre-existing neutralizing antibodies in humans compared with other commonly used AAV serotypes (van der Marel *et al.*, 2011), it has the potential to be developed as a selective viral vector for gene therapy of human liver cancers. The present example demonstrates that further augmentation of rAAV3-mediated transduction efficiency in human liver cancer cells can be achieved through the elimination of specific surface-exposed tyrosine (Y), serine (S), and threonine (T) residues on the viral capsids. No observed significant alteration in cellular receptor interaction *in vitro* and viral-tropism *in vivo* was associated with these modifications. Furthermore, significant inhibition of tumorigenesis in a human liver cancer xenograft model was achieved through systemic administration of optimized rAAV3 vectors carrying a novel therapeutic gene from traditional Chinese medicine, *Trichosanthin*.

MATERIALS AND METHODS

[0320] ***Chemicals, plasmids and primers.*** Grade I-A heparin sodium salt were purchased from Sigma-Aldrich. Recombinant hHGF was purchased from Life Technologies. Plasmid pHelper was purchased from Agilent Technologies. Plasmids pdsAAV-CBAp-EGFP and pAAV-CBAp-FLuc were obtained from Dr. Xiao Xiao, University of North Carolina at Chapel Hill. Plasmid pdsAAV-AFPp-EGFP has been previously described (Glushakova *et al.*, 2009). The TCS gene (Shaw *et al.*, 1994) was synthesized by Life Technologies, based on the published sequence (*T. kirilowii* trichosanthin (TCS) mRNA, complete cds; GenBank: M34858.1), and sub-cloned into plasmid pdsAAV-AFPp-EGFP using *Age*I and *Hin*dIII restriction sites. All plasmids were sequenced prior to use.

[0321] ***Construction of optimized rAAV3 capsid plasmids.*** A two-stage PCR procedure, using Turbo *Pfu* Polymerase (Stratagene) was performed to introduce site-specific mutations in the rAAV3 capsid, as describe previously (Zhong *et al.*, 2008; Markusic *et al.*, 2010; Aslanidi *et al.*, 2013).

Briefly, in stage one, two PCR extension reactions were performed in separate tubes for each mutant. One tube contained the forward PCR primer and the other contained the reverse primer. In stage two, the two reactions were mixed and a standard PCR mutagenesis assay was carried out according to the manufacturer's instructions. All mutant plasmids were sequenced before use.

[0322] ***Cell lines and cultures.*** Human hepatocellular carcinoma (Huh7) cells have been described previously, (Ling *et al.,* 2010; Cheng *et al.,* 2012) and were maintained in complete Dulbecco's modified Eagle medium (C-DMEM, Mediatech, Inc.) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Sigma-Aldrich), 1% penicillin and streptomycin (Lonza). A newly established human hepatoblastoma (Hep293TT) cell line, was obtained from Dr. Gail E. Tomlinson (University of Texas Health Science Center at San Antonio) and was maintained in complete RPMI medium 1640 (Life Technologies) supplemented with 15% heat-inactivated FBS (Sigma-Aldrich) and 1% P/S (Lonza). Cells were grown as adherent culture in a humidified atmosphere at 37°C in 5% CO_2 and were sub-cultured after treatment with trypsin-versene mixture (Lonza) for 2-5 min at room temperature, washed and re-suspended in complete medium.

[0323] ***rAAV vectors production.*** Highly purified stocks of rAAV vectors were generated by the triple- plasmid transfection protocol. Briefly, HEK293 cells were co-transfected with three plasmids using Polyethelenimine (linear, MW 25000, Polysciences, Inc.), and medium was replaced six hrs post-transfection. Cells were harvested 72-hrs' post-transfection, subjected to three rounds of freeze-thaw and then digested with Benzonase (Life Technology). Viral vectors were purified by iodixanol (Sigma-Aldrich) gradient ultra-centrifugation followed by ion exchange chromatography using HiTrap SP/Q HP (GE Healthcare), washed with phosphate-buffered saline (PBS, Mediatech, Inc.) and concentrated by centrifugal spin concentrators with a 150 Kda molecular-weight cutoff (MWCO). The physical genomic titers of recombinant vector stocks were determined by quantitative DNA slot-blot and Southern blot analyses.

[0324] ***rAAV vectors transduction in vitro.*** Cells were seeded in 96-well plates at 5,000 or 10,000 cells per well in C-DMEM. Twenty-four hours later, cells were mock-treated or treated with indicated chemicals for 2 hrs. rAAV infections (MOI: 5×10^3) were then performed in serum- and antibiotic-free DMEM medium for 2 hrs, with or without indicated chemicals, followed by extensive washes with PBS to remove the vector inoculum. Transgene expression was analyzed by either fluorescence microscopy or flow cytometry 72-hrs' post-transduction.

[0325] ***hHGF competition assay.*** Huh7 cells were transduced with scAAV3-CBAp-EGFP vectors at an MOI of 5×10^3 vgs/cell. Vectors were premixed with increasing concentrations of recombinant hHGF. Cells were analyzed for EGFP expression levels 72 hrs post-transduction.

[0326] ***Animal handling.*** All animal experiments were approved by the appropriate regulatory authorities, and were performed according to specified guidelines for animal care. Six- to ten-week old non-obese diabetic/severe combined immuno-deficient, IL2-gamma-deficient (NSG) male mice were purchased from Jackson Laboratory.

[0327] ***Human liver cancer xenograft model.*** Six- to ten-week old male NSG mice received subcutaneous injection of 5×10^6 Huh7 or Hep293TT cells on the ventral side of the neck between shoulder blades. Animals were kept in sterile cages until the end of the experiment.

[0328] ***In vivo FLuc assays.*** rAAV vectors were injected intravenously *via* tail-vein, or injected directly into tumors in NSG mice. For *in vivo* FLuc imaging, mice were weighed to calculate the volume of substrate, D-luciferin-K^+ salt (Caliper Life Sciences), according to the dose of 4 mg/kg of body weight and anesthetized. The calculated volume of the 5 mg/mL of stock substrate solution was mixed with 100 μL of PBS and injected intra-peritoneally. *In vivo* bioluminescence images were acquired immediately over a period of 5 min using a Xenogen machine equipped with a cooled couple-charged device camera (Xenogen). Signal intensity was quantified using the camera control program, Living Image software and presented as photons/second/cm^2/steridian (p/s/cm^2/sr).

[0329] *Statistical analysis.* Results are presented as mean ± standard deviation (SD). Differences between groups were identified using one-way ANOVA with Sidak's multiple comparison test in GraphPad Prism 6 software.

RESULTS

[0330] ***rAAV3 vectors selectively transduce human liver tumors in vivo.*** In the first set of studies, the transduction efficiency of rAAV3 and rAAV8 vectors in human HCC tumors was compared in a murine xenograft model *in vivo*. Non-obese, diabetic (NOD), severe-combined immune-deficient (*scid*), interleukin 2-deficient (IL2$^-$) (NSG) mice, both male and female ($n = 4$), were injected subcutaneously on the ventral side between shoulder blades with 5×10^6 human Huh7 cells, which formed tumors. Four-weeks later, mice were either mock injected, or injected *via* the tail vein with 1×10^{11} viral genomes (vgs)/mouse of rAAV3 or rAAV8 vectors carrying the firefly luciferase gene (FLuc) under the control of cytomegalovirus (CMV) enhancer/chicken β-actin hybrid promoter (CBAp) (FIG. 40A). Whole body bioluminescent imaging was performed 3 days post-vector administration. These results are shown in FIG. 36A. As can be seen, in both male and female mice injected with rAAV3 vectors, FLuc expression was restricted to the tumor, whereas in mice injected with rAAV8 vectors, FLuc expression was relatively more widespread, but predominantly localized to liver, and the transduction efficiency of rAAV8 vectors was significantly higher in male mice, an observation, consistent with previously published studies (Davidoff *et al.*, 2003). Quantitative data further demonstrated that rAAV3 vector-mediated transgene expression was restricted to tumors, and little transgene expression was detected in livers. In contrast, in rAAV8 vector-injected mice, only low-level transgene expression occurred in the tumors, whereas expression in the liver was significantly higher. To further corroborate these results, in the second set of experiments, rAAV3 and rAAV8 vectors were used in direct intra-tumor injections in male NSG mice bearing human Huh7 tumors ($n = 4$) at 1×10^{11} vgs/mouse, and whole body bioluminescence images were obtained 3 days post-vector injections. As can be seen in FIG. 36C, high-level transgene expression was localized to the tumor in mice injected with rAAV3 vectors, whereas in addition to the

tumor, significant transgene expression in the liver was also detected in mice injected with rAAV8 vectors. Thus, in contrast to rAAV8 vectors, rAAV3 vectors possess human liver tumor-tropism in this experimental model *in vivo*.

[0331] In preliminary experiments, a diminution in transgene expression was also noted in tumors 5-days' post-vector administration, which was due to the rapid growth of HCC tumors. Thus, to further corroborate these results, in the next set of experiments, direct intra-tumor injections was performed of rAAV3 and rAAV8 vectors at low ($L = 1 \times 10^{11}$ vgs/mouse) and high ($H = 1 \times 10^{12}$ vgs/mouse) doses. Whole-body bioluminescence imaging data obtained at both day 3 and day 7 are shown in FIG. 36D. It was evident that, even at a high dose, ectopic expression in the liver in rAAV3 vector-injected mice was minimal, whereas intra-tumor injection of rAAV8 vectors resulted in strong transgene expression in the liver in a dose- and time-dependent manner. At Day 7, post-vector injections, mice were sacrificed and the viral genome copy numbers persisting in the liver tissue samples were compared. These results, shown in FIG. 36E, indicated that a large amount of rAAV8 vector genomes were present in the liver, whereas the numbers of rAAV3 vector genomes were minimal, corroborating earlier results. These studies, together with earlier results (Ling *et al.*, 2010; Glushakova *et al.*, 2009; Cheng *et al.*, 2012; Ling *et al.*, 2011) provide a clear rationale for employing rAAV3 vector-mediated gene therapy for HCC.

[0332] Further augmentation of rAAV3 vector-mediated transgene expression in human liver cancer cells in vitro can be achieved through modifications of viral capsids. To further enhance the transduction efficiency of rAAV3 vectors, site-directed mutagenesis of rAAV3 capsids was performed. In addition to mutagenesis of surface-exposed tyrosine (Y) to phenylalanine (F) residues (Cheng et al., 2012), surface-exposed serine (S), threonine (T), and lysine (K) residues were also mutagenized to valine (V) and glutamic acid (E) or arginine (R) residues, respectively. Priority was given to the positions that are conserved among various AAV serotypes, and have previously been shown to augment the transduction efficiency of rAAV2 vectors (Zhong et al., 2008; Markusic et al., 2010; Aslanidi et al., 2013; Aslanidi et al., 2012). The wild type (WT) and all mutant rAAV3 vectors carrying the CBAp-driven enhanced green fluorescence protein (EGFP) reporter gene (FIG. 40A) were used to evaluate their transduction efficiencies in a human HCC cell line, Huh7, under identical conditions. A summary of these data is provided in FIG. 41. The transduction efficiency of two K-mutants (K528E; K533E) was reduced >10-fold, and that of several Y- and T-mutants (Y272F; Y444F; T251V; Y705+731F+T492V) was reduced >2-fold. The transduction efficiency of the rest of the mutants was increased, which ranged between <2-fold to >10-fold. The seven best mutants as well as the WT rAAV3 vectors carrying the CBAp-driven FLuc reporter gene were then used to transduce Huh7 cells under identical conditions. These results (shown in FIG. 37A) indicated that the transduction efficiency of Y705+731F and S663V+T492V+K533R mutants was increased by ~10-fold, and that of S663V+T492V mutants was increased by ~15-fold, compared with the WT rAAV3 vectors. To further validate the observed increased transduction efficiency of these mutants, the three

best mutant rAAV3 vectors carrying the CBAp promoter-driven EGFP reporter gene were used to transduce a different human HCC cell line, HepG2, under identical conditions. The results (shown in FIG. 37B), demonstrated that the transduction efficiency of S663V+T492V+K533R and Y705+731F mutants was increased by ~2- and ~3-fold, respectively, and that of S663V+T492V mutants was increased by ~5-fold, compared with the WT rAAV3 vectors. The transduction efficiency of the best mutant (S633V+T492V) was also evaluated in a more recently-established, human hepatoblastoma (HB) cell line, Hep293TT (Cheng et al., 2012) (FIG. 37C). Thus, the optimized rAAV3 vector may prove useful in the potential gene therapy of human liver cancer.

[0333] ***Modifications of specific amino acids on rAAV3 capsids do not alter the virus-cellular receptor interaction.*** Owing to the uncertainty of viral capsid amino acid(s) responsible for virus-receptor(s) interaction, the inventors also examined whether cellular heparan sulfate proteoglycan (HSPG) and human hepatocyte growth factor receptor (hHGFR), previously identified as receptor (Rabinowitz *et al.*, 2004) and co-receptor (Ling *et al.*, 2010) of WT rAAV3 vectors, were involved in transduction by our optimized rAAV3 vectors. The following three sets of studies were performed. First, transduction of Huh7 cells with rAAV3-CBAp-EGFP vectors or the two best mutant vectors were performed in the presence of either low (100 ng/mL) or high (100 μg/mL) doses of soluble heparin. These results (shown in FIG. 37D, indicated that both Y705+731F and S663V+T492V mutants performed in a similar manner as the WT rAAV3 vectors, in which the low-dose of heparin enhanced viral vector-mediated transduction efficiency, whereas the high-dose dramatically reduced it. Second, transduction assays were performed in the presence of 5 μg/mL human hepatocyte growth factor (hHGF), which was previously shown to significantly inhibit the transduction efficiency of WT rAAV3 vectors (Ling *et al.*, 2010). These results, shown in FIG. 37E, demonstrated that the transduction efficiency of both the WT and the mutant viral vectors was significantly affected. And third, the WT and the two mutant rAAV3 vectors were used to transduce a human breast cancer cell line, T47D, that expresses undetectable levels of endogenous hHGFR, as well as T47D cells stably transfected with a hHGFR expression plasmids (T47D+hHGFR). These results, shown in FIG. 37F, indicated that both the WT and the mutant rAAV3 vectors transduce T47D+hHGFR cells more efficiently (>5-fold) than the parental T47D cells. Taken together, these data confirmed that the optimized rAAV3 vectors also utilize cellular HSPG and hHGFR as receptor/co-receptor for their transduction.

[0334] ***Modifications of specific amino acids on rAAV3 capsids further enhance viral transduction efficiency in vivo following systemic administration.*** The inventors next evaluated the transduction efficiency of the two optimized rAAV3 vectors in a murine xenograft model in vivo. To this end, human Huh7 or Hep293TT tumor-bearing NSG mice were used (n = 4), and a relatively low dose (5×10^{10} vgs/mouse) of rAAV3-Y705+731F-CBAp-FLuc or rAAV3-S663V+T492V-CBAp-FLuc vectors were delivered *via* the tail-vein. Whole-body bioluminescent imaging was performed 3-days' post-vector injections. From the results shown in FIG. 38A, it was clear that both Huh7 and

Hep293TT tumors were efficiently targeted by the optimized rAAV3 vectors, and that transgene expression in both types of tumors was significantly enhanced by rAAV3-S663V+T492V vectors (FIG. 38B), compared with rAAV3-Y705+731F vectors, which have previously been shown to be significantly more efficient than the WT rAAV3 vectors *in vivo* (Cheng *et al.*, 2012). Three days' post-vector injections, mice were sacrificed, and liver and tumor tissues were harvested. Total DNA samples were isolated from both liver and tumor tissues, and subjected to qPCR analyses to determine the vector-genome copy numbers. From the data shown in FIG. 38C and FIG. 38D, it was apparent that the persisting vector genomes of rAAV3-S663V+T492V mutant in the tumor was significantly higher than those of rAAV3-Y705+731F mutant (FIG. 38C), presumably due to more efficient intracellular trafficking and nuclear entry (Aslanidi *et al.*, 2013), which also correlates well with the FLuc transgene expression (FIG. 38A and FIG. 38B). No significant difference in the persisting vector genomes of the two vectors in the liver was observed, which is also comparable to that reported previously (Zincarelli *et al.*, 2008). It is noteworthy, however, that despite the presence of a roughly 5-fold higher vector genome copy numbers in the liver, compared with the tumor, little transgene expression in the liver was detected with either of the two optimized rAAV3 vectors.

[0335] Suppression of tumorigenesis in the human liver cancer xenograft model in vivo following systemic administration of optimized rAAV3 vectors expressing a novel therapeutic gene. All of the studies described thus far were carried out with reporter genes, which lack therapeutic value. Thus, although the use of a number of well-established pro-apoptotic and "suicide" genes was contemplated, efforts were focused on a newly-identified therapeutic gene, which encodes trichosanthin (TCS), a ribosome-inactivating protein, isolated from a traditional Chinese medicinal herb, Trichosanthes kirilowii (Sha *et al.*, 2013). Although the nucleotide sequence of the TCS gene was determined more than 20 years ago (Shaw *et al.*, 1994), the delivery of a gene encoding TCS into cells has never been pursued. TCS gene-expressing cassettes under the control of the AFPp were synthesized (detailed FIG. 40A), whose intracellular expression significantly inhibited the growth of human HCC cell lines *in vitro*.

[0336]

ATGATCAGATTCTTAGTCCTCTCTTTGCTAATTCTCACCCTCTTCCTAACAACTCCTGCTGTGGAGGGCGATGTT
AGCTTCCGTTTATCAGGTGCAACAAGCAGTTCCTATGGAGTTTTCATTTCAAATCTGAGAAAAGCTCTTCCAAAT
GAAAGGAAACTGTACGATATCCCTCTGTTACGTTCCTCTCTTCCAGGTTCTCAACGCTACGCATTGATCCATCTC
ACAAATTACGCCGATGAAACCATTTCAGTGGCCATAGACGTAACGAACGTCTATATTATGGGATATCGCGCTGGC
GATACATCCTATTTTTTCAACGAGGCTTCTGCAACAGAAGCTGCAAAATATGTATTCAAAGACGCTATGCGAAAA
GTTACGCTTCCATATTCTGGCAATTACGAAAGGCTTCAAACTGCTGCAGGCAAAATAAGGGAAAATATTCCGCTT
GGACTCCCTGCTTTGGACAGTGCCATTACCACTTTGTTTTACTACAACGCCAATTCTGCTGCGTCGGCACTTATG
GTACTCATTCAGTCGACGTCTGAGGCTGCGAGGTATAAATTTATTGAGCAACAAATTGGGAAGCGTGTTGACAAA
ACCTTCCTACCAAGTTTAGCAATTATAAGTTTGGAAAATAGTTGGTCTGCTCTCTCCAAGCAAATTCAGATAGCG
AGTACTAATAATGGACAGTTTGAAAGTCCTGTTGTGCTTATAAATGCTCAAAACCAACGAGTCACGATAACCAAT
GTTGATGCTGGAGTTGTAACCTCCAACATCGCGTTGCTGCTGAATAGAAACAATATGGCAGCCATGGATGACGAT
GTTCCTATGACACAGAGCTTTGGATGTGGAAGTTATGCTATTTAG (SEQ ID NO:26)

[0337]

GAATTCATGATCAGATTCTTAGTCCTCTCTTTGCTAATTCTCACCCTCTTCCTAACAACTCCTGCTGTGGAGGGC
GATGTTAGCTTCCGTTTATCAGGTGCAACAAGCAGTTCCTATGGAGTTTTCATTTCAAATCTGAGAAAAGCTCTT
CCAAATGAAAGGAAACTGTACGATATCCCTCTGTTACGTTCCTCTCTTCCAGGTTCTCAACGCTACGCATTGATC
CATCTCACAAATTACGCCGATGAAACCATTTCAGTGGCCATAGACGTAACGAACGTCTATATTATGGGATATCGC
GCTGGCGATACATCCTATTTTTTCAACGAGGCTTCTGCAACAGAAGCTGCAAAATATGTATTCAAAGACGCTATG
CGAAAAGTTACGCTTCCATATTCTGGCAATTACGAAAGGCTTCAAACTGCTGCAGGCAAAATAAGGGAAAATATT
CCGCTTGGACTCCCTGCTTTGGACAGTGCCATTACCACTTTGTTTTACTACAACGCCAATTCTGCTGCGTCGGCA
CTTATGGTACTCATTCAGTCGACGTCTGAGGCTGCGAGGTATAAATTTATTGAGCAACAAATTGGGAAGCGTGTT
GACAAAACCTTCCTACCAAGTTTAGCAATTATAAGTTTGGAAAATAGTTGGTCTGCTCTCTCCAAGCAAATTCAG
ATAGCGAGTACTAATAATGGACAGTTTGAAAGTCCTGTTGTGCTTATAAATGCTCAAAACCAACGAGTCACGATA
ACCAATGTTGATGCTGGAGTTGTAACCTCCAACATCGCGTTGCTGCTGAATAGAAACAATATGGCAGCCATGGAT
GACGATGTTCCTATGACACAGAGCTTTGGATGTGGAAGTTATGCTATTCTCGAGGACTACAAGGATGACGATGAC
AAGGATTACAAAGACGACGATGATAAGGACTATAAGGATGATGACGACAAATAA (SEQ ID NO:27)

[0338] Then, rAAV3-S663V+T492 mutant vectors were generated carrying this novel therapeutic gene. In addition, to allow initiating the treatment at an early time-point, before the tumors are palpable, a genetically-modified human HCC cell line, Huh7-FLuc, was also generated in which the FLuc gene under the control of the CBAp promoter is stably transfected, which also allowed for monitoring the tumor growth by whole body bioluminescent imaging. NSG mice ($n = 10$) were subcutaneously injected on the ventral side between shoulder blades with 5×10^6 Huh7-FLuc cells. Four weeks post-xenografts, mice were divided into 2 groups, and 5×10^{10} vgs of rAAV3-S663V+T492V-AFPp-TCS vectors were injected *via* the tail-vein in the first group (Day 0). The second group of mice was injected with 5×10^{10} vgs of rAAV3-S663V+T492V-AFPp-EGFP vectors to serve as appropriate controls. Whole-body bioluminescent imaging of mice was performed at Day 0, Day 3, Day 8, and Day 11 post vector-administrations. These results, shown in FIG. 39A, document that whereas Huh7-FLuc tumors grew progressively in mice injected with rAAV3-S663V+T492V-AFPp-EGFP vectors, tumor growth in mice injected with rAAV3-S663V+T492V-AFPp-TCS vectors was significantly inhibited up until Day 11 ($p < 0.05$), with maximal growth inhibition at Day 8 ($p < 0.01$). Whole body bioluminescent images of mice performed at Day 8 post vector-administrations are shown in FIG. 39B. Moreover, on Day 11, all mice were sacrificed, and serum levels of aspartate transaminase (AST) and alanine transaminase (ALT) were determined. No significant differences were observed between TCS-treated and control groups, suggesting little liver injury in mice (FIG. 39C).

DISCUSSION

[0339] As stated above, HCC, which ranks fifth among solid tumors in humans, leads to ~695,900 deaths worldwide each year. Although patients with early diagnosis of HCC may benefit from surgical and/or chemotherapeutic interventions, relapse of the disease is a frequent occurrence, and the rate of treatment failure is high. For patients diagnosed with advanced HCC, the options are

limited largely to supportive care. Although the US Food and Drug Administration (FDA) has approved the use of Sorafenib in patients with advanced HCC, the median survival is increased by only ~3 months. Thus, it is readily clear that the development of novel therapeutic options for HCC is sorely needed, and the combination of gene therapy with tradition Chinese medicine (TCM) is a promising option. TCM medicine, as a main complementary and alternative medicinal therapy, has already become a commonly-used treatment for HCC in China (Zhai *et al.,* 2013). Recently, it has been reported that bioactive monomeric compounds extracted from TCM herbs have the ability to significantly enhance the therapeutic efficiency mediated by rAAV vectors (Zhang *et al.,* 2010; Mitchell *et al.,* 2013; Wang *et al.,* 2014; Ling *et al.,* 2014). Here, a novel strategy has been developed that combines gene therapy with TCM adminstration, in which a therapeutic suicide gene isolated from herbs is systemically delivered into malignant cells *in vivo* through rAAV vectors.

[0340] Recombinant AAV vector-mediated gene therapy of HCC has been attempted in the past. For example, the use of conventional, single-stranded (ss) rAAV2 vectors to target HCC *in vivo* has been reported (Su *et al.,* 2000), but the transduction efficiency of these vectors was low. In subsequent studies (Peng *et al.,* 2000), no transduction was observed in tumors larger than 2 mm following systemic administration. In more recent studies, the use of rAAV8 vectors to mediate delivery of specific miRNA-26A and miRNA-122 in mouse endogenous HCC tumor models was shown to result in inhibition of tumor growth (Kota *et al.,* 2009; Hsu *et al.,* 2012). However, rAAV8 vectors have a broad tropism to normal tissues other than the liver in murine models (Zincarelli *et al.,* 2008; Gao *et al.,* 2002; Wang *et al.,* 2005) and in non-human primates (Nathwani, *et al.,* 2006; Nathwani *et al.,* 2007). This example demonstrates that the remarkable tropism of rAAV3 vectors for human liver cancer cells *in vitro* can also be exploited to achieve targeted delivery of these vectors to human liver tumors in a xenograft mouse model *in vivo*. In addition, site-directed mutagenesis of specific amino acid residues on the rAAV3 capsid can further augment the transduction efficiency of rAAV3 vectors. Furthermore, the optimized rAAV3 vectors expressing a novel therapeutic gene can also be used to suppress human liver tumorigenesis in a murine xenograft model. It should be emphasized that these studies were carried out with well-established tumors, and the deliberate use of low vector doses to establish tumor-targeting. Thus, it is highly likely that the use of high vector doses and/or earlier intervention, before the tumor is well-established, it would be possible to achieve a more desirable therapeutic endpoint. It is also tempting to speculate that pending successful completion of additional studies with primary human liver tumor xenografts, especially in liver microenvironment, and safety and efficacy in large animal models, rAAV3-S663V+T492V vectors might prove useful in the potential gene therapy of human liver cancers.

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[0341] The following references, to the extent that they provide exemplary procedural or other details supplementary to those set forth herein, are specifically incorporated herein by reference:

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[0342] It should be understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and the scope of the appended claims.

[0343] All references, including publications, patent applications and patents cited herein are specifically incorporated herein by reference in their entirety to the same extent as if each reference was individually and specifically indicated to be incorporated by reference and was set forth in its entirety herein.

[0344] Recitation of ranges of values herein are merely intended to serve as a shorthand method of referring individually to each separate value falling within the range, unless otherwise indicated herein, and each separate value is incorporated into the specification as if it were individually recited herein.

[0345] All methods described herein can be performed in any suitable order, unless otherwise indicated herein, or otherwise clearly contradicted by context.

[0346] The use of any and all examples, or exemplary language (*e.g.*, "such as") provided herein, is intended merely to better illustrate the invention and does not pose a limitation on the scope of the invention unless otherwise indicated. No language in the specification should be construed as indicating any element is essential to the practice of the invention unless as much is explicitly stated.

[0347] The description herein of any aspect or embodiment of the invention using terms such as "comprising," "having," "including" or "containing" with reference to an element or elements is intended to provide support for a similar aspect or embodiment of the invention that "consists of," "consists essentially of," or "substantially comprises" that particular element or elements, unless otherwise stated or clearly contradicted by context (*e.g.*, a composition described herein as comprising a particular element should be understood as also describing a composition consisting of that element, unless otherwise stated or clearly contradicted by context).

[0348] All of the compositions and methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the compositions and methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents that are chemically and/or physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

Claims

1. An rAAV particle comprising a modified capsid protein that comprises any one of the following combinations of non-native amino acid substitutions:

(a) Y701F and Y705F, but not Y731F;

(b) T492V and S663V, but not Y444F, Y500F or Y730F; or

(c) Y705F, Y731F, and T492V;

of the wild-type AAV3 capsid protein as set forth in SEQ ID NO:3, or at the equivalent surface-exposed amino acid residues in any one of wild-type AAV1, AAV2, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, or AAV10 capsid proteins, as set forth in SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, or SEQ ID NO:10, respectively, or in any combination thereof;

or wherein the one or more amino acid substitutions are chosen from:

(d) Y444F and T550V;

(e) Y730F and T491V, but not Y444F, Y500F or S662V;

(f) S458F and S492V, but not S662V;

of the wild-type AAV2 capsid protein as set forth in SEQ ID NO:2, or at the equivalent surface-exposed amino acid residues in any one of wild-type AAV1, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, or AAV10 capsid proteins, as set forth in SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, or SEQ ID NO:10, respectively, or in any combination thereof.

2. The rAAV particle in accordance with claim 1, wherein the transduction efficiency of the particle is 2- to 50-fold, 6- to 40-fold, or 8- to 30-fold higher in a selected mammalian host cell than that of a particle that comprises a corresponding, unmodified, rAAV capsid protein; or

wherein the particle is less susceptible to ubiquitination when introduced into a mammalian cell than that of a particle that comprises a corresponding, unmodified, rAAV capsid protein.

3. The rAAV particle in accordance with claim 1, wherein the particle further comprises a nucleic acid segment that encodes a diagnostic, therapeutic, or chemotherapeutic agent operably linked to a promoter capable of expressing the nucleic acid segment in a suitable host cell comprising the rAAV particle.

4. The rAAV particle in accordance with claim 3, further comprising at least a first mammalian intron sequence operably linked to the nucleic acid segment.

5. The rAAV particle in accordance with claim 3, wherein the promoter is a heterologous promoter, a tissue-specific promoter, a cell-specific promoter, a constitutive promoter, an inducible promoter, or any combination thereof.

6. The rAAV particle in accordance with claim 3, wherein the nucleic acid segment expresses or encodes a polypeptide, a peptide, a ribozyme, a peptide nucleic acid, an siRNA, an RNAi, an antisense oligonucleotide, an antisense polynucleotide, an antibody, an antigen binding fragment, or any combination thereof.

7. The rAAV particle in accordance with claim 3, wherein the nucleic acid segment encodes a chemotherapeutic agent.

8. The rAAV particle in accordance with claim 3, wherein the diagnostic, therapeutic or chemotherapeutic agent is an agonist, an antagonist, an anti-apoptosis factor, an inhibitor, a receptor, a cytokine, a cytotoxin, an erythropoietic agent, a glycoprotein, a growth factor, a growth factor receptor, a hormone, a hormone receptor, an interferon, an interleukin, an interleukin receptor, a nerve growth factor, a neuroactive peptide, a neuroactive peptide receptor, a protease, a protease inhibitor, a protein decarboxylase, a protein kinase, a protein kinsase inhibitor, an enzyme, a receptor binding protein, a transport protein or an inhibitor thereof, a serotonin receptor, or an uptake inhibitor thereof, a serpin, a serpin receptor, a tumor suppressor, a cytotoxic agent, a cytostatic agent, an anti-inflammatory agent, or any combination thereof.

9. An isolated nucleic acid segment that encodes the modified capsid protein in accordance with any one of claims 1-8.

10. An isolated mammalian host cell comprising the rAAV particle in accordance with any one of claims 1-8.

11. A composition comprising:

(I) (a) the rAAV particle in accordance with any one of claims 1-8; or

(b) a nucleic acid segment that encodes the modified capsid protein of the rAAV particle of (a); and

(II) a pharmaceutically-acceptable buffer, diluent, or excipient.

12. The composition in accordance with claim 11, further comprising a lipid, a liposome, a lipid complex, an ethosome, a niosome, a nanoparticle, a microparticle, a liposphere, a nanocapsule, or any combination thereof.

13. The composition in accordance with claim 11, for use in therapy or prophylaxis.

14. A kit comprising:

(1) the rAAV particle in accordance with any one of claims 1-8; and

(2) instructions for using the rAAV particle in the diagnosis, prevention, treatment, or amelioration of one or more symptoms of liver cancer in a human.

15. A medicament for diagnosing, preventing, treating or ameliorating one or more symptoms of mammalian cancer, comprising a composition in accordance with claim 11.

16. A pharmaceutical composition for providing a mammal in need thereof with a diagnostically- or therapeutically-effective amount of a selected biological molecule, comprising the rAAV particle in accordance with any one of claims 1-8, which is provided to a cell, tissue or organ of a mammal in need thereof in an amount and for a

time effective to provide the mammal with a diagnostically- or a therapeutically-effective amount of the selected biological molecule.

17. A pharmaceutical composition for diagnosing, preventing, treating, or ameliorating at least one or more symptoms of liver cancer, including HCC, in a mammal, comprising the rAAV particle in accordance with any one of claims 1-8, which is to be administered to a mammal in need thereof in an amount and for a time sufficient to diagnose, prevent, treat or ameliorate the one or more symptoms of the liver cancer in the mammal.

18. A pharmaceutical composition for transducing a population of liver cells or liver tumor cells in a human comprising an effective amount of the rAAV particle in accordance with any one of claims 1-8, which is to be administered to the human for a time effective to transduce the population of liver cells or liver tumor cells.

19. The rAAV particle in accordance with claim 3, wherein the nucleic acid segment further comprises an enhancer, a post-transcriptional regulatory sequence, a polyadenylation signal, or any combination thereof, operably linked to the nucleic acid segment.

20. The rAAV particle in accordance with claim 5, wherein the tissue-specific promoter is a liver-specific promoter, a tumor cell-specific promoter, or a combination thereof.

21. The isolated mammalian host cell in accordance with claim 10, wherein the host cell is a stem cell, a hematopoietic cell, a blood cell, a neural cell, a retinal cell, an epithelial cell, an endothelial cell, a pancreatic cell, a cancer cell, a muscle cell, a vascular cell, a diaphragm cell, a stomach cell, a liver cell, a tumor cell, or a CD34+ cell.

22. The composition in accordance with claim 11, wherein the composition is comprised within a kit for diagnosing, preventing, treating or ameliorating one or more

symptoms of a mammalian disease, injury, disorder, trauma or dysfunction, including, but not limited to liver cancer.

23. The composition in accordance with claim 22, wherein the liver cancer is HCC.

24. The composition in accordance with claim 13, wherein the therapy or prophylaxis is for a human cancer.

25. The medicament in accordance with claim 15, wherein the mammalian cancer is human liver cancer.

26. The pharmaceutical composition in accordance with claim 17, wherein the mammal is human.

27. The pharmaceutical composition in accordance with claim 18, wherein the human is diagnosed with, having, or suspected of having HCC.