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WO-A1-2014/184528
WO-A1-2014/184529
WO-A2-92/09690
WO-A2-2007/067818
LI Z ET AL: "Cell-targeted phagemid particles preparation using Escherichia coli bearing ligand-pIII encoding helper phage genome", BIOTECHNIQUES RAPID DISPATCHES, vol. 41, no. 6, 1 December 2006 (2006-12-01), pages 706-707, XP055331093, US ISSN: 0736-6205, DOI: 10.2144/000112294
JIANG H ET AL: "Development of efficient RNA interference system using EGF-displaying phagemid particles", ACTA PHARMACOLOGICA SINICA, vol. 29, no. 4, 1 April 2008 (2008-04-01), pages 437-442, XP055331097, GB ISSN: 1671-4083, DOI: 10.1111/j.1745-7254.2008.00768.x
SUWAN KEITTISAK ET AL: "Next-generation of targeted AAVP vectors for systemic transgene delivery against cancer", PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, vol. 116, no. 37, 2 August 2019 (2019-08-02), pages 18571-18577, XP055786504, US ISSN: 0027-8424, DOI: 10.1073/pnas.1906653116 Retrieved from

Fortsættes ...

the Internet: URL:<https://www.pnas.org/content/pnas/116/37/18571.full.pdf>>

DESCRIPTION

[0001] The present invention relates to phagemid vectors and associated phagemid particles, and in particular to hybrid and recombinant phagemid vectors, particles and expression systems. Described herein is the use of such phagemid particles and expression systems as a research tool, and for the delivery of transgenes in a variety of gene therapy applications, DNA and/or peptide vaccine delivery and imaging techniques. The invention extends to in vitro methods for producing recombinant adeno-associated viruses (rAAV) and to genetic constructs used in such methods.

[0002] In the past decade, a number of viral and non-viral vectors have emerged as potential delivery vectors for industrial and therapeutic applications. An important property of vectors, in addition to being efficient at delivering genes, is that it must also be easily produced and commercially viable. Despite the large body of basic research that must underpin the vector design and refinement, vector production is an area that must be addressed in order for successful commercialization to occur. Adeno-associated virus (AAV)-mediated gene therapy is a case in point, as vector production has been a bottleneck for clinical translation. Recombinant AAV (rAAV) is an attractive vector in gene therapy. However, efficient vector production at commercial scales is not yet possible. A variety of expression systems have been developed for rAAV production. While transfection-based protocols have been the gold standard for high-purity laboratory-scale production, they cannot be efficiently translated to commercial-scale protocols. Current methods for commercial-scale production rely on the use of eukaryotic viruses to rescue AAV vectors from a producer cell line. Despite being more efficient, the use of infectious eukaryotic viruses is a major point of concern, not only when purifying viral particles, but also regarding safety for *in vivo* use.

[0003] AAV are non-enveloped viruses with a 4.7Kb wild type genome that is flanked by inverted terminal repeats (ITRs). The genome contains two open reading frames, rep and cap, which provide proteins necessary for replication and encapsidation of the viral genome. In nature, wild type AAV is found in the presence of adenovirus (Ad) as they provide adeno-helper proteins that are essential for packaging of the AAV genome in to icosahedral virions. Therefore, AAV production relies on three key elements: the ITR-flanked genome, rep and cap, and adeno-helper genes.

[0004] At present, laboratory scale production of rAAV uses DNA transfection to introduce all three genetic elements in to human embryonic kidney HEK293 cells, which is a suitable mammalian producer cell as they inherently express an adeno-helper protein from immortalization. Although laboratory scale production provides rAAV of high purity, transfection methods are not suitable for large-scale production and face major limitations, including inefficiency, which leads to low rAAV yields and high costs. Also, in many cases, live viruses, such as adenovirus or herpes simplex virus, are used to efficiently supply the helper functions, which present significant health and safety concerns for *in vivo* use.

[0005] Large-scale commercial production of rAAV is possible, but it comes at very high costs and results in the production of low purity rAAV. Alongside the cell factory system involving large-scale adherent culture of HEK293 cells, the baculovirus expression vectors (BEVS) and Sf9 insect cell system has served as the most reliable candidate system for large-scale rAAV production. Recent studies have shown that this system is amenable to genetic modification, and that transcriptional control can be used to regulate *rep* gene expression, which is toxic to the producer cell. Nevertheless, despite its advantageous capabilities, the BEVS/Sf9 paradigm remains costly and lacks refinement, as baculovirus contamination of rAAV preparations is almost unavoidable, and with a risk of high immunogenicity.

[0006] In 2006, Hajitou et al. attempted to fulfil the need for vectors by creating a hybrid between recombinant adeno-associated virus (rAAV) and filamentous bacteriophage (i.e. phage), called the Adeno-associated Virus/Phage (AAVP) (Nature protocols 2, 523-531 (2007); Cell 125, 385-398 (2006)). The AAVP is a hybrid phage vector in which gene expression is under the control of a eukaryotic transgene cassette flanked by internal terminal repeats (ITRs) of AAV2, and inserted in an intergenomic region of bacteriophage. This vector combines the specificity of bacteriophage vectors with the genetic characteristics of AAV, yielding a virus that can reproduce only in prokaryotic hosts and transduce mammalian cells with the expression profile similar to rAAV. Importantly, rAAV can be rescued from HEK293 cells transduced with AAVP following transfection with *rep*- and *cap*-expressing plasmid, and subsequent infection with wild-type adenovirus type 5. Hence, the AAVP vector possesses favourable characteristics of mammalian and prokaryotic viruses, and does not suffer from the disadvantages that those individual vectors normally carry.

[0007] However, the AAVP still has certain inherent limitations of bacteriophage and thus leaves room for significant improvement of AAVP or phage vectors in general, and so there is a need for designing novel superior phage-based vectors. For instance, AAVP are a hybrid between two virus species (i.e. bacteriophage and AAV), AAVP vectors contain the genome of both the eukaryotic and prokaryotic viruses. Despite being essential for viral reproduction, the prokaryotic genome is functionally or therapeutically irrelevant. Inclusion of the phage viral genome thus deleteriously affects vector efficiency and the production method, and ultimately leads to AAVP's relatively low gene transduction efficacy when compared to mammalian viruses. There is therefore a need to provide novel modified bacteriophage systems, which can be used both in gene therapy techniques and for large-scale production of recombinant viral vectors, such as adeno-associated virus (AAV) or lentivirus.

[0008] The research described herein has developed a so-called "hybrid phagemid viral vector system", with the new phagemid particle being referred to as Phagemid/Adeno-associated Virion (i.e. PAAV). Another name used by the inventors for the novel vectors they have created is "phasmid". Unlike the prior art AAVP genome, which consists of a rAAV cassette inserted in to the filamentous phage genome, the PAAV genome of the invention does not contain any structural bacteriophage genes, and so a prokaryotic helper virus is required to facilitate vector assembly in the host.

[0009] The invention is as defined by the claims.

[0010] In a first aspect of the invention, there is provided a composition for producing a recombinant adeno-associated virus (AAV)/phagemid particle from a prokaryotic host, the composition comprising:- (i) a first vector configured to persist inside a prokaryotic host, and comprising: a) at least one AAV transgene expression cassette flanked by Inverted Terminal Repeat sequences (ITRs), b) a bacteriophage packaging signal comprising a bacteriophage origin of replication for enabling replication of the particle into single-stranded DNA; and c) a bacterial origin of replication for enabling replication of double-stranded vector inside a prokaryotic host, wherein the first vector lacks structural genes which encode the bacteriophage capsid proteins; and (ii) a second vector comprising a nucleic acid encoding bacteriophage structural proteins, which are capsid proteins required for packaging the single-stranded DNA, resulting in the formation and extrusion of a recombinant AAV/phagemid particle from the prokaryotic host.

[0011] In a second aspect of the invention, there is provided an *in vitro* and/or *ex vivo* method for producing recombinant adeno-associated virus (AAV) vector, the method comprising introducing into, a eukaryotic host cell, a recombinant AAV/phagemid particle, and allowing the host cell to produce recombinant AAV vector, wherein the AAV/ phagemid particle comprises bacteriophage capsid proteins and an AAV/phagemid particle genome, wherein the AAV/phagemid particle genome comprises: i) at least one AAV transgene expression cassette, flanked by Inverted Terminal Repeat sequences (ITRs), which encodes an agent for delivery to the target cell; ii) a bacteriophage packaging signal comprising a bacteriophage origin of replication for enabling replication of the particle into single-stranded DNA; and iii) a bacterial origin of replication for enabling replication of double-stranded vector inside a prokaryotic host, wherein the AAV phagemid particle comprises a genome which lacks bacteriophage structural genes required for the formation, packaging or extrusion of the particle from a prokaryotic host, wherein the AAV phagemid particle lacks structural genes which encode the bacteriophage capsid proteins, wherein the method is not a method for treatment of the human or animal body by surgery or therapy.

[0012] In a third aspect of the invention, there is provided a recombinant vector comprising: i) a transgene expression cassette flanked by adeno-associated virus (AAV) Inverted Terminal Repeat sequences (ITRs); ii) AAV rep-cap genes; and iii) and adeno-helper genes.

[0013] In a fourth aspect of the invention, there is provided a recombinant adeno-associated virus (AAV)/phagemid particle comprising bacteriophage capsid proteins and AAV/phagemid particle genome, wherein the AAV/phagemid particle genome comprises: i) at least one AAV transgene expression cassette flanked by Inverted Terminal Repeat sequences (ITRs); ii) a bacteriophage origin of replication for enabling replication of the particle into single-stranded DNA; and iii) a bacterial origin of replication for enabling replication of double-stranded vector inside a prokaryotic host, wherein the AAV phagemid particle comprises a genome which lacks bacteriophage structural genes required for the formation, packaging or extrusion of the particle from a prokaryotic host, wherein the AAV phagemid particle genome lacks structural

genes which encode the bacteriophage capsid.

[0014] In a fifth aspect of the invention, there is provided the use of the vector according to the third aspect, or the particle according to the fourth aspect, to produce a recombinant AAV viral vector *in vitro*, or *ex vivo*, optionally wherein the recombinant phagemid particle comprises a targeting moiety for a target eukaryotic cell, and optionally wherein the eukaryotic host cell type is a diseased cell, optionally wherein the diseased cell is a malignant or benign tumour.

[0015] In a sixth aspect of the invention, there is provided a method for producing recombinant AAV viral vector, the method comprising introducing, into a eukaryotic host cell, the vector according to the third aspect or the particle according to the fourth aspect, and allowing the host cell to produce recombinant viral vector *in vitro* or *ex vivo*, optionally wherein the recombinant phagemid particle comprises a targeting moiety for the target host eukaryotic cell, and optionally wherein the eukaryotic host cell type is a diseased cell, optionally wherein the diseased cell is a malignant or benign tumour.

[0016] In a seventh aspect of the invention, there is provided a recombinant AAV/phagemid particle comprising a genome comprising: i) one or more transgene expression cassette, wherein the one or more transgene expression cassette comprises left and right Inverted Terminal Repeat sequences (ITRs) of an AAV serotype, wherein at least one transgene expression cassette encodes an agent for delivery to the target cell, ii) a bacteriophage origin of replication for enabling replication of the particle into single-stranded DNA; and iii) a bacterial origin of replication for enabling replication of double-stranded vector inside a prokaryotic host, wherein the AAV phagemid particle comprises a genome which lacks bacteriophage structural genes required for the formation, packaging or extrusion of the particle from a prokaryotic host, wherein the AAV phagemid particle genome lacks structural genes which encode the bacteriophage capsid proteins, wherein the AAV phagemid particle comprises bacteriophage structural capsid proteins, wherein a capsid minor coat protein is configured to display a cell-targeting ligand for enabling delivery of the particle to a target cell, wherein the cell-targeting ligand is the RGD4C targeting peptide of SEQ ID No: 7.

[0017] Thus, according to an example of the disclosure, there is provided a recombinant phagemid particle for expressing a transgene in a target cell transduced with the particle, the phagemid particle comprising at least one transgene expression cassette which encodes an agent which exerts a biological effect on the target cell, characterised in that the phagemid particle comprises a genome which lacks at least 50% of its bacteriophage genome.

[0018] Advantageously, re-engineering hybrid AAV vectors into the phagemid particle according to the disclosure, substantially lacking the phage genome from which the particle is derived, dramatically enhances the functional properties of the resultant vector (i.e. the phagemid particle). Altering the viral expression system to a phagemid-based system according to the invention expands the possibility of applying phagemid viral vectors in a much broader context. By eliminating at least 50% of the bacteriophage genome, which constitutes over 50% of the genome size, from the particle's genome, the resultant particle size of the

phagemid particle is dramatically reduced.

[0019] The term "phagemid particle" can refer to a hybrid phagemid genome encapsulated by phage-derived coat proteins. The hybrid phagemid genome is a "phagemid genome" (i.e. a genetic construct containing two origins of replication - one from bacteriophage (e.g. F1), and one from bacteria (e.g. pUC1)). In one embodiment, the phagemid genome may contain an incorporated "recombinant transgene cassette from AAV" (rAAV), and is therefore a hybrid and not a conventional phagemid genome with a normal (i.e. generic, non-viral) recombinant transgene expression cassette. The phagemid particle can refer to the hybrid phagemid genome (i.e. the invention) that has been encapsulated by phage proteins derived from a trans-acting agent (such as a helper phage).

[0020] While allowing additional capacities to incorporate very large or multiple transgene cassettes, these smaller phagemid particles also display added advantages in enhanced gene transfer, production yield, biodistribution and evasion from eukaryotic cellular barriers. Another significant advantage of using the phagemid particle of the invention is that they have the ability to accommodate extremely large and numerous transgene cassettes or gene inserts, such as genes of the three plasmids used for recombinant AAV production by transfection, as described hereinafter. Hence, by combining the genetic components for viral production in a single or multiple phagemid vector(s), an efficient commercial-scale virus-producing gene delivery system has been designed.

[0021] Preferably, the phagemid particle comprises a virion.

[0022] One preferred embodiment of the genome of the recombinant phagemid particle is illustrated on Figure 3, with preferred components being shown on Figures 4-6.

[0023] Preferably, the genome of the recombinant phagemid particle comprises a packaging signal for enabling replication of the phagemid genome into single-stranded DNA, which can subsequently be packaged into the phagemid particle inside a prokaryotic host. The packaging signal may preferably comprise an origin of replication. For example, the origin of replication preferably comprises an F1 ori, more preferably from an F1 bacteriophage. The DNA sequence of one embodiment of the F1 ori is represented herein as SEQ ID No: 1, as follows:

```
ACGCGCCCTGTAGCGCGCATTAAAGCGCGCGGGTGTGGTGGTTACGCGCAGCGTGACCGCTACACTTGC
CAGCGCCCTAGCGCCCGCTCCTTTTCGCTTTCTTCCCTTCTTCTCGCCACGTTGCGCGGCTTTCCTCGT
CAAGCTCTAAATCGGGGGCTCCCTTTAGGGTTCCGATTTAGTGCTTTACGGCACCTCGACCCCAAAAAAC
TTGATTTGGGTGATGGTTCACGTAGTGGGCCATCGCCCTGATAGACGGTTTTTCGCCCTTTGACGTTGGA
GTCCACGTTCCTTTAATAGTGGACTCTTGTTCCAAACCTGGAACAACACTCAACCCATCTCGGGCTATTCT
TTTGATTATAAGGGATTTTGGCGATTTTCGGCCTATTGGTTAAAAAATGAGCTGATTTAACAAAAATTTA
ACGCGAATTTTAACAAAATATTAACGTTTACAATTT
```

[SEQ ID NO:1]

[0024] Preferably, the genome of the recombinant phagemid particle comprises an origin of

replication for enabling replication of double-stranded vector inside a prokaryotic host. Preferably, the origin of replication enables high copy number replication of the vector inside the host. Preferably, the origin of replication comprises a pUC ori. The DNA sequence of one embodiment of the pUC ori is represented herein as SEQ ID No: 2, as follows:

```
TTGAGATCCTTTTTTCTGCGCGTAATCTGCTGCTTGCAAACAAAAAACCCGCTACCAGCGGTGGTT
TGTTTGCCGGATCAAGAGCTACCAACTCTTTTCCGAAGGTAAGTGGCTTCAGCAGAGCGCAGATACCAA
ATACTGTCCTTCTAGTGTAGCCGTAGTTAGGCCACCACTTCAAGAACTCTGTAGCACCGCCTACATACCT
CGCTCTGCTAATCCTGTTACCACTGGCTGCTGCCAGTGGCGATAAGTCGTGTCTTACCGGGTTGGACTCA
AGACGATAGTTACCGGATAAGGCGCAGCGTCGGGCTGAACGGGGGGTTCGTGCACACAGCCAGCTTGG
AGCGAACGACCTACACCGAACTGAGATACCTACAGCGTGAGCTATGAGAAAGCGCCACGCTTCCCGAAGG
GAGAAAGGCGGACAGGTATCCGTAAGCGGCAGGGTCGGAACAGGAGAGCGCACGAGGGAGCTTCCAGGG
GGAAACGCCTGGTATCTTTATAGTCCTGTGCGGGTTTCGCCACCTCTGACTTGAGCGTCGATTTTTGTGAT
GCTCGTCAGGGGGGCGGAGCCTATGGAAA
```

[SEQ ID NO:2]

[0025] Alternatively, in another embodiment, the phagemid particle may be designed such that it integrates into the genome of a host cell. In this case, nucleic acid sequences, which favour targeted integration (e.g. by homologous recombination) of the particle's genome are envisaged. Hence, the genome of the recombinant phagemid particle may comprise one or more DNA sequence, which favours targeted integration into a host genome.

[0026] In one embodiment, the phagemid particle may be used as an experimental research tool, and used *ex vivo* or *in vitro*.

[0027] In another example, preferably the phagemid particle may be used as a recombinant vector for the delivery of the transgene to a tissue specific target, irrespective of whether the vector is administered systemically or locally to a subject *in vivo*, applied to a mixture of cells *in vitro*, or applied to an organ *ex vivo*. Preferably, the at least one transgene expression cassette comprises a viral transgene expression cassette, more preferably a mammalian viral transgene expression cassette. For example, the at least one transgene expression cassette may, in one preferred example, comprise a lentivirus transgene expression cassette. The at least one transgene expression cassette is preferably an adeno-associated virus (AAV) transgene expression cassette.

[0028] The transgene expression cassette may comprise any nucleic acid encoding an agent, which may have therapeutic or industrial utility in the target cell or tissue. In one embodiment of the invention, the nucleic acid may be DNA, which may be genomic DNA or cDNA. Non-naturally occurring cDNA may be preferred in some embodiments. In another embodiment, the nucleic acid may be RNA, such as antisense RNA or shRNA.

[0029] In one preferred embodiment, the transgene expression cassette may comprise shRNA configured to target mTOR expression in a tumour cell. As shown in the Example 7, down-regulation of mTOR expression in tumour cells (e.g. medulloblastoma cells) may be achieved

with treatment with RGD4C-phagemid carrying the mTOR/shRNA (RGD4C-mTOR/shRNA).

[0030] The agent encoded by the nucleic acid may be a polypeptide or protein. For example, where the phagemid particle of the invention is used to treat cancer, the transgene may encode the Herpes simplex virus thymidine kinase gene, which may subsequently exert a therapeutic effect on the target tumour cell.

[0031] In another preferred embodiment, therefore, the transgene expression cassette may encode TNF α for expression in a tumour cell. As shown in Example 7, RGD4C-phagemid can successfully deliver TNF α to DIPG in a selective manner, resulting in apoptosis induction. Therefore, RGD4C-phagemid-TNF α has therapeutic potential for use in targeted therapy against DIPG.

[0032] However, it will be appreciated that the type of cell, which is targeted by the recombinant phagemid particle depends on the type of cell-targeting ligand expressed on the surface of the particle.

[0033] The transgene expression cassette may comprise one or more functional elements required for expression of the nucleic acid in the target cell. For example, preferably the transgene expression cassette comprises a promoter, such as the CMV promoter. The DNA sequence of one embodiment of the CMV promoter is represented herein as SEQ ID No: 3, as follows:

```
ACGCGTGGAGCTAGTTATTAATAGTAATCAATTACGGGGTCATTAGTTCATAGCCCATATATGGAGTTCC
GCGTTACATAACTTACGGTAAATGGCCCGCTGGCTGACCGCCCAACGACCCCGCCATTGACGTCAAT
AATGACGTATGTTCCCATAGTAACGTCAATAGGGACTTTCCATTGACGTCAATGGGTGGAGTATTTACGG
TAAACTGCCCACTTGGCAGTACATCAAGTGTATCATATGCCAAGTACGCCCCCTATTGACGTCAATGACG
GTAAATGGCCCGCTGGCATTATGCCCAGTACATGACCTTATGGGACTTTCCTACTTGGCAGTACATCTA
CGTATTAGTCATCGCTATTACCATGGTGATGCGGTTTTGGCAGTACATCAATGGGCGTGGATAGCGGTTT
GACTCACGGGGATTTCGAAGTCTCCACCCATTGACGTCAATGGGAGTTTGTGTTGCACCAAAATCAACG
GGACTTTCCAAAATGTCGTAACAACTCCGCCCCATTGACGCAAATGGGCGGTAGGCGTGACGGTGGGAG
GTCTATATAAGCAGAGCTCGTTTAGTGAACCGTCAGATCGCCTGGAGACGCCATCCACGCTGTTTTGACC
TCCATAGAAGACACCGGGACCGATCCAGCCTCC
```

[SEQ ID NO:3]

[0034] In another preferred embodiment, the transgene expression cassette comprises a grp78 promoter. The nucleic acid sequence of one embodiment of the grp78 promoter is represented herein as SEQ ID No: 8, as follows:

```
CCCGGGGGCCCAACGTGAGGGGAGGACCTGGACGGTTACCGGCGGAAACGGTTTCCAGGTGAGAGGTCAC
CCGAGGGACAGGCAGCTGCTCAACCAATAGGACCAGCTCTCAGGGCGGATGCTGCCTCTCATTGGCGGGCC
GTTAAGAAATGACCACTAGCCAATGAGTCGGCTGGGGGGCGCGTACCAGTGACGTGAGTTGCGGAGGAGGC
CGCTTCGAATCGGCAGCGGCCAGCTTGGTGGCATGAACCAACAGCGGCCTCCAACGAGTAGCGAGTTCA
CCAATCGGAGGCCTCCACGACGGGGCTGCGGGGAGGATATATAAGCCGAGTCGCGGACCGGCGCGCTCGA
TACTGGCTGTGACTACACTGACTTGGAC
```

[SEQ ID NO: 8]

[0035] Preferably, the transgene expression cassette comprises nucleic acid for encoding a

polyA tail attachable to the expressed agent. The DNA sequence of one embodiment of the nucleic acid for encoding a polyA tail is represented herein as SEQ ID No: 4, as follows:

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ACGGGTGGCATCCCTGTGACCCCTCCCCAGTGCCTCTCCTGGCCCTGGAAGTTGCCACTCCAGTGCCAC
CAGCCTTGTCTAATAAAATTAAGTTGCATCATTTTGTCTGACTAGGTGTCCTTCTATAATATTATGGGG
TGGAGGGGGGTGGTATGGAGCAAGGGGCAAGTTGGGAAGACAACCTGTAGGGCTGCGGGGTCTATTGGG
AACCAAGCTGGAGTGCAGTGGCACAATCTTGGCTCACTGCAATCTCCGCCCTCCTGGGTTCAAGCGATTCT
CCTGCCTCAGCCTCCCGAGTTGTGGGATTCCAGGCATGCATGACCAGGCTCAGCTAATTTTGTTTTTT
TGGTAGAGACGGGGTTTACCATATTGGCCAGGCTGGTCTCCAACCTCTAATCTCAGGTGATCTACCCAC
CTTGGCCTCCCAAATGCTGGGATTACAGGCGTGAACCACTGCTCCCTTCCCTGTCCTT
```

[SEQ ID NO:4]

[0036] Preferably, the transgene expression cassette comprises left and/or right Inverted Terminal Repeat sequences (ITRs). An ITR can be specific to an AAV serotype, and can be any sequence, so long as it forms a hairpin loop in its secondary structure. For example, the AAV serotype may be AAV1-9, but is preferably AAV1, AAV2, AAV5, AAV6 or AAV8. The DNA sequence of one embodiment (left ITR from a commercially available AAV plasmid) of the ITR is represented herein as SEQ ID No: 5, as follows:

```
CCTGCAGGCAGCTGCGCGCTCGCTCGCTCACTGAGGCCGCCGGGCGTCGGGCGACCTTTGGTCCGCCGG
CCTCAGTGAGCGAGCGAGCGCGCAGAGAGGAGTGGCCAACCTCCATCACTAGGGGTTCTT
```

[SEQ ID NO:5]

[0037] The DNA sequence of another embodiment (right ITR from a commercially available AAV plasmid) of the ITR is represented herein as SEQ ID No: 6, as follows:

```
AGGAACCCCTAGTGATGGAGTTGGCCACTCCCTCTCTGCGCGCTCGCTCGCTCACTGAGGCCGGGCGACC
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AAAGGTCGCCCGACGCCCGGGCTTTGCCCGGGCGGCCTCAGTGAGCGAGCGAGCGCGCAGCTGCCTGCAG
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G

[SEQ ID NO:6]

[0038] Preferably, the genome of the recombinant phagemid particle comprises a selection marker, which will depend on the host cell in which the vector is harboured, for example for conferring ampicillin resistance in a host cell, preferably a bacterium. The marker provides selection pressure during production of the phagemid particle in the host cell.

[0039] Preferably, the recombinant phagemid particle comprises one or more capsid minor coat protein. The recombinant phagemid particle may comprise a pIII capsid minor coat protein that is configured to display a cell-targeting ligand for enabling delivery of the particle to the target cell. Preferably, the recombinant phagemid particle comprises one or more capsid major coat protein. The recombinant phagemid particle may comprise at least one pVIII capsid major coat protein that is configured to display a foreign peptide thereon.

[0040] The recombinant phagemid particle may comprise a modification of the capsid structure, for example by treatment, or chemical or biochemical conjugation. Examples of suitable modifications may include cross-linking peptide residues on to the phagemid particle.

In another embodiment, the recombinant phagemid particle may comprise one or functional peptide attached to the capsid thereof. For example, a functional peptide may comprise a nuclear translocation signal. The phagemid particle may therefore be multifunctional, and use features disclosed in WO 2014/184528.

[0041] In another embodiment, the recombinant phagemid particle may be combined with a cationic polymer to form a complex having a net positive charge, as described in WO 2014/184529. The cationic polymer may be selected from a group consisting of: chitosan; poly-D-lysine (PDL); diethylaminoethyl (DEAE); diethylaminoethyl-dextran (DEAE.DEX); polyethyleneimine (PEI); polybrene; protamine sulphate; and a cationic lipid. Preferably, the cationic lipid is selected from the group consisting of fugene[®], lipofectamine[®], and DOTAP (N-[1-(2,3-Dioleoyloxy)propyl]-N,N,N-trimethylammonium methyl-sulfate). Preferably, the cationic polymer comprises DEAE, more preferably DEAE.DEX.

[0042] Preferably, the phagemid particle comprises a genome which substantially lacks the phage genome from which the particle is derived. Preferably, the genome of the recombinant phagemid particle lacks at least 60%, more preferably at least 70%, and even more preferably at least 80% of the bacteriophage genome from which it is derived. More preferably, the genome of the recombinant phagemid particle lacks at least 90%, more preferably at least 95%, and even more preferably at least 99% of the bacteriophage genome from which it is derived. Preferably, the genome of the recombinant phagemid particle lacks all of the bacteriophage genome from which it is derived. As discussed above, however, the genome of the phagemid viral particle may, in some embodiments, comprise the bacteriophage origin of replication for enabling replication of the particle into single-stranded DNA, i.e. F1 bacteriophage ori.

[0043] Preferably, the phagemid particle lacks bacteriophage structural genes in its genome required for the formation, packaging or extrusion of the particle from a prokaryotic host. Such structural genes encode the capsid proteins etc. Preferably, the phagemid particle comprises a genome which lacks a gene encoding a minor or major coat protein from which the particle is derived. Preferably, the phagemid particle comprises a genome which lacks a pIII capsid minor coat protein, or which lacks a pVIII capsid major coat protein. Most preferably, the phagemid particle comprises a genome which lacks both a pIII capsid minor coat protein, and a pVIII capsid major coat protein.

[0044] Thus, the recombinant phagemid particle preferably comprises a replication-deficient, virus-like-particle or virion constructed from, and displaying, the structural components, including but not limited to proteins and other conjugated compounds, derived from a bacteriophage, despite the genome of the particle not containing the structural genes of a bacteriophage from which it is derived.

[0045] Accordingly, given that the genome of the recombinant phagemid particle of the disclosure lacks the derivative phage genome, including the structural genes, an alternative system is required in order to provide the necessary structural (i.e. capsid) genes that are

required to package the recombinant phagemid genome in a bacteriophage capsid to produce the particle of the invention. Accordingly, the inventors have devised a novel system for producing the particles of the disclosure, involving the use of a separate so-called "helper virus" vector. In effect, therefore, the particle of the disclosure is a hybrid phagemid vector, which includes components of a phagemid and a eukaryotic virus.

[0046] Hence, in an example, there is provided a system for producing a recombinant phagemid particle from a prokaryotic host, the system comprising:-

1. (i) a first vector configured to persist inside a prokaryotic host, and comprising at least one transgene expression cassette, and a packaging signal for enabling replication of the vector into single-stranded DNA; and
2. (ii) a second vector comprising nucleic acid encoding structural proteins required for packaging the single-stranded DNA, resulting in the formation and extrusion of a recombinant phagemid particle from the prokaryotic host.

[0047] Advantageously, separating the reproductive elements of the phagemid particle into the first "therapeutic" vector carrying the transgene, and the second separate "helper" vector carrying the viral packaging structural genes substantially decreases the genome/vector size, and thereby significantly increases transgene capacity. In embodiments in which the phagemid particle is used therapeutically, this is a particularly useful advantage for gene therapy applications of the new system. Consequently, this results in an enhanced production yield, gene transduction efficiency and flexibility of the vector system for other applications.

[0048] The novelty of the system according to the disclosure is its ability to package the genome of AAV which is provided by the first vector, into a prokaryotic virus capsid (i.e. bacteriophage), which is provided by the second vector. Thus, while the prior art system (i.e. AAVP) is a chimera of two genomes, the system of the disclosure (i.e. PAAV) is a chimera between prokaryotic viral phenotypes and a eukaryotic viral genotype.

[0049] Preferably, the system of the disclosure is used to produce the recombinant phagemid particle according to the disclosure. Preferably, the first vector therefore comprises the genome of the recombinant phagemid particle. The packaging signal of the first vector may preferably comprise an origin or replication. Preferably, the origin of replication in the first vector comprises an F1 ori, more preferably from an F1 bacteriophage.

[0050] Preferably, the first vector comprises a second origin of replication for enabling replication of double-stranded vector inside a prokaryotic host. Preferably, the origin of replication enables high copy number replication of the vector inside the host. Preferably, the origin of replication comprises a pUC ori. Alternatively, the first vector may comprise one or more DNA sequence, which favours targeted integration into a host genome, thus removing the requirement for any origin of replication.

[0051] The transgene expression cassette comprises a viral transgene expression cassette, more preferably a mammalian viral transgene expression cassette. The at least one transgene expression cassette comprises AAV transgene expression cassette.

[0052] One preferred embodiment of the second vector is illustrated in Figure 7, with preferred components being shown in Figure 8. The second vector or "helper phage" is preferably a bacteriophage engineered specifically for rescuing the phagemid particles carrying the first vector (i.e. the phagemid particle's genome) from prokaryotic hosts, an embodiment of which is shown in Figure 3. The second vector (i.e. the helper phage) is therefore provided to lend its proteins and polypeptides to the first vector (i.e. the phagemid particle's genome), or any other DNA entity that contains a functional packaging signal and/or a single stranded origin or replication. The second vector is most preferably replication-defective. Preferably, the second vector comprises a disrupted packaging signal, which significantly deters its ability to package itself into phage particles. Preferably, the second vector comprises a disrupted origin of replication. In one embodiment, the disrupted origin of replication is a medium copy number origin, such as p15a. In another embodiment, the disrupted origin of replication is a low copy number origin, such as a pMB1. Preferably, the first vector (i.e. the phagemid particle's genome) is configured to outcompete with the second vector (i.e. the helper phage) in both replication and packaging.

[0053] The genome of the second vector may be engineered to give the resultant recombinant phagemid particle targeting properties (or multifunctional properties as described in WO 2014/184528). Hence, it provides the structural capsid proteins for phagemid particle assembly. Preferably, the second vector comprises nucleic acid encoding one or more capsid minor coat proteins, or one or more capsid major coat proteins. All capsid proteins may either be wild type or recombinant, present in single or multiple copies, and modified to display chimeric or synthetic peptides. This includes the display of antigens of other viruses for peptide vaccine delivery or as an adjuvant in the case that a DNA vaccine (delivered by the phagemid particle of the disclosure) is desired.

[0054] In one embodiment, therefore, the second vector may comprise a first nucleic acid sequence encoding a pIII capsid minor coat protein that is configured to display a cell-targeting ligand for enabling delivery of the recombinant phagemid particle to a target cell (e.g. a tumour). Therefore, it may be desired to induce a 9-amino acid mutation in the pIII minor coat protein of the recombinant phagemid particle in order to confer its specificity to tumour cells and angiogenic tumour-associated endothelial cells that express $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins. Thus, the genome of the second vector may comprise the RGD4C targeting peptide (CDCRGDCFC - SEQ ID No: 7).

[0055] In another embodiment, the second vector may comprise a second nucleic acid sequence encoding at least one pVIII capsid major coat protein that is configured to display a foreign peptide thereon. Thus, it may be desired to induce a mutation in the wild pVIII major coat protein of the recombinant phagemid particle in order to display a short peptide, for example less than 10 amino acids long. The short peptide may be a targeting moiety or have

inherent biological/chemical functionality in vivo or in vitro. For example, immune stimulation in vivo via antigen display, or binding to nanoparticles (e.g. gold) in vitro via displaying a gold-binding peptide.

[0056] The first vector is a member of the adeno-associated virus species.

[0057] Once the first vector (i.e. the phagemid particle's genome) and the second vector (i.e. the Helper phage) have been constructed, they are used together to produce, in a prokaryotic host, the recombinant phagemid particle of the disclosure. It will be appreciated that the packaging signal (e.g. the origin of replication) of the first vector, which is for enabling replication of the phagemid genome into single-stranded DNA, functions to signal the second vector (i.e. the helper phage) structural proteins to package the phagemid genome (i.e. they work together in trans in the host) to create the particle of the disclosure.

[0058] In one preferred embodiment, the first vector (phagemid particle genome) comprises a nucleic acid sequence substantially as set out in SEQ ID No: 9, or a fragment or variant thereof, wherein SEQ ID No: 9 is represented as follows:

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CCTGCAGGCAGCTGCGCGCTCGCTCGCTCACTGAGGCCGCCCGGGCGTCGGGCGACCTTTGGTCGCCCCG
CCTCAGTGAGCGAGCGAGCGCGCAGAGAGGGAGTGGCCAACTCCATCACTAGGGGTTCTGCGGCCGC--
-TRANSGENE---
AGGAACCCCTAGTGATGGAGTTGGCCACTCCCTCTCTGCGCGCTCGCTCGCTCACTGAGGCCGGGCGACC
AAAGGTCGCCCCAGCGCCCGGGCTTTGCCCGGGCGGCCTCAGTGAGCGAGCGAGCGCGCAGCTGCCTGCAG
GGGCGCCTGATGCGGTATTTTCTCCTTACGCATCTGTGCGGTATTTACACCGCATACTGTCAAAGCAACC
ATAGTACGCGCCCTGAGCGGCGCATTAAAGCGCGCGGGTGTGGTGGTTACGCGCAGCGTGACCGCTACA
CTTGCCAGCGCCCTAGCGCCCGCTCCTTTTCGCTTTCTTCCCTTCCCTTTCTCGCCACGTTTCGCGGCTTTC
CCCGTCAAGCTCTAAATCGGGGGCTCCCTTTAGGGTTCCGATTTAGTGCTTTACGGCACCTCGACCCCAA
AAAACCTTGATTTGGGTGATGGTTCACGTAGTGGGCCATCGCCCTGATAGACGGTTTTTCGCCCTTTTGACG
TTGGAGTCCACGTTCTTTAATAGTGGACTCTTGTTCCAACTGGAACAACACTCAACCCTATCTCGGGCT
ATTCTTTTGATTTATAAGGGATTTTGCCGATTTTCGGCCTATTGGTTAAAAAATGAGCTGATTAACAAA
ATTTAACCGGAATTTTAAACAAATATTAACGTTTACAATTTTATGGTGCACTCTCAGTACAATCTGCTCT
GATGCCGCATAGTTAAGCCAGCCCCGACACCCGCCAACACCCGCTGACGCGCCCTGACGGGCTTGCTGTC
TCCCGGCATCCGCTTACAGACAAGCTGTGACCGTCTCCGGGAGCTGCATGTGTGTCAGAGGTTTTCACCGTC
ATCACCGAAACGCGCGAGACGAAAGGGCCTCGTGATACGCTATTTTATAGGTTAATGTCATGATAATA
ATGGTTTCTTAGACGTCAGGTGGCACTTTTCGGGGAAATGTGCGCGGAACCCCTATTTGTTTATTTTCT
AAATACATTCAAATATGTATCCGCTCATGAGACAATAACCCCTGATAAATGCTTCAATAATATGAAAAAG
GAAGAGTATGAGTATTCACATTTCCGTGTGCGCCTTATTCCTTTTTCGCGCATTTTGCCCTTCCTGTT
TTTGCTCACCCAGAAACGCTGGTGAAGTAAAGATGCTGAAGATCAGTTGGGTGCACGAGTGGGTTACA
TGAAGTGGATCTCAACAGCGGTAAAGATCCTTGAGAGTTTTCGCCCGAAGAACGTTTTCCAATGATGAG
CACTTTTAAAGTTCGCTATGTGGCGGGTATTATCCCGTATTGACGCGGGCAAGAGCAACTCGGTGCG
CGCATACACTATTCTCAGAAATGACTTGGTTGAGTACTACCAGTACAGAAAAGCATCTTACGGATGGCA
TGACAGTAAGAGAATTATGCAGTGCTGCCATAACCATGAGTGATAAACAACGCGGCCAACTTACTTCTGAC
AACGATCGGAGGACCGAAGGAGCTAACCGCTTTTTTGCAACAATGGGGGATCATGTAACTCGCCTTGAT
CGTTGGGAACCGGAGCTGAATGAAGCCATAACCAACGACGAGCGTGACACCACGATGCCGTAGCAATGG
CAACAACGTTGCGCAAACTATTAACGCGGAACACTTACTCTAGCTTCCCGGCAACAATTAATAGACTG
GATGGAGGCGGATAAAGTTGCGAGGACCACTTCTGCGCTCGGCCCTTCCGGCTGGCTGGTTTATTGCTGAT
AAATCTGGAGCCGGTGAGCGTGGGTCTCGCGGTATCATTGCAGCACTGGGGCCAGATGGTAAGCCCTCCC
GTATCGTAGTTATCTACAGACGGGGAGTCAGGCAACTATGGATGAACGAAATAGACAGATCGCTGAGAT
AGGTGCCCTCACTGATTAAGCATTGGTAACGTGACACCAAGTTTACTCATATATACTTTAGATTGATTTA
AAACTTCATTTTAAATTTAAAGGATCTAGGTGAAGATCCTTTTGTATAATCTCATGACCAAAATCCCTT
AACGTGAGTTTTCTTCCACTGAGCGTCAGACCCCGTAGAAAAGATCAAAGGATCTTCTTGAGATCCTTT
TTTTCTGCGCGTAATCTGCTGCTTGCAACAAAAAACCACCGCTACCAGCGGTGGTTTGTGTGCGCGAT
CAAGAGCTACCAACTCTTTTTCCGAAGGTAACCTGGCTTCAGCAGAGCGCAGATACCAAACTACTGTCCTT
TAGTGTAGCCGTAGTTAGGCCACCACTTCAAGAACTCTGTAGCACCGCCTACATACCTCGCTCTGCTAAT
CCTGTTACAGTGGCTGCTGCCAGTGGCGATAAGTCTGTCTTACCAGGTTGGACTCAAGACGATAGTTA
CCGGATAAGGCGCAGCGGTGCGGCTGAACGGGGGGTTCGTGCACACAGCCAGCTTGGAGCGAACGACCT
ACACCGAACTGAGATACCTACAGCGTGAGCTATGAGAAAGCGCCACGCTTCCCGAAGGGAGAAAGGCGGA
CAGGTATCCGGTAAGCGGCAGGGTCCGAACAGGAGAGCGCACAGGGGAGCTTCCAGGGGGAAACGCCTGG
TATCTTTATAGTCCIGTCGGGTTTCGCCACCTCTGACTTGAGCGTCGATTTTTGTGATGCTCGTCAGGGG
GGCGGAGCCTATGGAACCAACGCGAGCAACGCGGCCTTTTACGGTTCCTGGCCTTTTGCTGGCCTTTTGC
TCACATGT
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[illegible]

TTTGTTCCTTGAATGTTTGTTCATCATCTCTTTTGTCTCAGGTAATGAAATGAATAATTCGCTCTGC
GCGATTTTGTAACTTGGTATTTCAAAGCAATCAGGCGAATCCGTTATGTTTCTCCCGATGTAAAAGGTAC
TGTTACTGTATATTCATCTGACGTTAAACCTGAAAATCTACGCAATTTCTTTATTTCTGTTTACGTGCT
AATAATTTTGATATGGTTGGTTCAATTCCTTCCATAATTCAGAAGTATAATCCAAACAATCAGGATTATA
TTGATGAATTGCCATCATCTGATAATCAGGAATATGATGATAATTCGCTCCTTCTGGTGGTTTCTTTGT

TCCGCAAAATGATAATGTTACTCAAACCTTTTAAATTAATAACGTTCCGGGCAAAGGATTTAATACGAGTT
GTCGAATTGTTTGTAAAGTCTAATACTTCTAAATCCTCAAATGTATATCTATTGACGGCTCTAATCTAT
TAGTTGTTAGTGCACCTAAAGATATTTTAGATAACCTTCTCAATTCCTTTCTACTGTTGATTGGCCAAAC
TGACCAGATATTGATTGAGGGTTTGATATTTGAGGTTTACGCAAGGIGATGCTTTAGATTTTTTCATTTGCT
GCTGGCTCTCAGCGTGGCACTGTTGAGGCGGTGTTAATACTGACCGCTCACCCTCTGTTTATCTTCTG
CTGGTGGTTCGTTCCGTTATTTTAAATGGCGATGTTTTAGGGCTATCAGTTCCGCGCATTAAGACTAATAG
CCATTCAAAAATATGTCTGTGCCACGTATCTTACGCTTTCAGGTCAGAAGGGTTCTATCTCTGTTGGC
CAGAATGTCCCTTTTATTACTGGTCTGTGTGACTGGTGAATCTGCCAATGTAAATAATCCATTTAGACGA
TTGAGCGTCAAAATGTAGGTATTTCCATGAGCGTTTTTCTGTTGCAATGGCTGGCGGTAATATTGTTCT
GGATATTACCAGCAAGGCCGATAGTTTGTAGTTCTTCTACTCAGGCAAGTGATGTTATTACTAATCAAAGA
AGTATTGCTACAACGGTTAATTTGCGTGATGGACAGACTCTTTTACTCGGTGGCTCACTGATTATAAAA
ACACTTCTCAAGATCTGGCGTACCGTTCTCTGTCTAAATCCCTTTAATCGGCCCTCTGTTAGCTCCCG
CTCTGATTCCCAACGAGGAAAGCACGTTATACGTGCTCTGTCAAAGCAACCATAGTACGCGCCCTGTAGCGG
CGCATTAAAGCGCGGCGGGTGTGGTGGTTACGCGCAGCGTGACCGCTACACTTGCAGCGCCCTAGCGCCC
GCTCCTTTTCGCTTTCTTCCCTTCTTCTCGCCACGTTCCGCGGCTTTCCCGCTCAAGCTCTAAATCGGG
GGCTCCCTTTAGGGTTCCGATTAGTGCTTTACGGCACCTCGACCCCAAAAACTTGATTGGGTGATGG
TTCACGTAGTGGGCCATCGCCCTGATAGACGGTTTTTCGCCCCTTGACGTTGGAGTCCACGTTCTTTAAT
AGTGGACTCTTGTTCCAAACCTGGAAACAACACTCAACCTATCTCGGGACGGATCGCTTCATGTGGCAGGA
GAAAAAAGGCTGCACCGGTGCGTCAGCAGAATATGTGATACAGGATATATCCGCTTCTCTGCTCACTGA
CTCGCTACGCTCGGTCTGCTGACTGCGGCGAGCGGAAATGGCTTACGAACGGGGCGGAGATTTCTTGGA
GATGCCAGGAAGATACTTAACAGGGAAGTGAGAGGGCCGCGGCAAGGCCGTTTTTCCATAGGCTCCGCC
CCCTGACAAGCATCACGAAATCTGACGCTCAAACTCAGTGGTGGCGAAACCCGACAGGACTATAAAGATA
CAGGCGTTTCCCTTGGCGGCTCCCTCGTGCCTCTCTGTTCTCTGCTTTTCGGTTTACCGGTGTCATT
CGCTGTTATGGCCGCTTTGTCTCATTCCACGCTGACACTCAGTTCCGGGTAGGCAGTTGCTTCAAGC
TGGACTGTATGCACGAACCCCGTTTCTAGTCCGACCGCTGCGCTTATCCGGTAACTATCGTCTTGAGTC
CAACCCGGAAGACATGCAAAAGCACCACTGGCAGCAGCCACTGGTAATTGATTAGAGGAGTTAGTCTT
GAAGTATGCGCGGTTAAGGCTAAACTGAAAGGACAAGTTTGGTGACTGCGCTCTCCAGCCAGTTA
CCTCGGTTCAAAGAGTTGGTAGCTCAGAGAACCTTCGAAAAACCGCCCTGCAAGGCGGTTTTTTCGTTTT
CAGAGCAAGAGATTACGCGCAGACCAAAACGATCTCAAGAAGATCATCTTATTAAGGGGTCTGACGCTCA
GTGGAACGAAAACCTCACGTTAAGGGATTTTGGTCTAGAGATTATCAAAAAGGATCTTACCTAGATCCTT
TTAAATTAATAATGAAGTTTTTAATCAATCTAAAGTATATATGAGTAAACTTGGTCTGACAGTTACCAAT
GCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTCTGTTTATCCATAGTTGCTTGACTCCCGT
CGTGTAGATACTACGATACGGGAGGGCTTACCATCTGGCCCAAGTGTGCAATGATACCGGAGACCA
CGCTCACCAGGCTCCAGATTTATCAGCAATAAACACGACCGGATTGAGCTCGCCCCGGGGATCGACCA
GTTGGTGATTTTGAACTTTGTCTTGGCACGGAACGGTCTGCGTTGTGCGGAAGATGCGTGATCTGATCC
TTCAACTCAGCAAAAGTTTCGATTATTTCAACAAAGCCCGCTCCCGTCAAGTCAGCGTAATGCTCTGCCA
GTGTTACAACCAATTAACCAATCTGATTAGAAAACTCATCGAGCATCAATGAAACTGCAATTTATCT
ATATCAGGATTATCAATACCATATTTTGTAAAAAGCCGTTTCTGTAAATGAAGGAGAAAACTACCGAGGC
AGTTCCATAGGATGGCAAGATCTGGTATCGGTCTGCGATTCCGACTCGTCCAAACATCAATACAACCTAT
TAATTTCCCTCTGTCAAAAATAAGGTTATCAAGTGAGAAATCACCATGAGTGACGACTGAATCCGGTGAG
AATGGCAAAAGCTTATGCATTTCTTCCAGACTTGTTCACAGGCCAGCCATTACGCTCGTCATCAAAAT
CACTCGCATCAACCAACCGTTATTCATTCTGTTGCGCTGAGCGAGACGAAATACGCGATCGCTGTT
AAAAGGACAATTACAACAGGAATCGAATGCAACCGGCGCAGGAACACTGCCAGCGCATCAACAATATTT
TCACCTGAATCAGGATATTTCTTAATACCTGGAATGCTGTTTTCCCGGGGATCGCAGTGGTGAGTAACC
ATGCATCATCAGGAGTACGGATAAAATGCTTGATGGTGGGAAGAGGCATAAATCCGTGACCCAGTTTAG
TCTGACCATCTCATCTGTAACATCATTTGGCAACGCTACCTTTGCCATGTTTCAGAAACAACCTTGGCGCA
TCGGGCTTCCCATACAATCGATAGATTGTGCGACCTGATTGCCCGACATTATCGCGAGCCCATTTATACC
CATATAAATCAGCAATCATGTTGGAATTTAATCGCGGCTCGAGCAAGACGTTTCCGTTGAATATGGCT
CATAACACCCCTTGTATTACTGTTTATGTAAGCAGACAGTTTTATTGTTTCAATGATGATATATTTTATCT
TGTGCAATGTAACATCAGAGATTTTGTAGACACAACGTGGCTTTCCCCCCCCCCCCCTGCAGGTCTCGGGC
TATTTCTTTGATTTATAAGGGATTTTGGCGATTTTCGGCTATTGGTTAAAAAATGAGCTGATTTAACAAA
AATTTAACCGGAATTTTAAACAAATATTAACGTTTACAAATTTAAATATTTGCTTATACAATCTTCTGTT
TTTGGGGCTTTTCTGATTATCAACCGGGGTACATATGATTGACATGCTAGTTTTACGATTACCGTTTCAT
GATTTCTTGTTTGTCTCAGACTCTCAGGCAATGACCTGATAGCCTTTGTAGACCTCTCAAAAATAGCTA
CCCTCTCCGGCATGAATTTATCAGCTAGAACGGTTGAATATCATATTGATGGTGATTTGACTGTCTCCGG
CCTTTCTCACCCTTTTGAATCTTTACCTACACATTACTCAGGCATTGCATTTAAATATATGAGGGTTCT
AAAAATTTTATCCTTGCCTTGAATAAAGGCTTCTCCCGCAAAAGTATTACAGGGTCATAATGTTTTTG
GTACAACCGATTAGCTTTATGCTCTGAGGCTTTATTGCTTAATTTTGCTAATTTCTTTGCCTTGCCTGTA
TGATTTATTGGATGTT

[SEQ ID No: 10]

[0060] In one preferred embodiment, the second vector (helper phage without RGD sequence) comprises a nucleic acid sequence substantially as set out in SEQ ID No: 11, or a fragment or variant thereof, wherein SEQ ID No: 11 is represented as follows:

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AACGCTACTACTATTAGTAGAATTGATGCCACCTTTTCAGCTCGCGCCCCAAATGAAAATATAGCTAAAC
AGGTTATTGACCATTTGCGAAATGTATCTAATGGTCAAACATAAATCTACTCGTTCGCAGAATTGGGAATC
AACGTGTTACATGGAATGAACTTCCAGACACCGTACTTTAGTTGCATATTTAAACATGTTGAGCTACAG
CACCAGATTGAGCAATTAAGCTCTAAGCCATCCGCAAAAATGACCTCTTATCAAAAGGAGCAATTAAGG
TACTCTCTAATCCTGACCTGTTGAGTTTGGCTTCCGGTCTGGTTTCGCTTTGAAGCTCGAATTAACCGCG
ATATTTGAAGTCTTTCCGGCTTCTCTTAATCTTTTGTATGCAATCCGCTTTGCTTCTGACTATAATAGT
CAGGGTAAAGACCTGATTTTTGATTATGGTCATTCTCGTTTTCTGAACTGTTTAAAGCATTGAGGGGG
ATTCAATGAATATTTATGACGATTCCGACGATTGGACGCTATCCAGTCTAAACATTTTACTATTACCCC
CTCTGGCAAAACTTCTTTTGCAAAAGCCTCTCGCTATTTTGGTTTTTATCGTCTGTTGGTAAACGAGGGT
TATGATAGTGTGTCTTACTATGCTCGTAATTCCTTTTGGCGTTATGTATCTGCATTAGTTGAATGTG
GTATTCCTAAATCTCAACTGATGAATCTTTCTACCTGTAATAATGTTGTTCCGTTAGTTTCGTTTATTAA
CGTAGATTTTTCTTCCCAACGCTCTGACTGGTATAATGAGCCAGTTCTTAAATCGCATAAGGTAATTCA
CAATGATTAAAGTTGAAATTAACCATCTCAAGCCCAATTACTACTCGTTCTGGTGTTCCTCGTCAGGG
CAAGCCTTATTCAGTGAATGAGCAGCTTTGTTACGTTGATTGGGTAAATGAATATCCGGTTCTTGTCAAG
ATTACTCTTGATGAAGGTGAGCCAGCCTATGCGCCTGGTCTGTACACCGTTTCACTGTCTCTTTCAAAG
TTGGTCAGTTCCGTTCCCTTATGATTGACCGTCTGCGCCTCGTTCCGGCTAAGTAACATGGAGCAGGTG
CGGATTTTCGACACAATTTATCAGGCGATGATACAAATCTCCGTTGTACTTTGTTTTCGCGCTTGGTATAAT
CGCTGGGGGTCAAAGATGAGTGTGTTTAGTGTATTTTCGCCCTCTTTTCGTTTTAGGTTGGTGCCTTCGTA
GTGGCATTACGTATTTTACCGGTTAATGGAACCTTCCCTCATGAAAAGTCTTTAGTCTCAAAGCCTCT
GTAGCCGTTGCTACCCCTCGTTCCGATGCTGCTTTTCGCTGCTGAGGGTGACGATCCCGCAAAAGCGGCCT
TTAACTCCCTGCAAGCCTCAGCGACCGAATATATCGGTTATGCGTGGCGGATGGTTGTTGTCTATGTGCGG
CGCAACTATCGGTATCAAGCTGTTTAAAGAAATTCACCTCGAAAGCAAGCTGATAAACCAGATACAATTA
GGCTCCTTTTGGAGCCTTTTTTTTTGGAGATTTTCAACGTGAAAAAATATTATTTCGCAATTCCTTTAGT
TGTTCCCTTCTATTCTCACTCCGCTGAAACTGTTGAAAGTTGTTTAGCAAAACCCCATACAGAAAAATCA
TTTACTAACGCTCTGGAAGACGACAAAACTTTAGATCGTTACGCTAACTATGAGGGTTGTCTGTGGAATG
CTACAGGCGTTGTAGTTTGTACTGGTGACGAACTCAGTGTACGGTACATGGGTTCTTATTGGGCTTGC
TATCCCTGAAAATGAGGGTGGTGGCTCTGAGGGTGGCGGTTCTGAGGGTGGCGGTTCTGAGGGTGGCGGT
ACTAAACCTCCTGAGTACGGTGATACACCTATTCGGGCTATACCTTATATCAACCTCTCGACGGCATT
ATCCGCCCTGGTACTGAGCAAAAACCCGCTAATCCTTCTCTTGAGGAGTCTCAGCCTCTTAATAC
TTTCAATGTTTCAAGATAATAGGTTCCGAAATAGGCAGGGGGCATTAACTGTTTATACGGGCACTGTTACT
CAAGGCACTGACCCCGTTAAACTTATTACCAGTACACTCCTGTATCATCAAAAGCCATGTATGACGCTT
ACTGGAACGGTAAATTCAGAGACTGCGCTTTCCATTCTGGCTTTAATGAGGATCCATTTCGTTTGTGAATA
TCAAGGCCAATCGTCTGACCTGCCTCAACCTCCTGTCAATGCTGGCGGCGGCTCTGGTGGTGGTTCTGGT
GGCGGCTCTGAGGGTGGTGGCTCTGAGGGTGGCGGTTCTGAGGGTGGCGGCTCTGAGGGAGGCGGTTCCG
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AAATGCCGATGAAAACGCGCTACAGTCTGACGCTAAAGGCAAACTTGATTCTGTGCTACTGATTACGGT
GCTGCTATCGATGGTTTCAATGGTGACGTTTCCGGCCTTGCTAATGGTAATGGTGTACTGGTGATTTTG
CTGGCTCTAATTCCTCAATAGGCTCAAGTCCGTTGACGGTGATAATTCACCTTTAATGAATAATTTCCGTCA
ATATTTACCTTCCCTCCCTCAATCGGTTGAATGTGCGCCTTTTGTCTTTAGCGCTGGTAAACCATATGAA
TTTTCTATTGATTGTGACAAAAATAAATTTATCCGTGGTGTCTTTGCGTTTTCTTTATATGTTGCCACCT
TTATGTATGTATTTTCTACGTTTGTCTAACATACTGCGTAATAAGGAGTCTTAATCATGCCAGTCTTTTG
GGTATTCGGTTATTATGCGTTTTCTCGGTTTCTTTCGTTAACTTTGTTTCGGCTATCTGCTTACTTTTC
TTAAAAAGGGCTTCGGTAAGATAGCTATTGCTATTTTCAATGTTTCTTGTCTTTATTTATTTGGGCTTAACTC
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AATTAGGCTCTGGAAGACGCTCGTTAGCGTTGGTAAGATTGAGGATAAAATTGTAGCTGGGTGCAAAAT
AGCAACTAATCTTGATTAAAGGCTTCAAAACCTCCCGCAAGTGGGAGGTTTCGCTAAAACGCGCTCGCGTT
CTTAGAATACCGGATAAGCCTTCTATATCTGATTGCTTGTCTATTGGGCGCGGTAATGATTCTACGATG
AAAAATAAACGGCTTGCTTGTCTCGATGAGTGCCTGTTGTTTTAATACCGGTTCTTGGAAATGATAA
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GGAAAGACAGCCGATTATTGATTGGTTTTCTACATGCTCGTAAATAGGATGGGATATTATTTTTCTTGTT
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TGGACAGAAATACCTTTTACCTTTTGTGCGTACTTTATATCTCTTATTACTGGCTCGAAAATGCCTCTGCC
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ATTCTTATTTAAGCCTTATTTATCACACGGTCCGTTATTTCAAACCATTAATTTAGGTCAGAAGATGAA
ATTAATAAAAAATATTTGAAAAAGTTTTCTCGCGTTCTTTGTCTTGGCATTGGATTGTCATCAGCATT
ACATATAGTTATATAACCCAACCTAAGCCGGAGGTTAAAAAGGTAGTCTCTCAGACCTATGATTTTGATA
AATTCATATTGACTCTTCTCAGCGTCTTAATCTAAGCTATCGCTATGTTTTCAAGGATTCTAAGGGAAA
ATTAATTAATAGCGACGATTTACAGAAGCAAGGTTATTCACCTACATATATTGATTTATGTACTGTTTCC
ATTAATAAAGGTAATTCAAATGAAATTTGTAATGTAATTAATTTGTTTTCTTGATGTTTGTTCATCA
TCTTCTTTTGTCTCAGGTAATTTGAAATGAATAATTCGCCCTCTGCGCGATTTTGTAACTGGTATTCAAAGC
AATCAGGCGAATCCGTTATTGTTTCTCCCGATGTAAAGGTACTGTTACTGTATATTCACTGACGTTAA
ACCTGAAAAATCTACGCAATTTCTTTATTTCTGTTTTACGTGCTAATAATTTTGATATGGTTGGTTCAATT
CCTTCCATAATTCAGAAGTATAATCCAAACAATCAGGATTATATTGATGAATTGCCATCATCTGATAATC
AGGAATATGATGATAATTCGCCCTCCTTCTGGTGGTTTTCTTTGTTCCGCAAAATGATAATGTACTCAAAC
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TTTTAAATTAATAACGTTCCGGGCAAAGGATTTAATACGAGTTGTGCAATTGTTTGTAAGTCTAATACT
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 TAGATAACCTTCTCAATTCTTCTACTGTTGATTTGCCAACTGACCAGATATTGATTGAGGGTTTGAT
 ATTTGAGGTTTACGCAAGGTGATGCTTTAGATTTTTTCATTTGCTGCTGGCTCTCAGCGTGGCACTGTTGCA
 GGCGGTGTTAAATACTGACCGCCTCACCTCTGTTTTATCTTCTGCTGGTGGTTTCGTTTCGGTATTTTTAAATG
 GCGATGTTTTAGGGCTATCAGTTCGCGCATTAAAGACTAATAGCCATTCAAAAATATTGTCTGTGCCACG
 TATTCCTACGCTTTCAGGTCTAGAAGGGTTCTATCTCTGTTGGCCAGAATGTCCTTTTTATTACTGGTCTG
 GTGACTGGTGAATCTGCCAATGTAAATAATCCATTTTCAGACGATTGAGCGTCAAAATGTAGGTATTTCCA
 TGAGCGTTTTTCTGTTGCAATGGCTGGCGGTAATATTGTTCTGGATATTACCAGCAAGGCCGATAGTTT
 GAGTTCCTTCTACTCAGGCAAGTGATGTTATTACTAATCAAAGAAGTATTGCTACAACGGTTAATTTGCGT
 GATGGACAGACTCTTTTACTCGTGGCCTCACTGATTATAAAAAACACTTCTCAAGATTCTGGCGTACCGT
 TCCTGTCTAAAATCCCTTTAATCGGCCCTCTGTTTAGCTCCCGCTCTGATTCCAACGAGGAAAGCACGTT
 ATACGTGCTCGTCAAAGCAACCTAGTACGCGCCCTGTAGCGCGCATTAAAGCGCGCGGGTGTGGTGGT
 TACGCGCAGCGTGACCGCTACACTTGCCAGCGCCCTAGCGCCCGCTCCTTTTCGCTTTCTTCCCTTCCTTT
 CTCGCCACGTTTCGCCGGCTTTCCCGTCAAGCTCTAAATCGGGGGTCCCTTTAGGGTTCCGATTATGTG
 CTTTACGGCACCTCGACCCCAAAAAACTTGATTTGGGTGATGGTTCACGTAGTGGGCCATCGCCCTGATA
 GACGGTTTTTTCGCCCTTTGACGTTGGAGTCCACGTTCTTTAATAGTGGACTCTTGTTCCAACTGGAACA
 ACACTCAACCTTATCTCGGGACGGATCGCTTCATGTGCGAGGAGAAAAAGGCTGCACCGGTGCGCTCAGC
 AGAATATGTGATACAGGATATATTCGCTTCTCGCTCACTGACTCGCTACGCTCGGTCTGCTGACTGCG
 GCGAGCGGAAATGGCTTACGAACGGGGCGGAGATTTCTGGAAGATGCCAGGAAGATACTTAACAGGGAA
 GTGAGAGGCGCGCGCAAAGCCGTTTTTCCATAGGCTCGCGCCCCCTGACAAGCATCACGAAATCTGACG
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 AAGGCTTTCTCCGCAAAAGTATTACAGGGTCATAATGTTTTTGGTACAACCGATTAGCTTTATGCTCTG
 AGGCTTTATTGCTTAATTTTGCTAATCTTTGCTTGGCTGTATGATTTATTGGATGTT

[SEQ ID No: 11]

[0061] As described in Example 1, the inventors have devised two alternative approaches (see Figures 9 and 10) for producing the recombinant phagemid particle of the invention in a prokaryotic host.

[0062] Hence, in an example, there is provided a method for producing a recombinant phagemid particle from a prokaryotic host, the method comprising:-

1. (i) introducing, into a prokaryotic host cell, a first vector configured to persist inside the prokaryotic host, and comprising at least one transgene expression cassette, and a packaging signal for enabling replication of the vector into single-stranded DNA;
2. (ii) introducing, into the host, a helper phage comprising nucleic acid encoding bacteriophage structural proteins; and
3. (iii) culturing the host under conditions which result in the single-stranded DNA being packaged by the structural proteins to form and extrude a recombinant phagemid particle from the prokaryotic host.

[0063] Advantageously, this embodiment (as shown in Figure 9) results in very high yields of particles. The first vector (i.e. the phagemid particle's genome) may be introduced into the host cell, for example by infection. The host cell may then be transformed with the helper phage, which results in the production of the recombinant phagemid particle. Preferably, the method comprises a purification step following the culturing step. Purification may comprise centrifugation and/or filtration.

[0064] In an example, there is provided a method for producing a recombinant phagemid particle from a prokaryotic host, the method comprising:-

1. (i) introducing into a prokaryotic host cell: (a) a first vector configured to persist inside the prokaryotic host, and comprising at least one transgene expression cassette, and a packaging signal for enabling replication of the vector into single-stranded DNA, and (b) a second vector comprising nucleic acid encoding structural proteins required for packaging the single-stranded DNA; and
2. (ii) culturing the host under conditions which result in the single-stranded DNA being packaged by the structural proteins to form and extrude a recombinant phagemid particle from the prokaryotic host.

[0065] Advantageously, this example (as shown in Figure 10) results in improved safety. The second vector (i.e. the helper phage) may be introduced into the host cell, for example by infection. The host cell may then be transformed with the first vector (i.e. the phagemid particle's genome), which results in the production of the recombinant phagemid particle. Preferably, the method comprises a purification step following the culturing step. Purification may comprise centrifugation and/or filtration.

[0066] In an example, there is provided use of a helper phage comprising nucleic acid encoding viral vector structural proteins to produce the recombinant phagemid particle according to the disclosure from a prokaryotic host.

[0067] In an example, there is provided a host cell comprising the first and/or second vector as defined in the disclosure.

[0068] The host cell is preferably prokaryotic, more preferably a bacterial cell. Examples of suitable host cells include: (i) TG1 (Genotype: K-12 *supE thi-1 Δ(lac-proAB) L1(mcrB-hsdSM)5*, (*r_K⁻m_K⁻*), Plasmids: F' [*traD36proAB⁺ lacI^q lacZΔM15*]), (ii) DH5αF'IQ[™] (Genotype: F-φ80*lacZΔM15 Δ(lacZYA-argF) U169 recA1 endA1 hsdR17 (rk⁻, mk⁺) phoA supE44 λ⁻ thi-i gyrA96 relA1*, Plasmids: F' *proAB⁺ lacIqZΔM15 zzf::Tn5 [KmR]*; and (iii) XLI-Blue MRF' (Genotype: *Δ(mcrA)183 Δ(merCB-hsdSMR-mrr)173 endA1 supE44 thi-i recA1 gyrA96 relA_i lac*, Plasmids: F' *proAB lacIqZΔM15 Tn10 (Tetr)*.

[0069] In another example, there is provided the recombinant phagemid particle or the system according to the disclosure, for use as an experimental research tool.

[0070] For example, the particle or system can be used *ex vivo* or *in vitro*.

[0071] Preferably, however, the particle is for use in therapeutically or in diagnostic methods, preferably *in vivo*.

[0072] Thus, in an example, there is provided the recombinant phagemid particle or the system according to the disclosure, for use in therapy or diagnosis.

[0073] The invention may be used for the treatment of a wide variety of diseases due to the target-specific nature and the improved transduction efficiency of the recombinant phagemid particle of the invention. Consequently, the therapeutic opportunities of recombinant bacteriophages used in gene therapy may be significantly increased by the invention due to its ability to carry one or more transgene expression cassettes. The invention may be used prophylactically to prevent disease, or after the development of a disease, to ameliorate and/or treat it.

[0074] Hence, in an example, there is provided recombinant phagemid particle or the system according to the disclosure, for use in a gene therapy technique.

[0075] In an example, there is provided a method of treating, preventing or ameliorating a disease in a subject using a gene therapy technique, the method comprising administering, to a subject in need of such treatment, a therapeutically effective amount of the recombinant phagemid particle or the system according to the disclosure.

[0076] It will be appreciated that the invention may be used to create a variety of different recombinant phagemid particles that can be used for the treatment and/or diagnosis of a variety of diseases depending on the nature of the particles and the displayed foreign proteins. For example, in an embodiment where the recombinant phagemid particle comprises a tumor-targeting ligand and/or which comprises a transgene expressing an anti-tumor gene (e.g. the

HSVtk gene), then it may be used to treat cancer. The target cell in the gene therapy technique is preferably eukaryotic, and preferably mammalian. The gene therapy technique therefore is preferably used to treat, prevent or ameliorate cancer. Tumours may be in the brain, e.g. medulloblastoma, or diffuse intrinsic pontine glioma (DIPG). The recombinant phagemid particle may be used in combination with conventional treatments, such as chemotherapeutic drugs (i.e. doxorubicin, temozolomide, lomustine), radiation therapy, or other drugs/xenobiotic compound, including but not limited to inhibitors of histone deacetylases (HDAC inhibitors), proteasome inhibiting drugs and anticancer products from natural and dietary sources (i.e. genistein).

[0077] The inventors believe that the recombinant phagemid particle of the invention will have a significant commercial value in the delivery of peptide and/or DNA and/or adjuvant vaccines.

[0078] Thus, in an example, there is provided a vaccine comprising the recombinant phagemid viral particle or the system according to the disclosure.

[0079] In an example, there is provided the recombinant phagemid viral particle or the system according to the disclosure, for use in vaccine delivery to a subject.

[0080] Preferably, the vaccine is a peptide vaccine. The vaccine is preferably a DNA vaccine. The vaccine preferably comprises a suitable adjuvant. In an embodiment, the recombinant phagemid particle may be used to carry a transgene or DNA cassette encoding an antigen to stimulate the body's immune system. The recombinant phagemid particle may also be used to directly display and express the antigen of interest on the major pVIII coat proteins, thus providing an efficient platform for the simultaneous delivery, by a single phage particle, of numerous antigens as vaccine DNA vaccines, or proteins, or adjuvants readily expressed on the phage surface. The subject may be mammalian, and is preferably human.

[0081] In an example, therefore, there is provided the recombinant phagemid particle or the system according to the disclosure, for use in delivering and targeting a foreign antigen to a tumour in a vaccinated subject. Animals will first be vaccinated against foreign antigens, or already vaccinated against the antigen used, then the tumour-targeted phagemid will be administered to the vaccinated animals to deliver the foreign antigens to tumours, in order to induce an immune attack against these tumours.

[0082] The inventors also believe that the recombinant phagemid particle of the invention can also be used in a variety of different genetic-molecular imaging techniques, such as positron emission tomography (PET), Ultrasound (US), SPECT imaging, functional magnetic resonance imaging, or bioluminescence imaging.

[0083] Hence, in an example, there is provided use of the recombinant phagemid particle or the system according to the disclosure, in a genetic-molecular imaging technique.

[0084] The transgene harboured by the phagemid particle may encode HSVtk and/or the

sodium/iodide symporter (NIS), and the particle is preferably used in combination with a radiolabelled substrate. For example, the human sodium/iodide symporter (*NIS*) imaging gene is preferably used in combination with I^{124} for clinically applicable positron emission tomography (PET) imaging, or with $I^{125/99m}$ -Tc-pertechnetate for clinically applicable SPECT imaging.

[0085] Alternatively, the HSVtk gene is preferably used in combination with radiolabeled nucleoside analogues such as the 20-[^{18}F]-fluoro-20-deoxy-1- β -D-arabino-furanosyl-5-ethyluracil ([^{18}F]FEAU).

[0086] It will be appreciated that the recombinant phagemid particles and systems described herein (i.e. referred to hereinafter as "agents") may be used in a medicament which may be used in a monotherapy, or as an adjunct to, or in combination with, known therapies for treating, ameliorating, or preventing disease, such as cancer. For example, a combined therapeutic approach using the phagemid particles and systems with existing chemotherapeutics, such as Temozolamide, Doxorubicin or Genistein, is preferred.

[0087] In another preferred example, therapy may comprise the combination of the recombinant phagemid particle and system with an extracellular matrix degrading agent, such as enzyme or losartan. The inventors believe that extracellular matrix degrading agents should enhance phagemid diffusion in the subject being treated, and especially within a solid tumour.

[0088] The agents (i.e. the recombinant phagemid particle, or the system) may be combined in compositions having a number of different forms depending, in particular, on the manner in which the composition is to be used. Thus, for example, the composition may be in the form of a powder, tablet, capsule, liquid etc. or any other suitable form that may be administered to a person or animal in need of treatment. It will be appreciated that the vehicle of medicaments according to the disclosure should be one which is well-tolerated by the subject to whom it is given. Medicaments comprising the agents according to the disclosure may be used in a number of ways. For instance, oral administration may be required, in which case the agents may be contained within a composition that may, for example, be ingested orally in the form of a tablet, capsule or liquid. Compositions comprising agents may be administered by inhalation (e.g. intranasally). Compositions may also be formulated for topical use. For instance, creams or ointments may be applied to the skin.

[0089] Agents according to the disclosure may also be incorporated within a slow- or delayed-release device. Such devices may, for example, be inserted on or under the skin, and the medicament may be released over weeks or even months. The device may be located at least adjacent the treatment site. Such devices may be particularly advantageous when long-term treatment with agents is required and which would normally require frequent administration (e.g. at least daily injection).

[0090] In a preferred example, agents and compositions according to the disclosure may be administered to a subject by injection into the blood stream or directly into a site requiring

treatment. Injections may be intravenous (bolus or infusion), subcutaneous (bolus or infusion), intradermal (bolus or infusion) or enhanced by convection (convection enhanced delivery - relevant to local injections at disease site).

[0091] It will be appreciated that the amount of the agent that is required is determined by its biological activity and bioavailability, which in turn depends on the mode of administration, the physiochemical properties of the agent (i.e. recombinant phagemid viral particle or the system), and whether it is being used as a monotherapy, or in a combined therapy. The frequency of administration will also be influenced by the half-life of the agent within the subject being treated. Optimal dosages to be administered may be determined by those skilled in the art, and will vary with the particular agent in use, the strength of the pharmaceutical composition, the mode of administration, and the advancement of the disease. Additional factors depending on the particular subject being treated will result in a need to adjust dosages, including subject age, weight, gender, diet, and time of administration.

[0092] Generally, a daily dose of between 0.01µg/kg of body weight and 500mg/kg of body weight of the agent may be used. More preferably, the daily dose is between 0.01mg/kg of body weight and 400mg/kg of body weight, and more preferably between 0.1mg/kg and 200mg/kg body weight.

[0093] As discussed in the Examples, the agent may be administered before, during the or after the onset of disease. For example, the agent may be administered immediately after a subject has developed a disease. Daily doses may be given systemically as a single administration (e.g. a single daily injection). Alternatively, the agent may require administration twice or more times during a day. As an example, the agent may be administered as two (or more depending upon the severity of the disease being treated) daily doses of between 25mg and 7000 mg (i.e. assuming a body weight of 70 kg). A patient receiving treatment may take a first dose upon waking and then a second dose in the evening (if on a two dose regime) or at 3- or 4-hourly intervals thereafter. Alternatively, a slow release device may be used to provide optimal doses of agents to a patient without the need to administer repeated doses.

[0094] Known procedures, such as those conventionally employed by the pharmaceutical industry (e.g. *in vivo* experimentation, clinical trials, etc.), may be used to form specific formulations comprising the particles or systems and precise therapeutic regimes (such as daily doses of the agent and the frequency of administration).

[0095] Hence, there is provided a pharmaceutical composition comprising the recombinant phagemid viral particle or the system according to the disclosure, and a pharmaceutically acceptable vehicle.

[0096] The composition can be used in the therapeutic amelioration, prevention or treatment of any disease in a subject that is treatable with gene therapy, such as cancer.

[0097] The disclosure also provides, in an example, a process for making the pharmaceutical

composition according to the disclosure, the process comprising contacting a therapeutically effective amount of the recombinant phagemid particle or the system according to the disclosure, and a pharmaceutically acceptable vehicle.

[0098] A "subject" may be a vertebrate, mammal, or domestic animal. Hence, agents, compositions and medicaments may be used to treat any mammal, for example livestock (e.g. a horse), pets, or may be used in other veterinary applications. Most preferably, however, the subject is a human being.

[0099] A "therapeutically effective amount" of agent (i.e. recombinant phagemid viral particle) is any amount which, when administered to a subject, is the amount of drug that is needed to treat the target disease, or produce the desired effect, e.g. result in effective delivery of the transgene to a target cell or tissue, such as result in tumor killing.

[0100] For example, the therapeutically effective amount of agent used may be from about 0.01 mg to about 800 mg, and preferably from about 0.01 mg to about 500 mg.

[0101] A "pharmaceutically acceptable vehicle" as referred to herein, is any known compound or combination of known compounds that are known to those skilled in the art to be useful in formulating pharmaceutical compositions.

[0102] In one embodiment, the pharmaceutically acceptable vehicle may be a solid, and the composition may be in the form of a powder or tablet. A solid pharmaceutically acceptable vehicle may include one or more substances which may also act as flavouring agents, lubricants, solubilisers, suspending agents, dyes, fillers, glidants, compression aids, inert binders, sweeteners, preservatives, dyes, coatings, or tablet-disintegrating agents. The vehicle may also be an encapsulating material. In powders, the vehicle is a finely divided solid that is in admixture with the finely divided active agents. In tablets, the active agent (e.g. the particle or system of the disclosure) may be mixed with a vehicle having the necessary compression properties in suitable proportions and compacted in the shape and size desired. The powders and tablets preferably contain up to 99% of the active agents. Suitable solid vehicles include, for example calcium phosphate, magnesium stearate, talc, sugars, lactose, dextrin, starch, gelatin, cellulose, polyvinylpyrrolidone, low melting waxes and ion exchange resins. In another embodiment, the pharmaceutical vehicle may be a gel and the composition may be in the form of a cream or the like.

[0103] However, the pharmaceutical vehicle may be a liquid, and the pharmaceutical composition is in the form of a solution. Liquid vehicles are used in preparing solutions, suspensions, emulsions, syrups, elixirs and pressurized compositions. The particles or system may be dissolved or suspended in a pharmaceutically acceptable liquid vehicle such as water, an organic solvent, a mixture of both or pharmaceutically acceptable oils or fats. The liquid vehicle can contain other suitable pharmaceutical additives such as solubilisers, emulsifiers, buffers, preservatives, sweeteners, flavouring agents, suspending agents, thickening agents, colours, viscosity regulators, stabilizers or osmo-regulators. Suitable examples of liquid

vehicles for oral and parenteral administration include water (partially containing additives as above, e.g. cellulose derivatives, preferably sodium carboxymethyl cellulose solution), alcohols (including monohydric alcohols and polyhydric alcohols, e.g. glycols) and their derivatives, and oils (e.g. fractionated coconut oil and arachis oil). For parenteral administration, the vehicle can also be an oily ester such as ethyl oleate and isopropyl myristate. Sterile liquid vehicles are useful in sterile liquid form compositions for parenteral administration. The liquid vehicle for pressurized compositions can be a halogenated hydrocarbon or other pharmaceutically acceptable propellant.

[0104] Liquid pharmaceutical compositions, which are sterile solutions or suspensions, can be utilized by, for example, intramuscular, intrathecal, epidural, intraperitoneal, intravenous and particularly subcutaneous injection. The particles or system (i.e. hybrid vector) may be prepared as a sterile solid composition that may be dissolved or suspended at the time of administration using sterile water, saline, or other appropriate sterile injectable medium.

[0105] The recombinant phagemid particle, system and pharmaceutical compositions may be administered orally in the form of a sterile solution or suspension containing other solutes or suspending agents (for example, enough saline or glucose to make the solution isotonic), bile salts, acacia, gelatin, sorbitan monoleate, polysorbate 80 (oleate esters of sorbitol and its anhydrides copolymerized with ethylene oxide) and the like. The particles and system can also be administered orally either in liquid or solid composition form. Compositions suitable for oral administration include solid forms, such as pills, capsules, granules, tablets, and powders, and liquid forms, such as solutions, syrups, elixirs, and suspensions. Forms useful for parenteral administration include sterile solutions, emulsions, and suspensions.

[0106] It will be appreciated that adeno-associated virus (AAV) is often the vector of choice for gene therapy. As a gene delivery vector, lentiviral vectors also have key several advantages over other systems. Firstly, they have a large packaging capacity of at least 8 Kb of DNA, which is an important feature when packaging sizeable expression cassettes of tissue-specific promoters and transgenes. Secondly, they differ from simpler retroviruses not only in the genome organisation, but also in that they are able to transduce non-dividing cells, which is a very useful quality when considering application as a gene therapy vector to non-proliferating tissues such as muscle, neurons and haematopoietic stem cells. In addition, lentivectors have reduced immunogenicity compared to adenoviral vectors, making it possible to consider systemic delivery routes. However, barrier of using AAV or lentivirus for laboratory and clinical research include their extremely high production cost and low yields.

[0107] The inventors have shown that in addition to exhibiting useful applications in gene therapy, imaging and vaccine delivery, the recombinant phagemid particle of the invention can also be used to produce recombinant AAV in vitro. Phage-guided AAV production utilizes the ability of the phagemid particles to package large amounts of single-stranded ssDNA. A typical AAV production system consists of three major elements: rAAV, rep-cap and adenohelper genes, which function together to produce rAAV particles.

[0108] Thus, in an example, there is provided use of the phagemid particle or the system according to the disclosure, to produce a recombinant viral vector comprising or derived from the viral genome within the genome of the phagemid particle.

[0109] In an example, there is provided a method for producing recombinant viral vector, the method comprising introducing into, a eukaryotic host cell, the recombinant phagemid particle or the system according to the disclosure, and allowing the host cell to produce recombinant viral vector.

[0110] The recombinant virus product is rAAV. Preferably, the phagemid viral particle or the system according to the disclosure is used in cis and/or trans together with the delivery and/or presence of other genetic elements required for the production of mammalian viruses, as determined by the phagemid particle's genome, inside the eukaryotic host cell. The method used to assist or enhance gene transfer to the host cell by the phagemid particle includes those described in WO 2014/184528 (i.e. multifunctional) and WO 2014/184529 (i.e. combination with a cationic polymer to form a complex having a net positive charge).

[0111] The eukaryotic host cell may be mammalian. The host cell may comprise or be derived from Human Embryonic Kidney Cells (HEK293), *Spodoptera frugiperda* pupal ovarian tissue (Sf9), or Chinese Hamster Ovary (CHO). Insect cells are also envisaged.

[0112] In one embodiment, the host cell may be transformed with one or more phagemid particle genome carrying genes selected from the group consisting of: rAAV, capsid, replication, helper protein encoding genes, and any other genes required for the expression and packaging of mammalian viruses.

[0113] For example, in hybrid phagemid particle-guided rAAV production, the rAAV gene may be carried by the recombinant phagemid viral particle according to the disclosure, as shown in Figure 3, and the adeno-helper and rep-cap genes may be carried on separate vectors, or be integrated into the eukaryotic host genome. For example, Figure 12 shows the adeno-helper genes on one vector, and Figure 13 shows the rep-cap on a separate vector. Any combinations of the rAAV, rep-cap and adeno-helper genes may be carried on one or more vectors, i.e. in cis or trans configurations. Alternatively, rep-cap or adeno-helper proteins, in the context of rAAV production, could also be integrated or introduced into the eukaryotic host as a stably expressed accessory DNA (e.g. a plasmid), whereby the hybrid phagemid particle supplies the recombinant viral genome for packaging into a recombinant virus, as determined by the transgene cassette inside the phagemid particle's genome.

[0114] In one preferred embodiment, rAAV, rep-cap and adeno-helper genes are carried on a single vector, as shown in Figures 14 and 15. The inventors believe that this is the first time that all three sets of genes have been harboured on the same vector.

[0115] Hence, in an example, there is provided a recombinant vector comprising comprising rAAV, rep-cap and adeno-helper genes.

[0116] In an example, there is provided a recombinant phagemid particle comprising the vector of the disclosure.

[0117] In an example, there is provided use of the vector or the particle of the disclosure, to produce a recombinant AAV viral vector comprising or derived from the viral genome of the phagemid particle.

[0118] In an example, there is provided a method for producing recombinant AAV viral vector, the method comprising introducing into, a eukaryotic host cell, the vector or the particle of the disclosure, and allowing the host cell to produce recombinant viral vector.

[0119] When introduced into the same eukaryotic host cell (see Figures 11 and 14), the rep-cap and adenohelper genes on the vector behave as trans-acting or cis-acting or a combination of both elements that facilitate packaging of the rAAV genome in the AAV virus capsid, in the context of rAAV production. This production process is comparable to transient co-transfection of multiple plasmids, and usually involving three plasmids. However, in this embodiment, the plasmids are replaced with the recombinant phagemid particles of the invention, which are targeted to eukaryotic cells (preferably mammalian cells), which also carry the same elements.

[0120] The method may be carried out in vivo, in vitro, ex vivo, or in situ. For in situ production, the recombinant phagemid particles preferably comprise a targeting moiety for the target eukaryotic cell that is the designated eukaryotic host. Preferably, in the context of in situ, ex vivo and in vivo virus production, the designated eukaryotic host cell type is a diseased cell. Preferably, the diseased cell is a malignant or benign tumour. In the context of in vitro virus production, preferably the eukaryotic host is a derivative of any of the eukaryotic hosts listed above. The application of the recombinant phagemid particles and genetic elements required for the production of recombinant virus (as determined by the transgene cassette in the hybrid phagemid particle), could be in any fashion as indicated earlier, either in cis-acting or trans-acting combinations, inside the eukaryotic host cell.

[0121] It will be appreciated that the disclosure extends to any nucleic acid or peptide or variant, derivative or analogue thereof, which comprises substantially the amino acid or nucleic acid sequences of any of the sequences referred to herein, including functional variants or functional fragments thereof. The terms "substantially the amino acid/polynucleotide/polypeptide sequence", "functional variant" and "functional fragment", can be a sequence that has at least 40% sequence identity with the amino acid/polynucleotide/polypeptide sequences of any one of the sequences referred to herein, for example 40% identity with the nucleic acids identified herein.

[0122] Amino acid/polynucleotide/polypeptide sequences with a sequence identity which is greater than 65%, more preferably greater than 70%, even more preferably greater than 75%, and still more preferably greater than 80% sequence identity to any of the sequences referred

to is also envisaged. Preferably, the amino acid/polynucleotide/polypeptide sequence has at least 85% identity with any of the sequences referred to, more preferably at least 90% identity, even more preferably at least 92% identity, even more preferably at least 95% identity, even more preferably at least 97% identity, even more preferably at least 98% identity and, most preferably at least 99% identity with any of the sequences referred to herein.

[0123] The skilled technician will appreciate how to calculate the percentage identity between two amino acid/polynucleotide/polypeptide sequences. In order to calculate the percentage identity between two amino acid/polynucleotide/polypeptide sequences, an alignment of the two sequences must first be prepared, followed by calculation of the sequence identity value. The percentage identity for two sequences may take different values depending on:- (i) the method used to align the sequences, for example, ClustalW, BLAST, FASTA, Smith-Waterman (implemented in different programs), or structural alignment from 3D comparison; and (ii) the parameters used by the alignment method, for example, local vs global alignment, the pair-score matrix used (e.g. BLOSUM62, PAM250, Gonnet etc.), and gap-penalty, e.g. functional form and constants.

[0124] Having made the alignment, there are many different ways of calculating percentage identity between the two sequences. For example, one may divide the number of identities by: (i) the length of shortest sequence; (ii) the length of alignment; (iii) the mean length of sequence; (iv) the number of non-gap positions; or (iv) the number of equivalenced positions excluding overhangs. Furthermore, it will be appreciated that percentage identity is also strongly length dependent. Therefore, the shorter a pair of sequences is, the higher the sequence identity one may expect to occur by chance.

[0125] Hence, it will be appreciated that the accurate alignment of protein or DNA sequences is a complex process. The popular multiple alignment program ClustalW (Thompson et al., 1994, Nucleic Acids Research, 22,4673-4680; Thompson et al., 1997, Nucleic Acids Research, 24, 4876-4882) is a preferred way for generating multiple alignments of proteins or DNA in accordance with the invention. Suitable parameters for ClustalW may be as follows: For DNA alignments: Gap Open Penalty = 15.0, Gap Extension Penalty = 6.66, and Matrix = Identity. For protein alignments: Gap Open Penalty = 10.0, Gap Extension Penalty = 0.2, and Matrix = Gonnet. For DNA and Protein alignments: ENDGAP = -1, and GAPDIST = 4. Those skilled in the art will be aware that it may be necessary to vary these and other parameters for optimal sequence alignment.

[0126] Preferably, calculation of percentage identities between two amino acid/polynucleotide/polypeptide sequences is then calculated from such an alignment as $(N/T) \times 100$, where N is the number of positions at which the sequences share an identical residue, and T is the total number of positions compared including gaps but excluding overhangs. Hence, a most preferred method for calculating relative percentage identity between two sequences comprises (i) preparing a sequence alignment using the ClustalW program using a suitable set of parameters, for example, as set out above; and (ii) inserting the values of N and T into the following formula:-Sequence Identity = $(N/T) \times 100$.

[0127] Alternative methods for identifying similar sequences will be known to those skilled in the art. For example, a substantially similar nucleotide sequence will be encoded by a sequence which hybridizes to a nucleic acid sequence described herein, or their complements under stringent conditions. By stringent conditions, we mean the nucleotide hybridises to filter-bound DNA or RNA in 3× sodium chloride/sodium citrate (SSC) at approximately 45°C followed by at least one wash in 0.2× SSC/0.1% SDS at approximately 20-65°C. Alternatively, a substantially similar polypeptide may differ by at least 1, but less than 5, 10, 20, 50 or 100 amino acids from the sequences shown herein.

[0128] Due to the degeneracy of the genetic code, it is clear that any nucleic acid sequence could be varied or changed without substantially affecting the sequence of the protein encoded thereby, to provide a functional variant thereof. Suitable nucleotide variants are those having a sequence altered by the substitution of different codons that encode the same amino acid within the sequence, thus producing a silent change. Other suitable variants are those having homologous nucleotide sequences but comprising all, or portions of, sequence, which are altered by the substitution of different codons that encode an amino acid with a side chain of similar biophysical properties to the amino acid it substitutes, to produce a conservative change. For example small non-polar, hydrophobic amino acids include glycine, alanine, leucine, isoleucine, valine, proline, and methionine. Large non-polar, hydrophobic amino acids include phenylalanine, tryptophan and tyrosine. The polar neutral amino acids include serine, threonine, cysteine, asparagine and glutamine. The positively charged (basic) amino acids include lysine, arginine and histidine. The negatively charged (acidic) amino acids include aspartic acid and glutamic acid. It will therefore be appreciated which amino acids may be replaced with an amino acid having similar biophysical properties, and the skilled technician will know the nucleotide sequences encoding these amino acids.

[0129] All of the features described herein (including any accompanying claims, abstract and drawings), and/or all of the steps of any method or process so disclosed, may be combined with any of the above aspects in any combination, except combinations where at least some of such features and/or steps are mutually exclusive.

[0130] For a better understanding of the invention, and to show how embodiments of the same may be carried into effect, reference will now be made, by way of example, to the accompanying Figures, in which:-

Figure 1 is a table showing features of the phagemid-AAV (PAAV) virus particle according to the invention compared to prior art AAVP virus particles;

Figure 2 shows schematic illustrations of embodiments of a Helper Phage and a Phagemid genome (PAAV) according to the invention, and a phagemid-AAV (PAAV) particle that is created by the Helper and phagemid. Structural genes are integral to packaging of DNA in to virus particles, and are supplied by the replication-defective Helper phage. The phagemid genome is extremely parasitic to the Helper phage. Ultimately, the PAAV particles are produced at yields that far surpass prior art systems;

Figure 3 is a schematic representation of one embodiment of a phagemid genome (PAAV);

Figure 4 shows the respective locations of *fi ori* and *pUC ori* on the phagemid genome shown in Figure 3;

Figure 5 shows the location of a selection marker gene (*AmpR*) on a recombinant adeno-associated virus (rAAV) transgene cassette on the phagemid genome shown in Figure 3;

Figure 6 shows the rAAV transgene cassette on the phagemid genome shown in Figure 3, which contains a gene of interest (e.g. GFP), the expression of which is driven by a CMV promoter and/or enhancer sequences, and tailed with a polyA signal. The entire transgene cassette is flanked by Inverted Terminal Repeat sequences (ITRs) from AAV;

Figure 7 shows an embodiment of the Helper phage which is a bacteriophage engineered for rescuing phagemid particles from prokaryotic hosts carrying a phagemid genome, such as that shown in Figure 3;

Figure 8 shows a section of the genome of the helper phage shown in Figure 5 comprising the RGD4C targeting peptide in the pIII minor coat protein;

Figure 9 shows a first embodiment of a method for producing phagemid-AAV (PAAV) particles;

Figure 10 shows a second embodiment of a method for producing phagemid-AAV (PAAV) particles;

Figure 11 shows one embodiment of a phage-based approach for in vitro AAV production showing the three vectors, (i) phagemid-AAV (PAAV), (ii) Rep-Cap phagemid, and (iii) adenohelper phagemid;

Figure 12 shows the genome map of an embodiment of the adenohelper phagemid vector shown in Figure 11;

Figure 13 shows the genome map of an embodiment of a Rep-Cap phagemid vector shown in Figure 11;

Figure 14 shows an embodiment of a unified adenohelper-Rep-cap phagemid-AAV (PAAV) vector;

Figure 15 shows the genome map of an embodiment of the unified adenohelper-Rep-Cap phagemid vector shown in Figure 11;

Figure 16 shows an embodiment of in situ AAV production using either the three phagemid vectors shown in Figures 11-13, or the unified adenohelper-Rep-Cap-AAV phagemid vector shown in Figures 14 and 15;

Figure 17 shows Transmission Electron Microscopy (TEM) of known AAVP vectors and PAAV vectors according to the invention. **(A)** RGD.AAVP.GFP filament (pink) is typically 1455.02nm in length. **(B)** RGD. PAAV.GFP filament (blue) is typically 729.96nm in length; helper phage

present in virus sample (green) is typically 1186.03nm in length;

Figure 18 shows internalisation of known AAVP vectors and PAAV vectors according to the invention in: **(A)** 293AAV and **(B)** U87 cells after 2 and 4 hours. Flow cytometric analysis was used with gating threshold set at 20000 events of total cell population. (n=3) *=p<0.05, **=p<0.01;

Figure 19 shows quantification of GFP-positive cells 9 days post-transduction in **(A)** 293AAV, **(B)** 293AAV with the addition of DEAE.DEXTRAN, **(C)** U87 and **(D)** U87 with the addition of DEAE.DEXTRAN. Flow cytometric analysis was used with gating threshold set at 20000 events of total cell population. (n=3) *=p<0.05, **=p<0.01;

Figure 20 shows quantification of genome copy numbers of rAAV-GFP from cell lysates following phagemid-guided gene transfer **(A)** or transfection **(B)** of rAAV expression elements. (Experiment A: n=1; Experiment B: n=3);

Figure 21 shows immunofluorescence staining of UW228 and DAOY human medulloblastoma cells to demonstrate expression of α_V , β_3 and β_5 integrin subunits, receptor for RGD4C-phagemid. Tumour cells were stained using primary rabbit anti- α_V , β_3 or β_5 antibodies (diluted 1:50 in PBS-1%BSA), then with goat anti-rabbit AlexaFluor-488 secondary antibody (showed in green) and counterstained with 0.05 μ g/ml DAPI (in blue). Images were taken using a confocal microscope;

Figure 22 shows targeted gene delivery to paediatric medulloblastoma cells by RGD4C-phagemid. Medulloblastoma cells (UW228) were grown on 96 well-plates, then transduced with RGD4C-phagemid vector carrying the Luciferase gene (RGD). Untreated cells or cells treated with the non-targeted vector (M13) were used as negative controls. Luciferase expression was monitored over a time course from day 2 to 4 after transduction;

Figure 23 shows Western blot analyses showing down regulation of mTOR expression in paediatric UW228 and DAOY medulloblastoma cells following treatment with RGD4C-phagemid carrying the mTOR/shRNA (RGD4C-mTOR/shRNA)). Cell lysates were collected at day 4 post vector treatment, and total proteins were measured by BCA assay. Western blot was probed with a monoclonal antibody to human mTOR (Cell Signalling). Untreated cells (CTR) and cells treated with RGD4C-phagemid, lacking mTOR/shRNA, (RGD4C) were used as negative controls;

Figure 24 shows combination treatment of temozolomide (TMZ) and RGD4C-phagemid carrying shRNA for mTOR in medulloblastoma. Medulloblastoma cells (UW228 and DAOY) were transduced with RGD4C-phagemid (RGD4C) or RGD4C-phagemid carrying mTOR/shRNA (RGD4C-mTOR/shRNA). Untreated cells were also used as controls. At day 7 post vector treatment, temozolomide (TMZ, 100 μ M) was added in a few treated wells to assess effect of combination of vectors with chemotherapy. Images were taken at day 8 after vector treatment;

Figure 25 shows treatment of medulloblastoma cells with TNF α vectors. UW228 cells were

treated with RGD4C-phagemid-TNF α (RGD4C/TNF α) and non-targeted (ctr). **A)** Expression of TNF α in the medium of vector-treated cells, measured using human TNF α ELISA Max. **B)** Cell viability, using MTT assay, following expression of TNF α . Error bars: mean $\pm$ SEM;

Figure 26 shows immunofluorescence staining of DIPG cells to demonstrate expression of α_v , β_3 and β_5 integrin subunits, receptor for RGD4C-phagemid. Cells were stained using primary rabbit antibodies then with goat anti-rabbit AlexaFluor-488 secondary antibody. Control cells received secondary antibody alone. Images were taken using a confocal microscope;

Figure 27 shows selective and dose dependent delivery of gene expression to DIPG cells by RGD4C-phagemid/AAV. Increasing vector dose 1×10^6 or 2×10^6 TU/cell of RGD4C-phagemid-Luc (RGD4C) carrying the reporter Luc (luciferase) gene was used to treat DIPG cells. Luc expression was measured daily. Non-targeted vector lacking RGD4C (ctr) was used as negative control for targeting. Error bars: mean $\pm$ SEM;

Figure 28 shows Treatment with RGD4C-phagemid-TNF α . DIPG cells were transduced with 2×10^6 TU/cell RGD4C-phagemid-TNF α (RGD4C) and non-targeted vector as negative control (ctr). Apoptotic activity was measured at day 9 post-vector treatment using caspase-Glo assay (caspase 3/7, caspase 8, and caspase9). Error bars: mean $\pm$ SEM. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$;

Figure 29 shows luciferase expression after transduction with RGD.PAAV at various concentrations of transducing units;

Figure 30 shows luciferase expression after transduction with NT.PAAV at various concentrations of transducing units; and

Figure 31 shows the percentage of PAAV vectors bound to the cell surface of 293 AAV cells. RGD.PAAV vectors had 58.2% binding efficiency, whereas M13.PAAV vectors had 7.1% binding efficiency relative to their respective controls.

Background

[0131] The development of gene delivery technology is instrumental to successful translation of basic research to the society. In the past decade, a number of viral and non-viral vectors have emerged as potential delivery vectors for industrial and therapeutic applications. An important property of vectors, in addition to being efficient at delivering genes, is that it must also be easily produced and commercially viable. In 2006, Hajitou et al. attempted to fulfil the need for such vectors by creating a hybrid between recombinant adeno-associated virus (rAAV) and filamentous bacteriophage (phage), called the Adeno-associated Virus/Phage (AAVP) (Nature protocols 2, 523-531 (2007); Cell 125, 385-398 (2006)). The resulting AAVP vector possesses favourable characteristics of mammalian and prokaryotic viruses, but does

not suffer from the disadvantages that those individual vectors normally carry. However, there are certain aspects of the AAVP vector that still leaves room for significant improvement. Above all, this includes the genetic design of the vector, which carries ramifications in its production and therapeutic properties. Ultimately, this leads to AAVP's relatively low gene transduction efficacy when compared to mammalian viruses.

[0132] The research described herein relates to the design of the most advanced version of phage gene delivery vectors and their superiority to the known and existing phage vector, AAVP, by using a so-called "phagemid system", with the new phagemid vector being referred to as Phagemid/Adeno-associated Virion Phagemid (i.e. PAAV). Unlike the AAVP genome, which consists of a rAAV cassette inserted in to the filamentous phage genome, the PAAV genome does not contain any structural phage genes - a prokaryotic helper virus is required to facilitate vector assembly (Mol Ther 3, 476-484; Pharmaceutical research 27, 400-420 (2010)). Separating the reproductive and therapeutic elements of the virus in to a therapeutic vector carrying the transgene and a separate helper virus carrying the structural genes substantially decreases the genome/vector size and thereby significantly increases transgene capacity, a useful advantage for gene therapy applications of the new system. Consequently, this results in the encapsidation of a eukaryotic virus genome into the capsid of a prokaryotic virus, resulting in a vector as hybrid between eukaryotic genome and prokaryotic capsid with enhanced production yield, gene transduction efficiency and flexibility of the vector system for other applications.

[0133] As described in the Examples below, the inventors have:-

1. 1. Designed and constructed a hybrid Phagemid - AAV Vector (PAAV) particle expression system;
2. 2. Characterised and determined whether the phagemid/AAV vector (PAAV) is more efficient at gene transduction than the known AAVP system at various stages, including but not limited to:
 1. a. Binding to the cell surface,
 2. b. Internalisation of the vector from the cell surface,
 3. c. Translocation of the vector genome to the host nuclei, and
 4. d. Recombinant transgene expression.
3. 3. Determined whether the hybrid phagemid PAAV vector system is capable of producing rAAV from a mammalian producer cell-line.

[0134] Referring first to Figure 1, there is shown a table comparing features of the phagemid-AAV (PAAV) particles according to the invention (i.e. virions) with the prior art AAVP viral particles. As can be seen, the PAAV particles (6kb) of the invention are much smaller than the known AAVP particles (14kb), i.e. 42% less DNA, and 50% shorter viral particles, and the PAAV particles are produced at yields that far surpass prior art systems (100X) the yield of AAVP). As a result, PAAV particles of the invention can carry larger payloads, which is very useful for delivering multiple transgenes in gene therapy approaches. The inventors have therefore

demonstrated that the modified bacteriophage expression system (PAAV) can be used as a highly viral vector for gene therapy, or for large-scale production of viral vectors.

Example 1 - Phagemid - AAV Vector (PAAV) Construction

[0135] Referring to Figure 2, there is shown an embodiment of a Helper Phage genome and a Phagemid genome (PAAV DNA) according to the invention, which are used together upon expression in a prokaryote to produce the phagemid-AAV (PAAV) particle, also shown in Figure 1. Structural genes are integral to packaging of DNA in to virus particles, and are supplied by the replication-defective Helper phage, which is discussed in detail below. The phagemid genome is extremely parasitic to the Helper phage, meaning it outcompetes the replication-defective helper phage in both replication and packaging.

A) Phagemid/AAV Vector

[0136] Referring now to Figure 3, there is shown one embodiment of the phagemid genome which is a plasmid containing two origins of replication and two other genetic elements. Phagemid genomes require two origins of replication to facilitate both its replication inside the prokaryotic (e.g. bacterial) host and packaging into phagemid particles when rescued by a helper virus.

[0137] Referring to Figure 4, the first origin of replication (ori) is a high-copy number origin of replication (pUC ori) that enables replication of the double-stranded phagemid (dsDNA) inside the prokaryotic host at large quantities. The second origin of replication is a phage origin of replication (f1 ori) that enables replication of the plasmid into single-stranded DNA, which can subsequently be packaged into a phagemid vector particle (PAAV).

[0138] Referring to Figure 5, the phagemid genome includes a selection marker gene. In order for the phagemid genome to replicate efficiently inside the prokaryotic host, a selection marker (e.g. ampicillin resistance) is used to ensure expression and provides selective pressure to prevent loss of the phagemid genome in the form of an antibiotic resistance gene (with its own promoter). This ensures expression (and replication) of the phagemid genome when the prokaryotic host is cultured in the presence of the antibiotic that the selection marker confers resistance to.

[0139] Referring to Figure 6, the phagemid genome further includes a recombinant (adeno-associated virus, AAV) transgene cassette which contains a transgene of interest. This can include, but is not limited to, polypeptides/proteins, short hairpin/small interfering/short guiding RNAs, or a combination of both. By way of example only, the transgene shown in Figure 6 encodes GFP and human Beta-globin. Expression of the transgene is driven by a viral promoter (e.g. CMV) and/or enhancer sequences, and tailed with a polyA signal to prevent

degradation. The promoter can also be a mammalian and tumour specific promoter in cancer gene therapy applications (i.e. promoter of the Glucose Regulated Protein [grp78]). The entire transgene cassette is flanked by Inverted Terminal Repeat sequences (ITRs) from AAV, which form a protective hairpin structure allowing the transgene cassette to be stably maintained as concatameric episomal (extra-chromosomal) DNA in the mammalian cell nucleus transduced by the phagemid particle. The ITRs enable AAV transgene cassettes to be stably expressed over a long period of time.

[0140] The phagemid, despite having a small genome, is unable to package itself into particles as it lacks structural phage genes. As a result, it requires "rescuing" by a helper virus, as shown in Figure 7, which provides structural (i.e. capsid) proteins required for formation and extrusion of particles from the prokaryotic host. Conventionally speaking, genetic elements in the vector are generic and used widely in genetic engineering.

B) Helper phage

[0141] Referring to Figure 7, the helper phage (referred to herein as M13KO7) is a bacteriophage engineered specifically for rescuing phagemid particles (i.e. PAAV) from prokaryotic hosts carrying and/or containing the phagemid genome shown in Figure 3. The helper phage contains a disrupted origin of replication (p15a, medium copy number) and packaging signal, which significantly deters its ability to package itself into phage particles. Consequently, the phagemid genome will outcompete the helper phage in both replication and packaging.

[0142] In order to give the phagemid targeting properties (or multifunctional properties as described in WO 2014/184528), the genome of the helper phage must be engineered to do so, as it provides the structural capsid proteins for phagemid particle assembly. For example, the helper genome may encode a pIII capsid minor coat protein that is configured to display a cell-targeting ligand for enabling delivery of the resultant PAAVP particle to a desired target cell (e.g. tumour). It can also encode at least one pVIII capsid major coat protein that is configured to display a foreign peptide on the resultant PAAV particle. In one embodiment, therefore, it is desired to induce a 9-amino acid mutation in the pIII minor coat protein to confer specificity to angiogenic tumour cells and tumour endothelial cells that express $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins. Thus, referring to Figure 8, the genome of the helper phage comprises the RGD4C targeting peptide (CDCRGDCFC - SEQ ID No: 7).

[0143] Once the PAAVP phagemid genome and the Helper phage have been constructed, they are used together to produce, in a prokaryotic host, the Phagemid - AAV Vector (PAAV) particle, as discussed below.

Example 2 - Phagemid - AAV Vector (PAAV) Production

[0144] The inventors have devised two different methods (Methods 1 and 2) for producing the Phagemid - AAV Vector (PAAV) particle, and these are illustrated in Figures 9 and 10.

Notes:

[0145]

- TG1: a strain of *E. coli* that carries the fertility factor (F' pilus).
- 2XYT: liquid broth used to culture TG1 *E. coli*.
- Kanamycin: antibiotic resistance selection marker present on the helper phage.
- Ampicillin: antibiotic resistance selection marker present on the phagemid vector.
- TYE top agar: solid media used to culture TG1 *E. coli*, adapted from 2x TY by the addition of 1.25% bacteriological agar.

Phaemid/AAV Vector (PAAV) Production Method 1: Infective Rescue

[0146] With reference to Figure 9:

1. 1. Add 4-5 ml of TG1 *E. coli* carrying PAAV genome pre-culture (overnight) to 60 ml 2xYT (100µg/ mL Ampicillin) supplemented with 1% glucose.
2. 2. Incubate culture at 37° in shaker (250 RPM).
3. 3. Once OD₆₀₀ is in the range of 0.5 to 0.8 (log phase), add at least 1×10¹⁰ transducing units of helper phage (M13KO7) to culture.
4. 4. Invert to mix. Incubate at 37° for 30 minutes.
5. 5. Pour the infected starter culture from step 3 in to a 2L flask with 2x YT (100µg/mL Ampicillin + 25µg/mL Kanamycin) supplemented with 1% glucose to a final volume of 400-450mL.
6. 6. Incubate overnight in an orbital shaker at 37°, 250rpm for 16-20 hours.
7. 7. Purify phagemid (PAAV) particles from culture supernatant.

[0147] The benefits of Method 1 are its very high yields.

Phagemid/AAV Vector (PAAV) Production Method 2: Stable producer cell-line With reference to Figure 10:

Part 1: Competent producer cell-line production

[0148]

1. Transform and plate TG1 competent E.coli (Zymo Research, USA) with ssDNA genome from helper hage M13KO7 in TYE top agar (50µg/mL Kanamycin)
1. Pick individual colonies and inoculate 5mL 2xYT media (50µg/mL Kanamycin) supplemented with 1% glucose.
2. Incubate overnight in an orbital shaker at 37°, 250rpm for 16-20 hours
3. Check for true positive transformants by extracting DNA from the 5mL overnight cultures using a commercial extraction kit (QIAGEN, Netherlands) and run on 1% agarose gel (100volts, 2.5mA) against a DNA ladder.
4. Prepare chemically competent cells from the correct transformant identified in step 4 using a published protocol (adapted from that published by Krantz et al., UC Berkeley)

Part 2: PAAV Phagemid Particle Production**[0149]**

1. 1. Transform competent cell-line created in Part 1 with a Phagemid/AAV genome and plate on TYE top agar (100µg/mL Ampicillin + 50µg/mL Kanamycin)
2. 2. Pick a colony and inoculate 5mL 2xYT (100µg/mL Ampicillin + 50µg/mL Kanamycin) supplemented with 1% glucose.
3. 3. Incubate in an orbital shaker at 37°, 250rpm for 4 hours
4. 4. Pour the infected starter culture from step 3 in to a 2L flask with 2xYT (100µg/mL Ampicillin + 25µg/mL Kanamycin) supplemented with 1% glucose to a final volume of 400-450mL
5. 5. Incubate overnight in an orbital shaker at 37°, 250rpm for 16-20 hours
6. 6. Purify phagemid particles from culture supernatant

PAAV Phagemid Particle Purification**[0150]**

1. 1. Transfer the warm overnight culture to centrifuge bottles and pellet the bacteria by centrifugation at 3300G, 4° for 30 minutes.
2. 2. Discard the pellet and transfer supernatant to a clean centrifuge bottle.

3. 3. Add 30% volume of supernatant in each bottle with ice-cold 20% PEG-8000/2.5M NaCl and swirl to mix.
4. 4. Incubate on ice for 4-24 hours
5. 5. Precipitate phagemid particles by centrifugation at 10000G, 4° for 30 minutes. Discard the supernatant.
6. 6. Dry the phagemid particle pellet by centrifugation at 10000G, 4° for 1 minute.
7. 7. Remove remaining supernatant with PEG/NaCl
8. 8. Resuspend the phagemid particle pellet in 0.5-2mL PBS
9. 9. Filter the resuspended phagemid particle preparation using a 0.45 micron filter.
10. 10. Keep the preparation at 4°. The preparation is stable for up to 2 years 4°. A 25% glycerol stock can be stored indefinitely at -80°.

Example 3 - Use of Phagemid - AAV Vector (PAAV) for gene therapy techniques

[0151] Examples 1 and 2 describe the components of the invention (i.e. phagemid genome shown in Figure 3 and helper phage shown in Figure 7) required to produce the Phagemid - AAV Vector (PAAV) particle and two methods of production. Once produced and purified, the PAAV particles can have a range of uses, such as in gene therapy.

[0152] As an example, the PAAVP particles described herein carry the GFP transgene, as it is readily detectable in known assays to show successful delivery to a target cell. In therapy, any transgene may be selected and engineered into the phagemid genome shown in Figure 3, to be carried in the resultant PAAV particles. For example, the transgene may be any gene encoding a protein, which may have therapeutic or industrial utility. For example, the transgene may encode dystrophin, a blood coagulation factor, insulin or a cytokine receptor sub-unit. The transgene may also encode a short hairpin/small interfering/short guiding RNA molecule using in RNAi therapy. The transgene may encode multiple polypeptides, nucleic acids, or a combination of both, fused together using an internal ribosomal entry site (IRES) or a viral fusion peptide (T2A peptides for in-frame fusion).

Example 4 - Use of Phagemid - AAV Vector (PAAVP) for in vitro AAV production

[0153] In addition to gene therapy, the PAAVP particles described herein can be used in novel methods for producing adeno-associated virus (AAV). Phage-guided AAV production utilizes the ability of the phagemid particles to package large amounts of dsDNA. A typical AAV production system consists of three major elements: rAAV, rep-cap and adenohelper genes, which function together to production recombinant AAV particles. The inventors have devised two different strategies.

[0154] With reference to Figure 11, the first strategy employed is to produce three different

phagemid vectors that carry the rAAV-producing elements. These are the Phagemid - AAV Vector (PAAV) (see Figure 3), the adenohelper phagemid particle (see Figure 12), and the rep-cap phagemid particle (see Figure 13). The basic structures of these particles are similar, as they contain two origins of replication and a selection marker, as described in the phagemid/AAV construction section. The key difference, however, is the transgene cassette. While the Phagemid - AAV (PAAV) genome contains an AAV transgene cassette, as shown in Figure 3, the adenohelper and rep-cap particles contain the adenohelper transgene or rep-cap transgene, as shown in Figures 12 and 13, respectively.

[0155] In another embodiment, the inventors have genetically engineered a so-called "unified construct" that contains all of the required elements inside a single vector genome, as shown in Figures 14 and 15.

[0156] When introduced into the same mammalian producer cell (see Figures 11 and 14), either on separate vectors or on the same unified vector, the rep-cap and adenohelper genes behave as trans-acting elements that facilitate packaging of the rAAV genome in the phagemid/AAV vector. This production process is comparable to transient co-transfection of three plasmids. However, in this case, the plasmids are replaced with phagemid vectors carrying the very same elements.

[0157] Below is described a protocol for PAAV/phagemid-guided production of adeno-associated virus (AAV).

Notes:

[0158]

DMEM: Dulbecco's Modified Eagle Medium.

FBS: Foetal Bovine Serum, a growth supplement.

Complete media: DMEM + 10% FBS.

EDTA: Ethyl-diamine tetra-acetic acid, an ion chelator used to dissociate cells by sequestering calcium ions required for tight junction formation.

GlutaMax: a growth supplement, analogue of L-Glutamine.

Protocol for phagemid-guided AAV production:

[0159]

1. 1. Seed and grow HEK293 cells in complete media (DMEM supplemented with 10% FBS, 20mM GlutaMax, Penicillin/Streptomycin and Non-Essential Amino Acids) in a 15cm tissue culture plate for a minimum of 48 hours until 80% confluence is achieved.
2. 2. Mix Phagemid/AAV, rep-cap phagemid and adeno-helper phagemid to achieve a 1:1:1 transducing unit ratio under 5mL total volume OR Aliquot a unified vector (single vector containing all three elements in a single particle) to achieve imillion transducing units per cell.
3. 3. Add an equal volume of serum-free DMEM (supplemented with 20mM GlutaMax) to the transduction mixture made in step 3.
4. 4. Invert to mix. Incubate at room temperature for 15 minutes.
5. 5. Wash the HEK293 cells plated in step 1 with PBS, repeat 3 times.
6. 6. Add the transduction mixture and swirl gently to distribute the mixture evenly.
7. 7. Incubate at 37°, 5% CO2 in a cell culture incubator for 72 hours
 1. a. After 6 hours of incubation with the transduction mixture, supplement with an equal volume of complete media (DMEM supplemented with 10% FBS, 20mM GlutaMax, Penicillin/Streptomycin and Non-Essential Amino Acids).
 2. b. After **24** hours, replace media with complete media (DMEM supplemented with 10% FBS, 20mM GlutaMax, Penicillin/Streptomycin and Non-Essential Amino Acids).

rAAV Purification:

[0160]

1. 1. Add 0.5M EDTA solution to the medium in the tissue culture plate to a final concentration of 0.010M, incubate for 5 minutes at room temperature.
2. 2. Collect the cells and media by aspiration and trituration and transfer to a 50mL centrifuge tube.
3. 3. Pellet the cells by centrifugation at 1500RPM, 5 minutes, Room temperature.
 1. a. Optional: collect the supernatant for further AAV purification.
4. 4. Resuspend the cell pellet in 2-5mL serum-free DMEM.
5. 5. Lyse the cells in the suspension by subjecting to 4 freeze-thaw cycles in an ethanol-dry ice bath and a water bath set to 37°.
6. 6. Centrifuge the cell lysate at 10000G, 10 minutes at Room temperature.
 1. a. Aliquot the supernatant for quantification/further purification/ concentration.
 2. b. Discard the pellet (debris).

Example 5 - Use of Phagemid - AAV Vector (PAAV) for in situ AAV production

[0161] Referring to Figure 16, the inventors have devised a method for the in situ production of AAV particles using the PAAV.

[0162] Firstly, an optimal dose (or multiple doses) of the three phagemid vectors or the unified vector are introduced in vivo through intravenous/thecal/peritoneal or intramuscular/subcutaneous (or any of the aforementioned routes of administration). The diseased tissue is a tumour displaying the relevant integrins and so the targeting moiety on the phagemid PAAV particles is the RGD4C sequence. The tumour should start to produce rAAV containing the viral transgene encoded in the hybrid phagemid particle and not wild-type AAV. These AAV particles should autoinfect nearby sites, as they naturally have high affinity to mammalian tissue, and eradicate the tumour over a given time.

Example 6 - Engineering Pseudovirions for Large-scale Targeted Gene Transfer and Recombinant Adeno-associated Virus Production

Transmission Electron Microscopy

[0163] In characterising the particles, the inventors imaged PAAV particles to show that vector size is substantially reduced when using the phagemid-based vector system. Using Transmission Electron Microscopy, the inventors imaged and measured the length of PAAV of the invention and known AAVP particles on mesh copper TEM grids after negative staining with uranyl acetate (see Figure 17). It was found that the average AAVP particle was 1455.02nm in length (Fig. 17A), while a typical PAAV particle according to the invention is only 729.96nm in length (Fig. 17B) - which equates to approximately 50% reduction in particle size. Compared to the helper phage that is used to produce PAAV particles (typically 1186.03nm, Fig. 17B), the relative vector size is approximately 38% shorter than the helper virus.

[0164] The difference in vector size forms the basis of the theory that PAAV may be more efficient as a gene delivery vector than the AAVP, not only in terms of production yield, but also in subsequent infection processes when entering and expressing genes in mammalian cells. As such, the inventors probed vector efficiency at various stages of infection, including binding, internalisation, and gene expression in 293AAV (a derivative of Human Embryonic Kidney 293) and U87 glioblastoma cell lines.

Vector Internalisation

[0165] Following binding, vectors undergo receptor-mediated endocytosis by the target cell. To investigate potential differences in vector internalisation, the inventors assayed the number of internalised vectors in target cells at two time-points (2 hours, 2H; 4 hours, 4H) using flow cytometry (see Fig. 18). It was found that PAAV vectors were internalised more efficiently at 2

hours (Median Fluorescence Intensity (MFI) = 1031.7, 335 higher than AAVP, $p < 0.05$) and to a greater overall extent at 4 hours when compared to AAVP in both cell lines. The MFI at 2 hours for PAAV was significantly higher than AAVP by 335 for 293AAV and 207 for U87 cells ($p < 0.05$). At 4 hours post-transduction, this difference became substantially greater for 293AAV (829 MFI, $p < 0.05$), but less so for U87 (157 MFI, non-significant). Overall, the MFI peaked at 2092 (293AAV, $p < 0.05$, Fig 18A) and 1137 (U87, Fig 18B) for PAAVi-treated cells, which was significantly higher than AAVP, which respectively peaked at 1063 (293AAV) and 980 (U87). The data demonstrates that PAAV performed consistently better than AAVP in rate and extent of internalisation for both time-points in both cell-lines.

Green Fluorescent Protein Expression following AAVP and PAAV-mediated Gene Transfer

[0166] To investigate whether the differences in vector internalisation translates to increased gene expression, the inventors performed a GFP-expression assay using RGD and NT PAAV.GFP and AAVP.GFP vectors (see Fig 19). In this experiment, they also tested whether addition of the cationic polymer DEAE.DEXTRAN (Dex) could enhance gene transfer by increasing the bioavailability and endosome-escape of PAAV vectors, as described in WO2014/184529. Nine days post-transduction, cells were trypsinised, and counted and analysed using a flow cytometer. It was found that transgene expression was generally higher in 293AAV cells than U87, regardless of whether Dex was used to assist vector transduction. When vector alone is used, the targeted RGD.PAAV.GFP vector transduces target cells with higher efficacy (7.7%, $p < 0.01$ and 1.4%, $p < 0.05$ GFP +ve cells in 293AAV and U87 cells, respectively) - compared to AAVP, this translates to a 2.44 and 1.56 fold increase respectively in 293AAV and U87 cells (Fig 19A, C).

[0167] When Dex is added however, gene expression increases dramatically for RGD.AAVP and RGD.PAAV vectors. In 293AAV cells, GFP expression in RGD.AAVP.GFP treated cells increased to 25% while RGD. PAAV.GFP treated cells experience a substantial increase to 50% (all $p < 0.01$); addition of Dex resulted in an increase in gene expression of 7.9-fold for RGD.AAVP and 6.5-fold for RGD. PAAV (Fig 19B, D). In U87 cells, which is regarded as highly resilient to transduction, Dex was able to augment gene expression by over 3.6-fold in RGD.PAAV.GFP to 4.8% GFP+ve cells ($p < 0.01$) - this was not the case for RGD.PAAV.GFP, as Dex increased gene expression by only 1.5-fold to 1.3% GFP+ve cells ($p < 0.05$). Interestingly, Dex enabled transduction by NT. PAAV (non-targeted) vectors in 293AAV cells (7.34%), but not with U87.

Phagemid-guided Recombinant Adeno-associated Virus Production

[0168] To assess whether PAAV and phagemid-derived vectors could be used to produce rAAV in a commercial producer cell-line, the inventors transduced 293AAV cells with three targeted

vectors, which are normally plasmids that require transfection for gene transfer. They were able to harvest rAAV particles from the cell lysate and quantify the rAAV gene copy number (GC) per mL over three time-points after phagemid-guided transduction (Fig. 20A). When compared to conventional transfection with FuGene6 (transfection reagent, 3.99e11 GC/mL, Fig 20B), phagemid-guided rAAV production provides over 1.9-fold increase at 168 hours (7.69e11 GC/mL, Fig 21A) in rAAV yield. Because phagemid-guided gene transfer requires extensive intracellular processing (unlike transfection), it requires a longer time for viral genes to be expressed and packaged in to functional particles. When yields are compared at the same 72-hour time-point however, transfection produced 1.76e11 GC/mL higher than phage-guided rAAV production. The rAAV yield per mL culture supernatant from transfection or phagemid-guided production dishes at all time points were approx. 8-9e10 GC/mL with no observable trends (data not shown).

Example 7 - Construction and uses of RGD4C-phagemid

[0169] The tripeptide, RGD, is found in proteins of the extracellular matrix, including fibronectin. The integrins act as receptors for fibronectin by binding to the RGD motif located in fibronectin in the site of cell attachment to $\alpha_v\beta_3$ integrin, and so the inventors induced a 9-amino acid mutation in the pIII minor coat protein of the recombinant phagemid particle in order to confer its specificity to tumour cells and angiogenic tumour-associated endothelial cells that express $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins. Thus, the genome of the second vector comprises the RGD4C targeting peptide (CDCRGDCFC - SEQ ID No: 7).

[0170] Referring to Figure 21, there is shown immunofluorescence staining of UW228 and DAOY human medulloblastoma cells, which demonstrates the expression of α_v , β_3 and β_5 integrin subunits, receptor for RGD4C-phagemid. These data demonstrate that the phagemid vector containing the RGD4C targeting peptide can be used for targeted gene delivery and gene therapy in the paediatric brain tumor, medulloblastoma.

[0171] Referring to Figure 22, there is shown targeted gene delivery to paediatric medulloblastoma cells by the RGD4C-phagemid, over a time course of 4 days. The data show that RGD4C-phagemid mediated efficient and selective gene delivery that increased overtime in medulloblastoma.

[0172] Figure 23 shows Western blot analyses showing down-regulation of the mammalian target of rapamycin (mTOR) expression in paediatric UW228 and DAOY medulloblastoma cells following treatment with RGD4C-phagemid carrying the mTOR/shRNA (RGD4C-mTOR/shRNA)). These data demonstrate that the RGD4C-phagemid can be successfully used to deliver shRNA in tumor cells to knock down expression of the therapeutic target mTOR in a selective and efficient way.

[0173] Figure 24 shows combination treatment of temozolomide (TMZ) and RGD4C-phagemid

carrying shRNA for mTOR in medulloblastoma cells, known for their resistance to temozolomide. The data demonstrate that targeted the RGD4C-mTOR/shRNA can re-sensitize medulloblastoma cells to TMZ and achieve complete tumor cell eradication. Therefore, targeted knockdown of mTOR expression by the RGD4C-phagemid is an efficient strategy to use in combination with temozolomide against chemoresistant tumor cells, such as medulloblastoma.

[0174] Figure 25 shows treatment of medulloblastoma cells with TNF α vectors. Therefore, RGD4C/TNFC1 has therapeutic potential for use in targeted tumor killing such as medulloblastoma. Figure 26 shows immunofluorescence staining of DIPG cells to demonstrate expression of α_v , β_3 and β_5 integrin subunits, receptor for RGD4C-phagemid. These data demonstrate that the phagemid vector containing the RGD4C targeting peptide can be used for targeted gene delivery and gene therapy in the paediatric brain tumors, DIPG.

[0175] Figure 27 shows selective and dose dependent delivery of gene expression to DIPG cells by RGD4C-phagemid/AAV. These data prove that RGD4C-phagemid can successfully deliver gene expression to DIPG in a dose-dependent and selective way. Figure 28 shows treatment with RGD4C-phagemid-TNF α . These data demonstrate that RGD4C-phagemid can successfully deliver TNF α to DIPG in a selective manner, resulting in apoptosis induction. Therefore, RGD4C-phagemid-TNF α has therapeutic potential for use in targeted therapy against DIPG.

Example 8 - Luciferase expression of RGD4C-phagemid

Protocol:

[0176] HEK cells were plated in a 48-well plate in complete media (DMEM, 10% FCS, 1% glutamine, 1% penicillin/streptomycin) and incubated for at least 48 hours until 70-80% confluence was reached. Cells were then washed with PBS and transduced with hybrid phage/phagemid vectors suspended in serum-free media (DMEM) for 12 hours before the media was supplemented with complete media. Luciferase expression was measured by adding 10uL of culture media to 50uL of prepared Quanti-luc (InvivoGen, USA) reagent. The emission of photos was measured using a plate reader equipped with a luminometer (promega, USA).

[0177] Figure 29 shows luciferase expression after transduction with RGD.PAAV at various concentrations of transducing units, and Figure 30 shows luciferase expression after transduction with NT.PAAV at various concentrations of transducing units. The graphs demonstrate a dose-dependent exponential relationship between time and expression of luciferase after incubation with hybrid phage/phagemid vectors at various concentrations. The figures demonstrate that quantifiable gene expression can be achieved by phagemid vectors

via an assay for secreted luciferase.

Example 9 - Binding of RGD.PAAV vector to 293 AAV cells

Protocol:

[0178] 293AAV cells were seeded on 24-well plates in complete media (DMEM +10%FCS, 1%Glutamine, 1% Penicillin/Streptomycin), and were left to reach 70-90% confluence for a minimum of 48 hours. The cells were washed twice with 500uL PBS and placed on ice before being transduced with 200000 TU/cell (transducing units/cell) of PAAV vectors suspended in 200uL of serum-free DMEM. After 1 hour of incubation on ice, the media was recovered from the wells and the amount of phagemid particles were titrated on TG1 *E.coli* and quantified by colony-counting.

[0179] Referring to Figure 31, there is shown the percentage of PAAV vectors bound to the cell surface of 293 AAV cells. RGD.PAAV vectors had 58.2% binding efficiency, whereas M13. PAAV vectors had 7.1% binding efficiency relative to their respective controls.

Discussion

[0180] There is strong evidence to suggest that targeted PAAV vectors are more efficient than AAVP vectors at gene transduction in both commercial and disease cell lines. Both internalisation and gene expression data concordantly indicate that PAAV are more efficient than AAVP. Evidence is also provided to suggest a strong synergistic effect between Dex and PAAV vectors on gene transduction that surpasses that of AAVP. Although these data suggest that PAAV are superior to AAVP, it must also be considered that PAAV vector samples contain helper phage contamination. Despite efforts in optimising experimental conditions during vector production, helper phage contamination (in this case, approx. 1/10) is unavoidable and will competitively inhibit transduction as it too displays the RGD targeting sequence on its minor coat protein. Taking this into account, the internalisation and gene expression data may very well be underestimating the 'true' efficacy of RGD.PAAV. Additionally, because the internalisation assay utilises staining of intracellular phage capsid for signal detection, the smaller overall size (and available capsid protein per particle) of the PAAV means that the proportional number of particles internalised cannot be compared directly to that of AAVP, which we have shown using TEM is twice in length compared to PAAV particles. Accordingly, methods of the invention involve a purification step (e.g. FPLC) to remove the helper phage.

[0181] It is essential that in addition to providing mechanistic insight, future work must encompass replication of all experiments using pure PAAV samples. In particular, phagemid-guided rAAV production may benefit greatly from decreased competitive inhibition by helper

phage contamination and yield multiple fold higher rAAV particles compared to conventional transfection protocols.

Summary

[0182] Large-scale production of recombinant adeno-associated virus (rAAV) has been a major hurdle for research, development and commercialization of genetic therapy. Despite being well-researched, rAAV production has been restricted to laboratory scales due to scalability limitations. By far, transient transfection of 'producer' cells has been the most popular technique, yielding high-purity rAAV vectors with no infectious contaminants, despite being extremely costly. Thus, an alternative method for gene transfer in rAAV-production systems is greatly warranted.

[0183] Hybrid phagemid vectors that are highly efficient at gene transfer to mammalian cells are described. By combining a rAAV transgene cassette to the phage capsid, it is possible to create a vector system that is easily produced at commercial scales. These phagemid/AAV (PAAV) vectors have very large cloning capacities and are targeted to mammalian cells, meaning transfection reagents are not required. As it is possible to clone all genetic elements for AAV production in to single or multiple phagemid vectors, the inventors have developed this platform technology for large-scale rAAV production. A novel large-scale rAAV production system using PAAV and bacteriophage vectors has been developed, in both adherent cells and in cell-suspensions. This platform technology will enable commercial virus production for clinical translation at GMP standards and pave the way for commercial production of other biosynthetics.

SEQUENCE LISTING

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1				5					10					15	

Val Glu Ser

REFERENCES CITED IN THE DESCRIPTION

Cited references

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Patent documents cited in the description

- [WO2014184528A](#) [0040] [0053] [0142]
- [WO2014184529A](#) [0041] [0110] [0166]
- [WO2014184528I](#) [0110]

Non-patent literature cited in the description

- Nature protocols, 2007, vol. 2, 523-531 [0006] [0131]
- Cell, 2006, vol. 125, 385-398 [0006] [0131]
- THOMPSON et al. Nucleic Acids Research, 1994, vol. 22, 4673-4680 [0125]
- THOMPSON et al. Nucleic Acids Research, 1997, vol. 24, 4876-4882 [0125]
- Mol Ther, vol. 3, 476-484 [0132]
- Pharmaceutical research, 2010, vol. 27, 400-420 [0132]

PATENTKRAV

1. Sammensætning til fremstilling af en rekombinant adeno-associeret virus- (AAV)/phagemidpartikel fra en prokaryotisk vært, hvilken sammensætning omfatter:

(i) en første vektor, der er konfigureret til at bestå inde i en prokaryotisk vært, og som omfatter:

a) mindst én AAV-transgenekspressionskassette flankeret af inverterede, terminale gentagelsessekvenser (ITR),

b) et bakteriofag-emballeringsignal, der omfatter en bakteriofag-replikationsoprindelse for at muliggøre replikation af partiklen til enkelt-strengt DNA; og

c) en bakteriereplikationsoprindelse for at muliggøre replikation af en dobbeltstrengt vektor inde i en prokaryotisk vært,

hvor den første vektor er uden strukturgener, der koder for bakteriofag-capsidproteiner; og

(ii) en anden vektor, der omfatter en nukleinsyre, der koder for bakteriofag-strukturproteiner, der er capsidproteiner krævet til emballering af det enkeltstrengede DNA, hvilket resulterer i dannelse og ekstrudering af en rekombinant AAV/phagemidpartikel fra den prokaryotiske vært.

2. Fremgangsmåde *in vitro* og/eller *ex vivo* til fremstilling af en rekombinant adeno-associeret virus- (AAV) vektor, hvilken fremgangsmåde omfatter indføring i, en eukaryotisk værtscelle, af en rekombinant AAV/phagemidpartikel, og at tillade, at værtscellen frembringer en rekombinant AAV-vektor, hvor AAV/phagemidpartiklen omfatter bakteriofag-capsidproteiner og et AAV/phagemidpartikelgenom, hvor AAV/phagemidpartikelgenomet omfatter:

i) mindst én AAV transgen-ekspressionskassette, flankeret af inverterede, terminale gentagelsessekvenser (ITR), der koder for et middel til indgivelse i målcellen;

ii) et bakteriofag-emballeringsignal, der omfatter en bakteriofag-replikationsoprindelse for at muliggøre replikation af partiklen til enkelt-strengt DNA; og

iii) en bakteriereplikationsoprindelse for at muliggøre replikation af en dobbeltstrengt vektor inde i en prokaryotisk vært,

og hvor AAV/phagemidpartikelgenomet er uden bakteriofag-strukturgener krævet til dannelse, emballering eller ekstrudering af partiklen fra en prokaryotisk vært, hvor AAV-phagemidpartiklen er uden strukturgener, der koder for bakteriofag-capsidproteiner.

3. Fremgangsmåde ifølge krav 2, hvor den eukaryotiske værtscelle er en pattedyre- eller en insektcelle, eventuelt hvor værtscellen omfatter humane embryonyreceller (HEK293), *Spodoptera frugiperda*-puppeovarievæv (Sf9) eller kinesisk hamsterovarie (CHO).

4. Fremgangsmåde ifølge enten krav 2 eller krav 3, hvor værtscellen transformeres til at omfatte AAV-rep-cap- og adenohelper-gener.

5. Rekombinant vektor, der omfatter:

- i) en transgenekspressionskassette flankeret af inverterede, terminale gentagelsessekvenser (ITR) af adeno-associeret virus (AAV);
- ii) AAV-rep-cap-gener og
- iii) og adenohelper-gener.

6. Rekombinant adeno-associeret virus (AAV)/phagemidpartikel, der omfatter bakteriofag-capsidproteiner og AAV/phagemidpartikelgenom, hvor AAV/phagemidpartikelgenomet omfatter:

i) mindst én AAV-transgenekspressionskassette flankeret af inverterede, terminale gentagelsessekvenser (ITR);

ii) en bakteriofag-replikationsoprindelse for at muliggøre replikation af partiklen til enkelt-strengt DNA og

iii) en bakteriereplikationsoprindelse for at muliggøre replikation af en dobbeltstrengt vektor inde i en prokaryotisk vært,

hvor AAV-phagemidpartiklen omfatter et genom, der ikke har bakteriofag-strukturgener krævet til dannelse, emballering eller ekstrudering af partiklen fra en prokaryotisk vært, hvor AAV-phagemidpartikelgenomet er uden strukturgener, der koder for bakteriofag-capsidet.

7. Anvendelse af vektoren ifølge krav 5, eller partiklen ifølge krav 6, til fremstilling af en rekombinant AAV-virusvektor *in vitro*, eller *ex vivo*, eventuelt hvor den rekombinante phagemidpartikel omfatter en targeteringsdel for en eukaryotisk målcelle, og eventuelt hvor den eukaryotiske værtscelletype er en syg celle, eventuelt hvor den syge celle er en ondartet eller godartet.

8. Fremgangsmåde til fremstilling af en rekombinant AAV-virusvektor, hvilken fremgangsmåde omfatter indføring, i en eukaryotisk værtscelle, af vektoren ifølge krav 5 eller partiklen ifølge krav 6, og at tillade, at værtscellen frembringer en rekombinant virusvektor *in vitro* eller *ex vivo*, eventuelt hvor den rekombinante phagemidpartikel omfatter en targeteringsdel for den eukaryotiske målværtscelle, og eventuelt hvor den eukaryotiske værtscelletype er en syg celle, eventuelt hvor den syge celle er en ondartet eller godartet tumor.

9. Rekombinant AAV/phagemidpartikel, der omfatter et genom, omfattende:

i) en eller flere transgen-ekspressionskassetter, hvor den ene eller flere transgen-ekspressionskassetter omfatter venstre og højre inverterede, terminale gentagelsessekvenser (ITR) af en AAV-serotype, hvor mindst én transgen-ekspressionskassette koder for et middel til indgivelse i målcellen,

ii) en bakteriofag-replikationsoprindelse for at muliggøre replikation af partiklen til enkelt-strengt DNA og

iii) en bakteriereplikationsoprindelse for at muliggøre replikation af en dobbeltstrengt vektor inde i en prokaryotisk vært,

hvor AAV-phagemidpartiklen omfatter et genom, der ikke har bakteriofag-strukturgener krævet til dannelse, emballering eller ekstrudering af partiklen fra en prokaryotisk vært, hvor AAV-phagemidpartikelgenomet ikke har strukturgener, der koder for bakteriofag-capsidproteiner, hvor AAV-phagemidpartiklen omfatter bakteriofag-strukturcapsidproteiner, hvor et mindre kappe capsidprotein er konfigureret til at vise en celletargeteringsligand for at muliggør indgivelse af partiklen til en målcelle, hvor celletargeteringsliganden er RGD4C-targeteringspeptidet ifølge SEQ ID NO: 7.

10. Partikel ifølge krav 9, hvor den rekombinante phagemidpartikel er kombineret med en kationisk polymer for at dannet et kompleks med en positiv nettoladning, eventuelt hvor den kationiske polymer er valgt fra en gruppe bestående af: chitosan; poly-D-lysin (PDL); diethylaminoethyl (DEAE); diethylaminoethyl-dextran (DEAE.DEX); polyethylenimin (PEI); polybren; protaminsulfat og et kationisk lipid.

11. Rekombinant vektor ifølge krav 5, rekombinant phagemidpartikel ifølge krav 6, eller rekombinant phagemidpartikel ifølge enten krav 9 eller krav 10, til anvendelse i terapi eller diagnose.

12. Rekombinant vektor ifølge krav 5, rekombinant phagemidpartikel ifølge krav 6, eller rekombinant phagemidpartikel ifølge enten krav 9 eller krav 10, til anvendelse i en genterapiteknik.
- 5 13. Rekombinant vektor ifølge krav 5, rekombinant phagemidpartikel ifølge krav 6, eller rekombinant phagemidpartikel ifølge enten krav 9 eller krav 10, til anvendelse i behandling, forebyggelse eller bedring af cancer.
- 10 14. Rekombinant vektor ifølge krav 5, rekombinant phagemidpartikel ifølge krav 6, den rekombinante phagemidpartikel ifølge enten krav 9 eller krav 10, til anvendelse i vaccineindgivelse til et individ, eventuelt hvor vaccinen er en peptidvaccine eller en DNA-vaccine.

DRAWINGS

Figure 1

Comparing AAVP and PAAV

	Next-generation PAAV vectors
Relative genome size	c. 6000 bases (42% of AAVP)
Relative virus size	~60% shorter than AAVP
Production yield	up to 400,000X current AAVP yields
Payloads per particle	Multiple (AAVP can only carry 1 payload)
<u>Biodistribution</u>	<i>Potentially better</i>

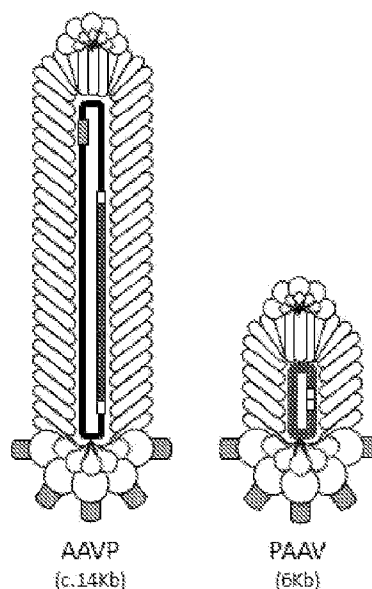
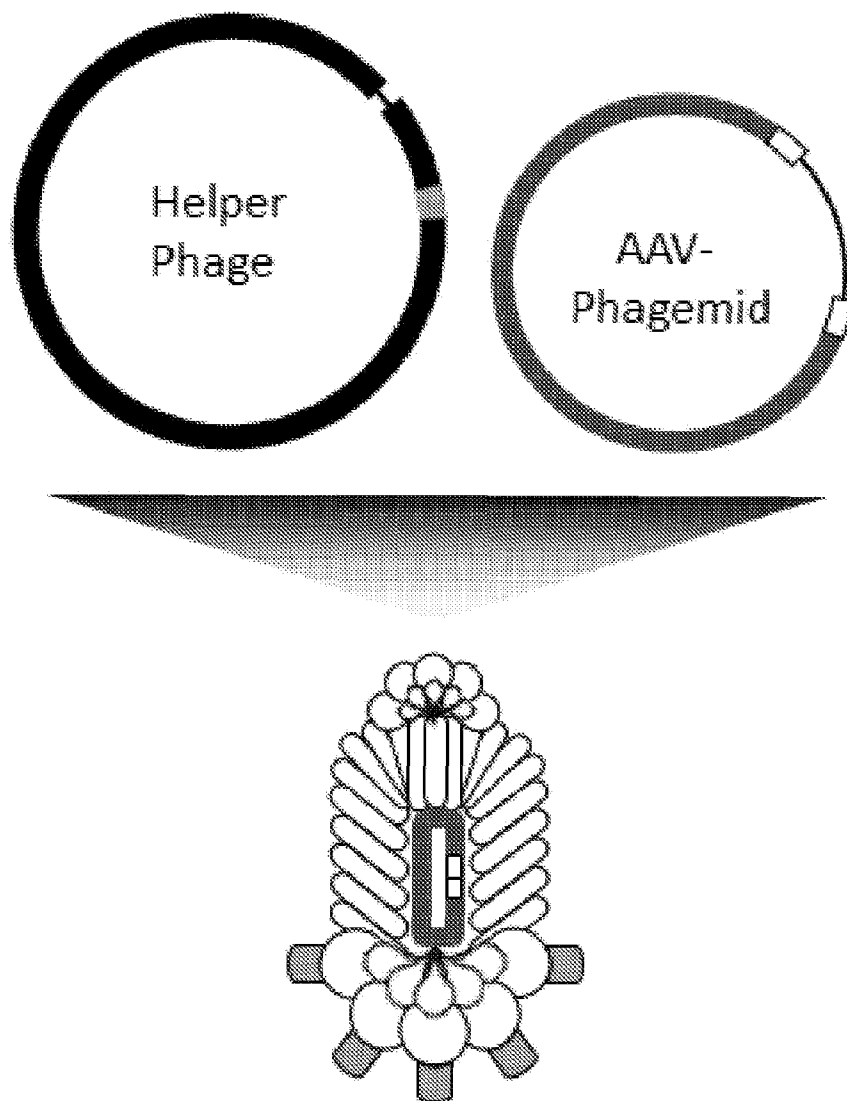


Figure 2

***Phagemid/Adeno-associated
Virion (PAAV)***

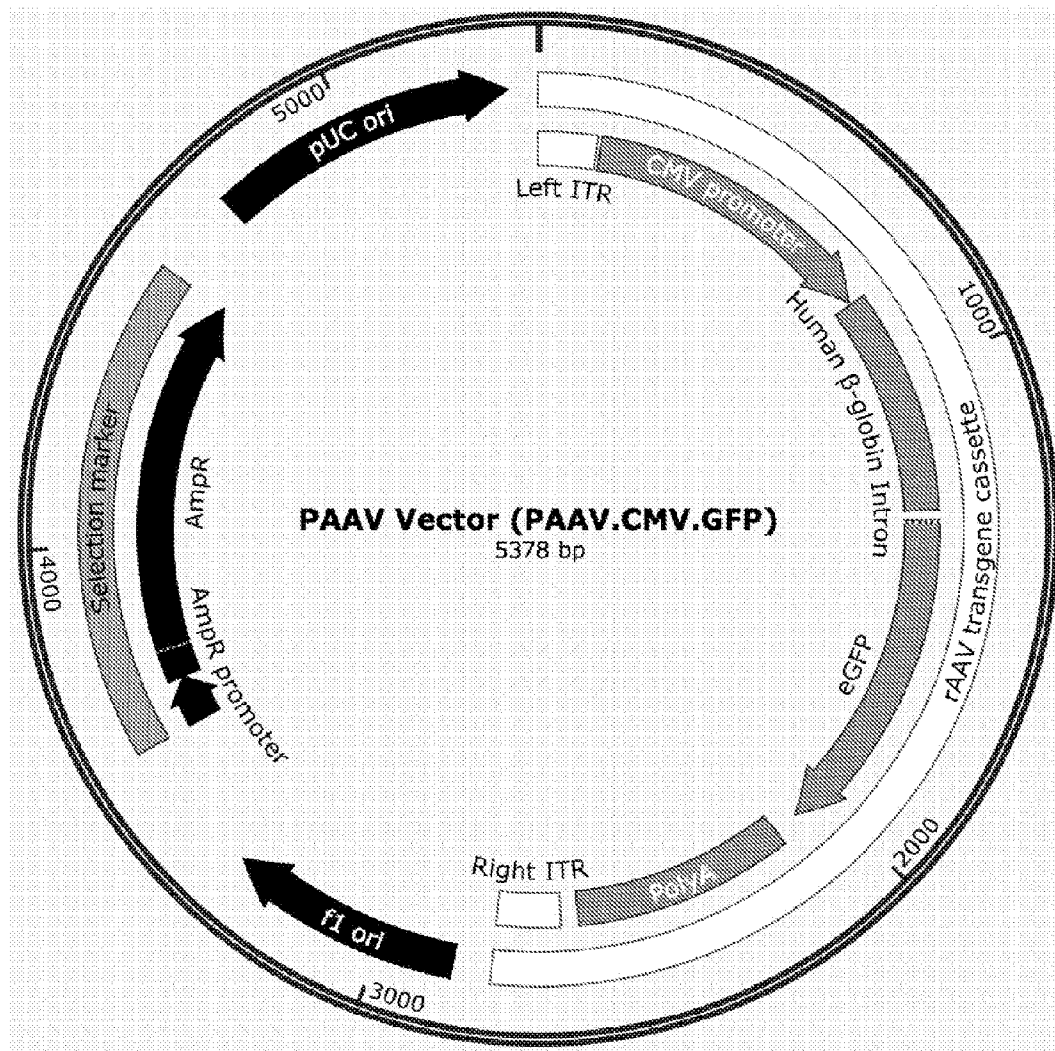
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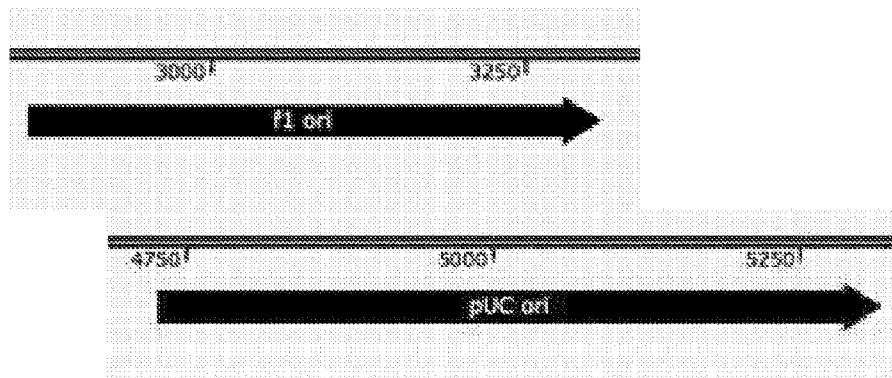
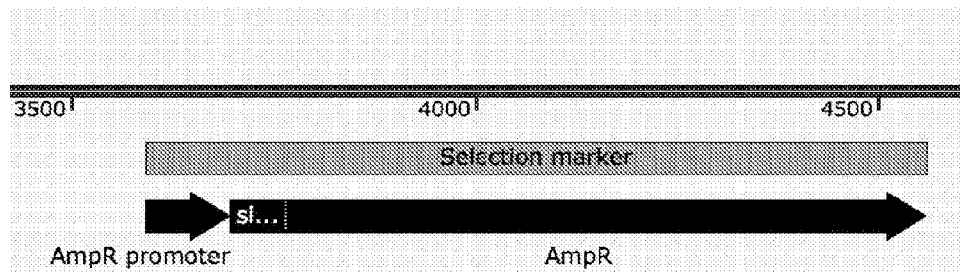
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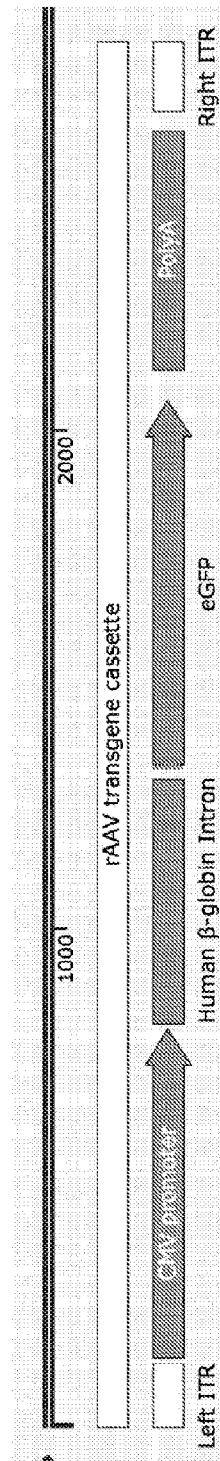
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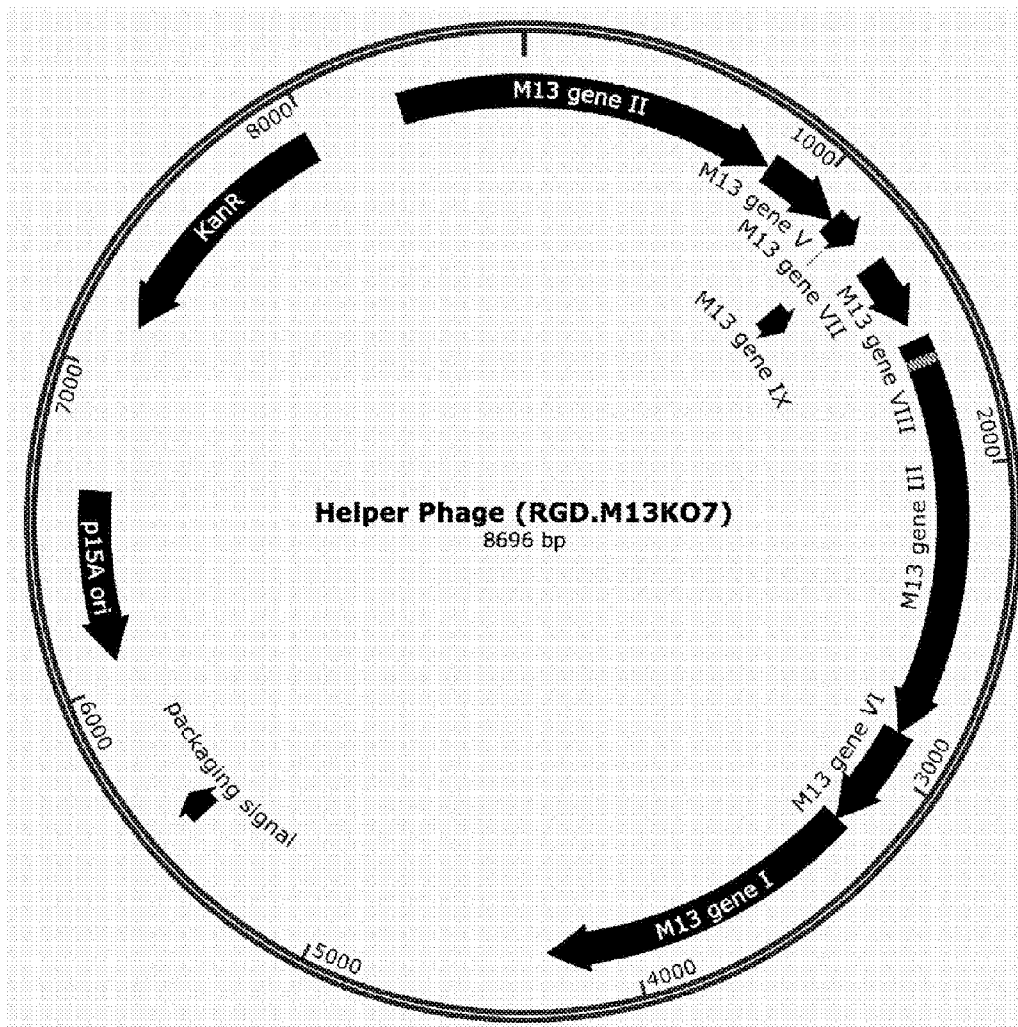
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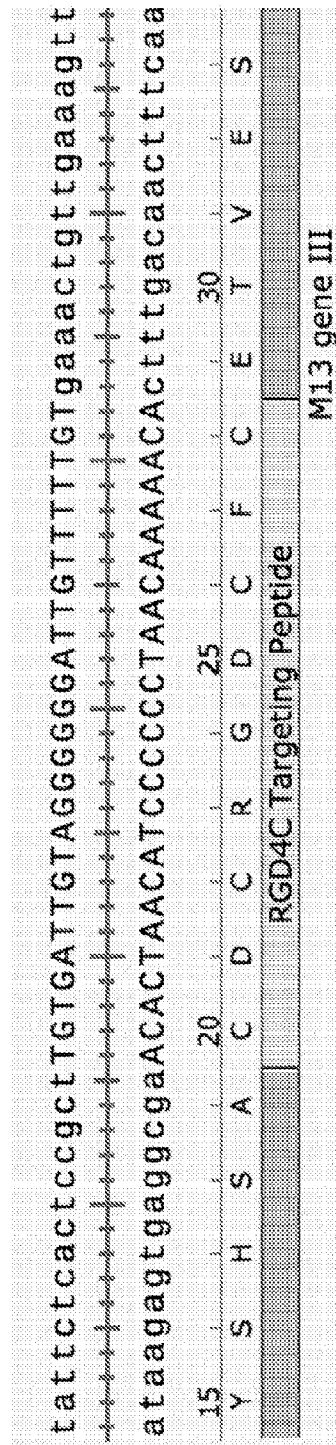


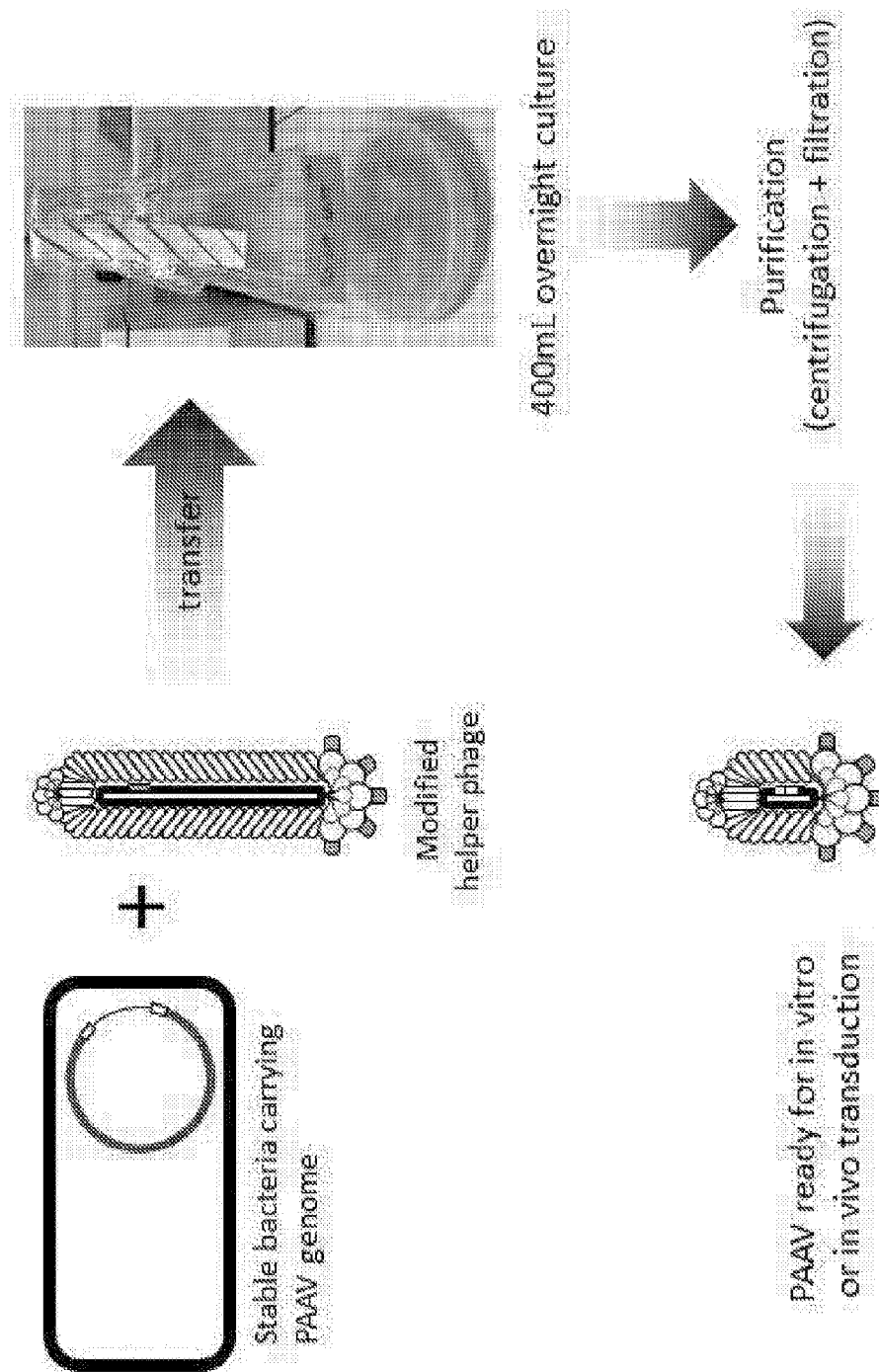
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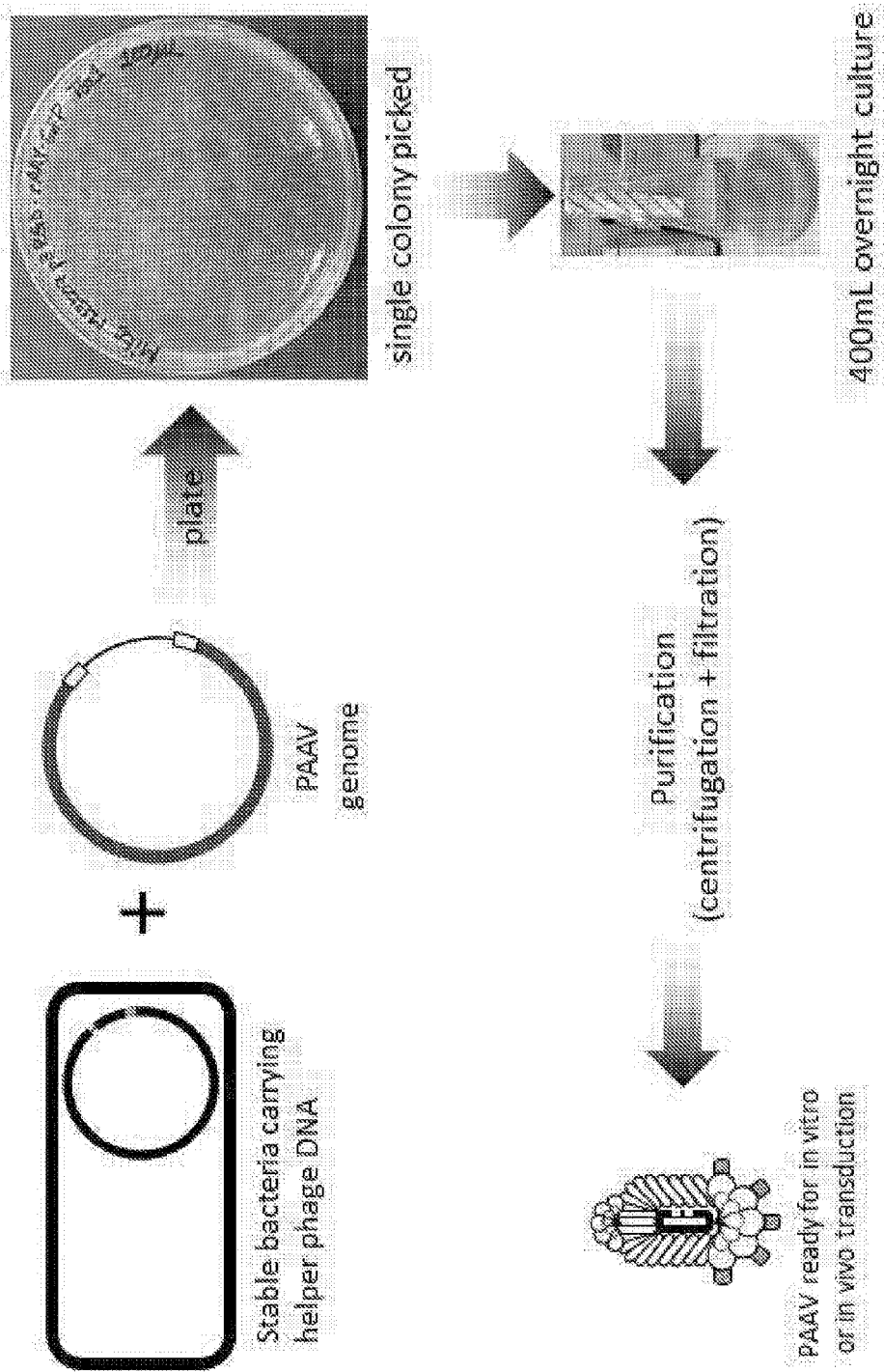
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Figure 11

Phagemid-guided rAAV production – methodology

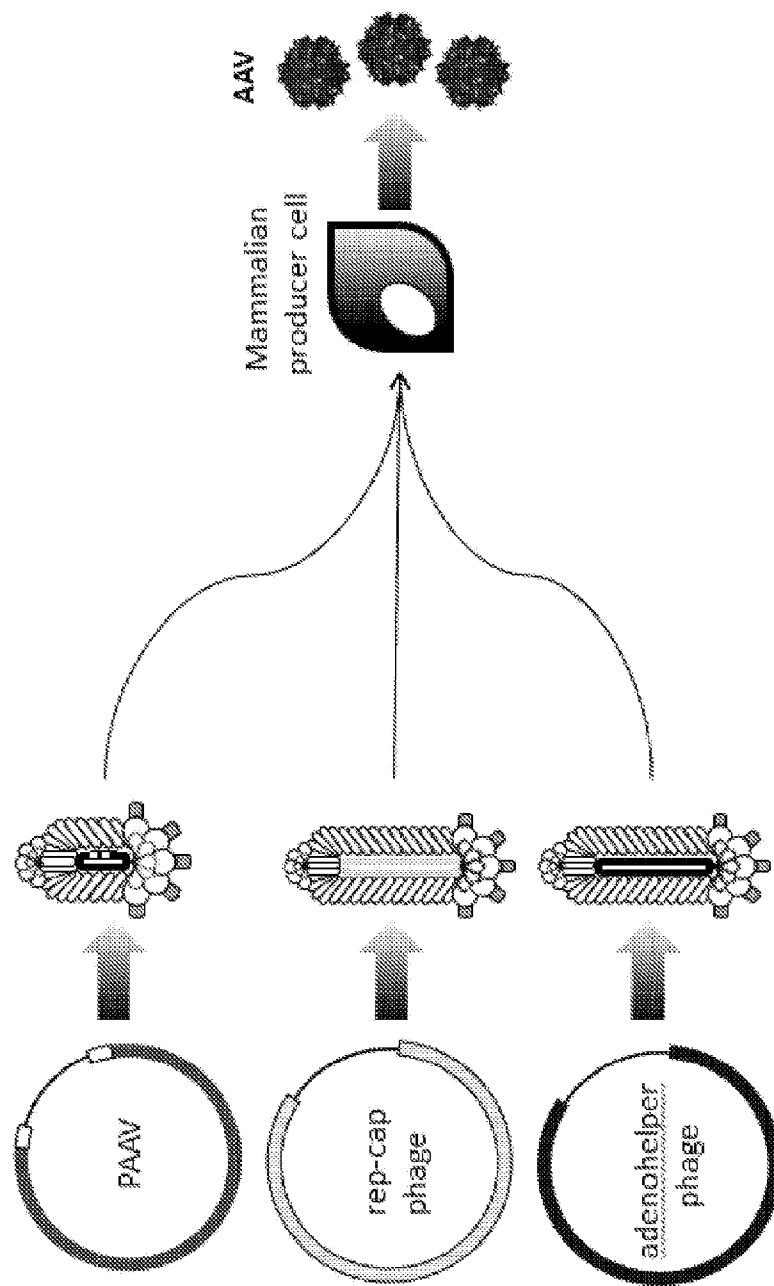


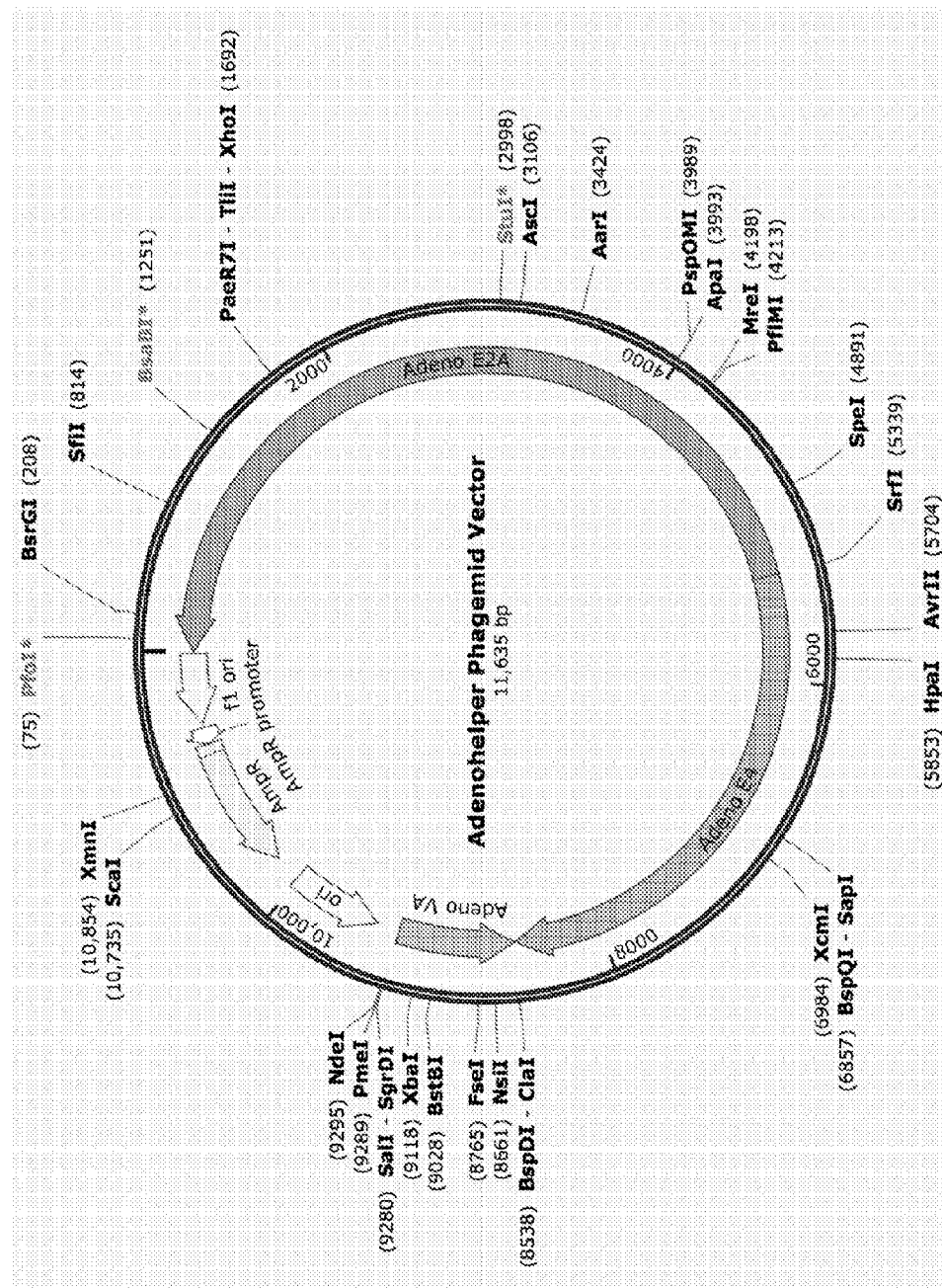
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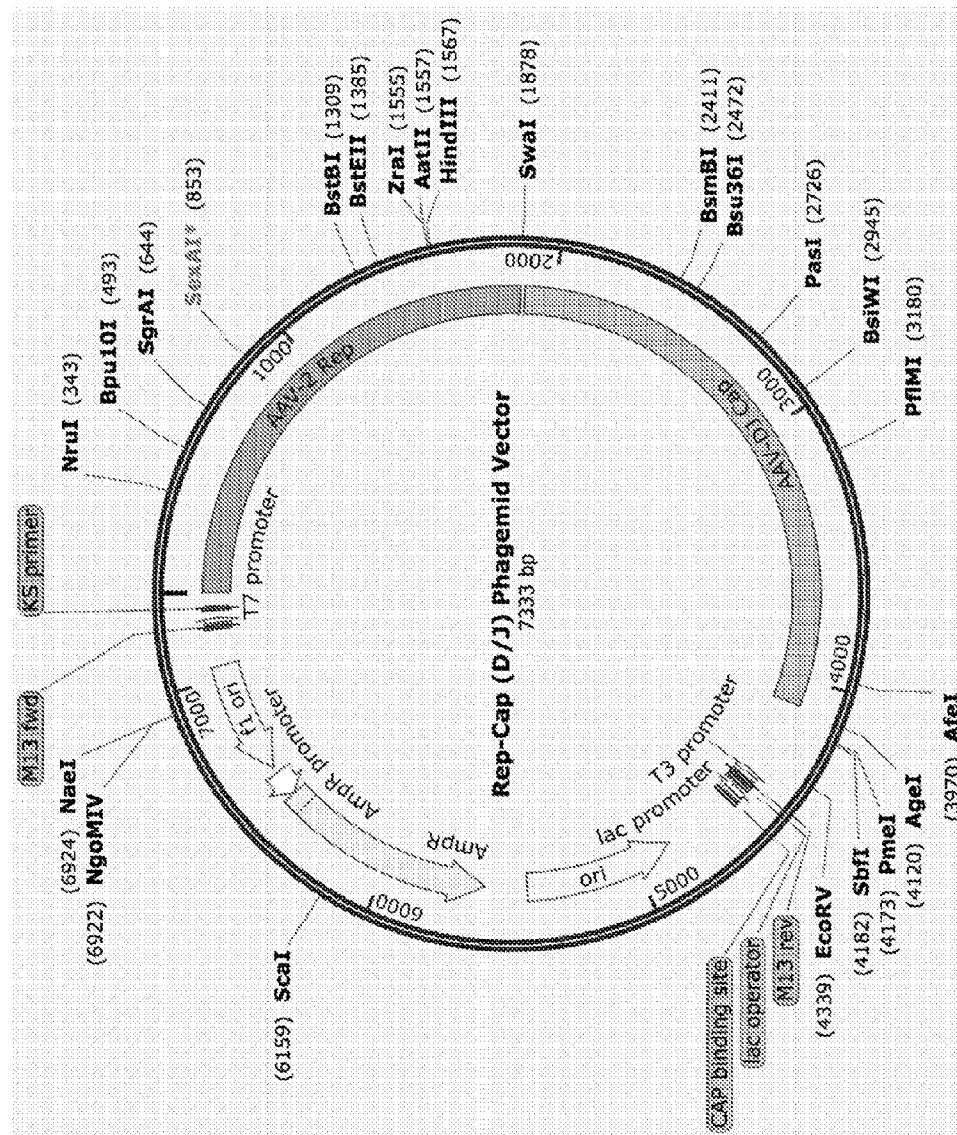
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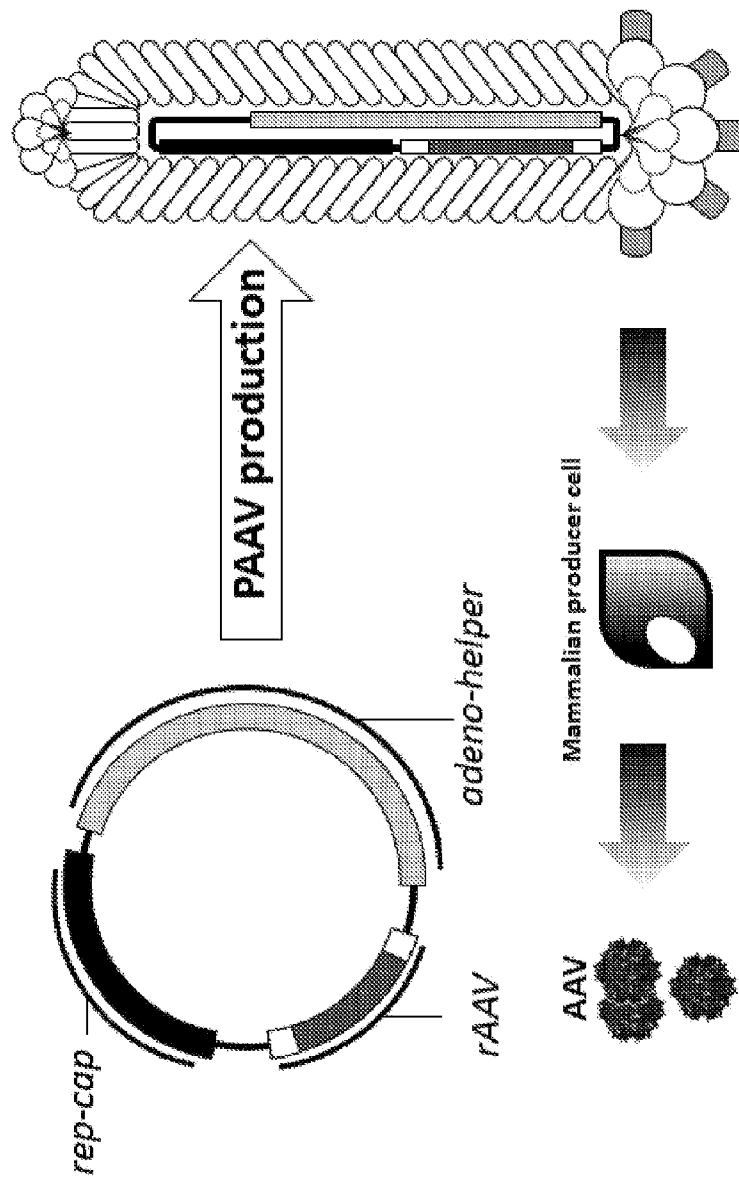
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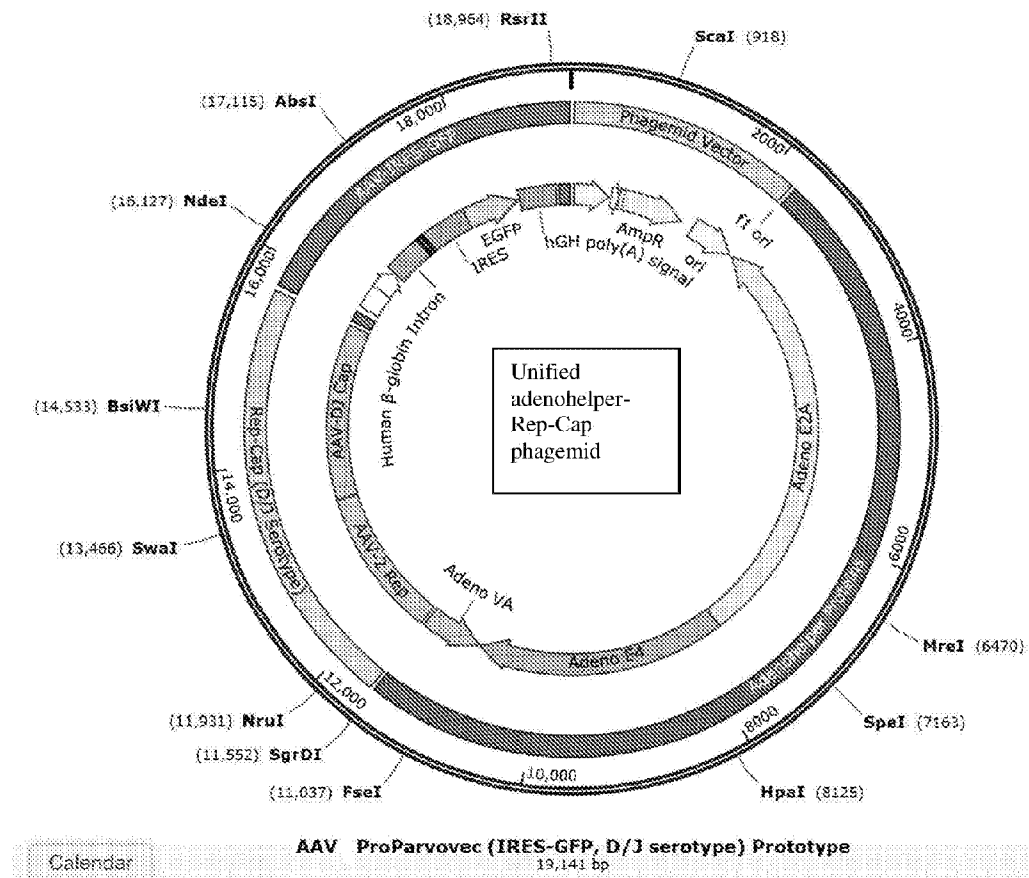
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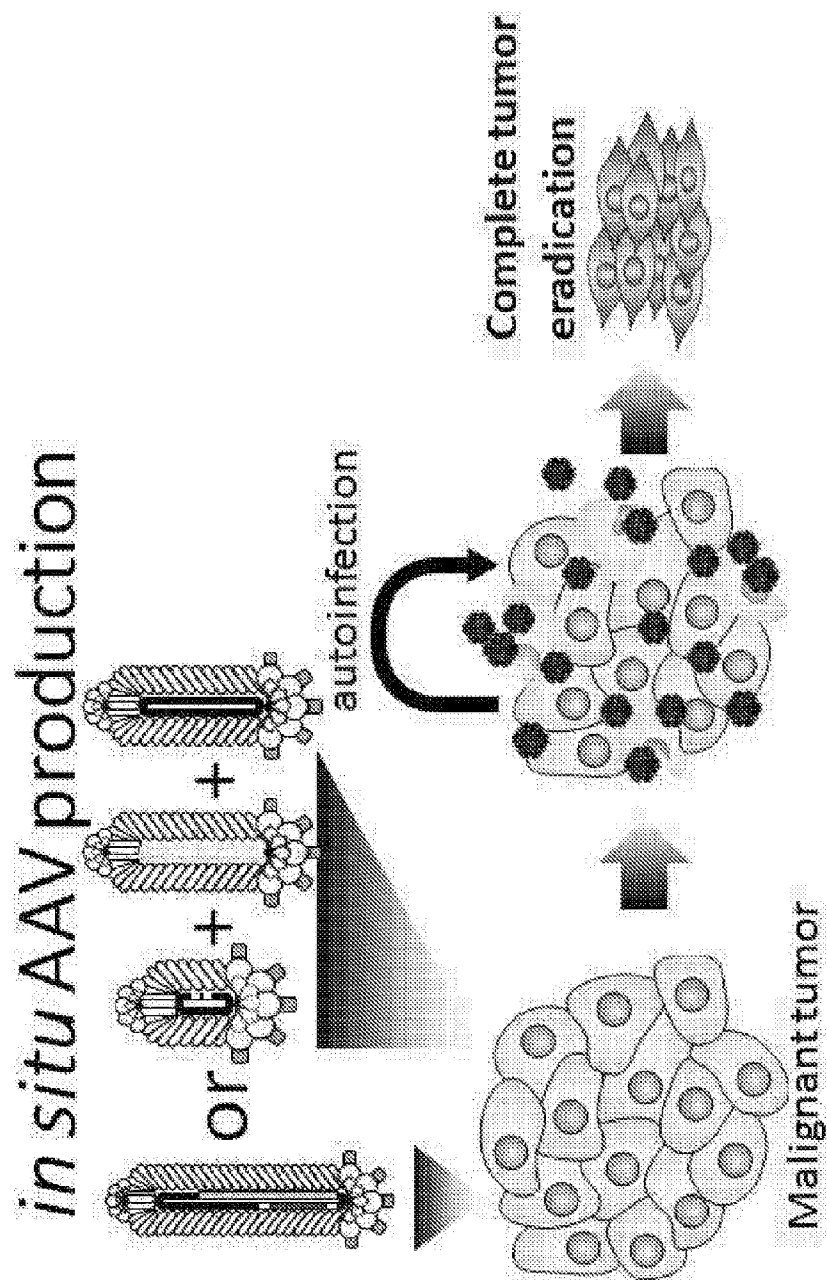
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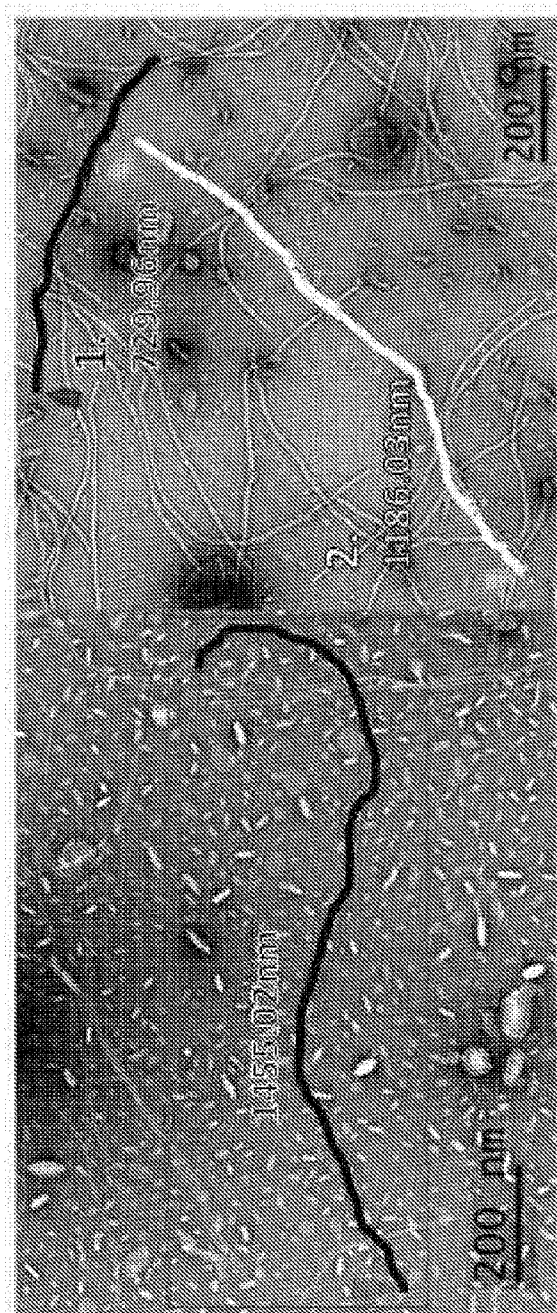
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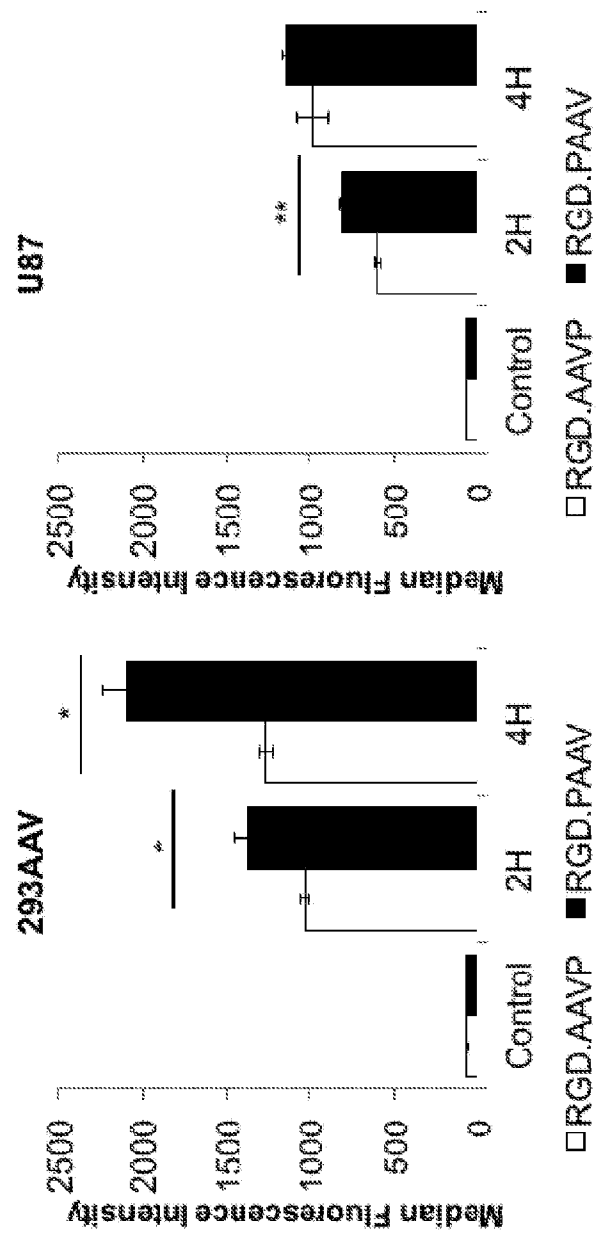
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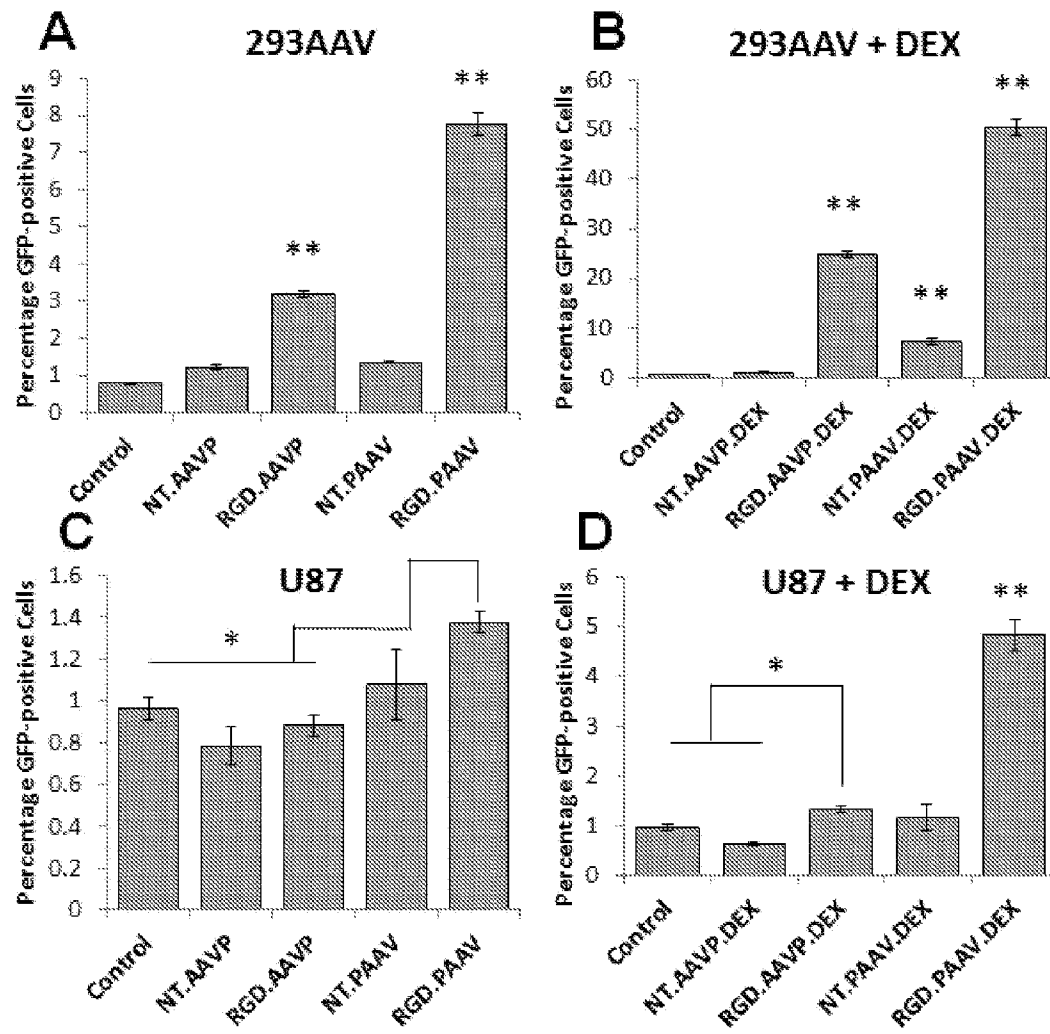
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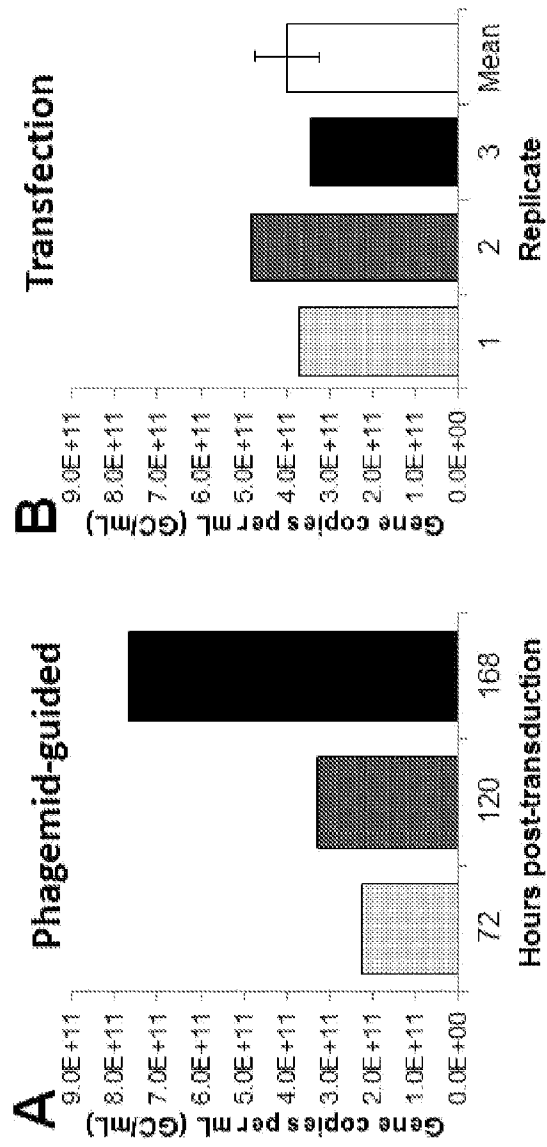


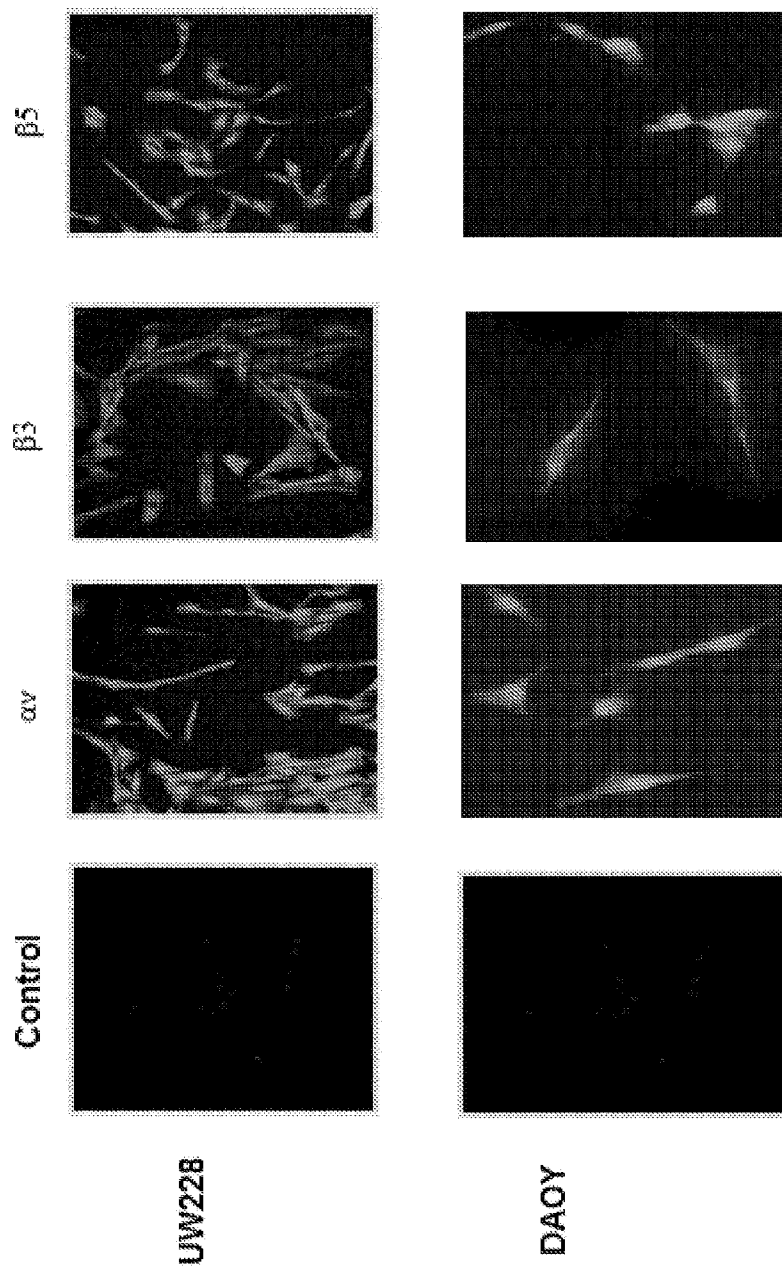
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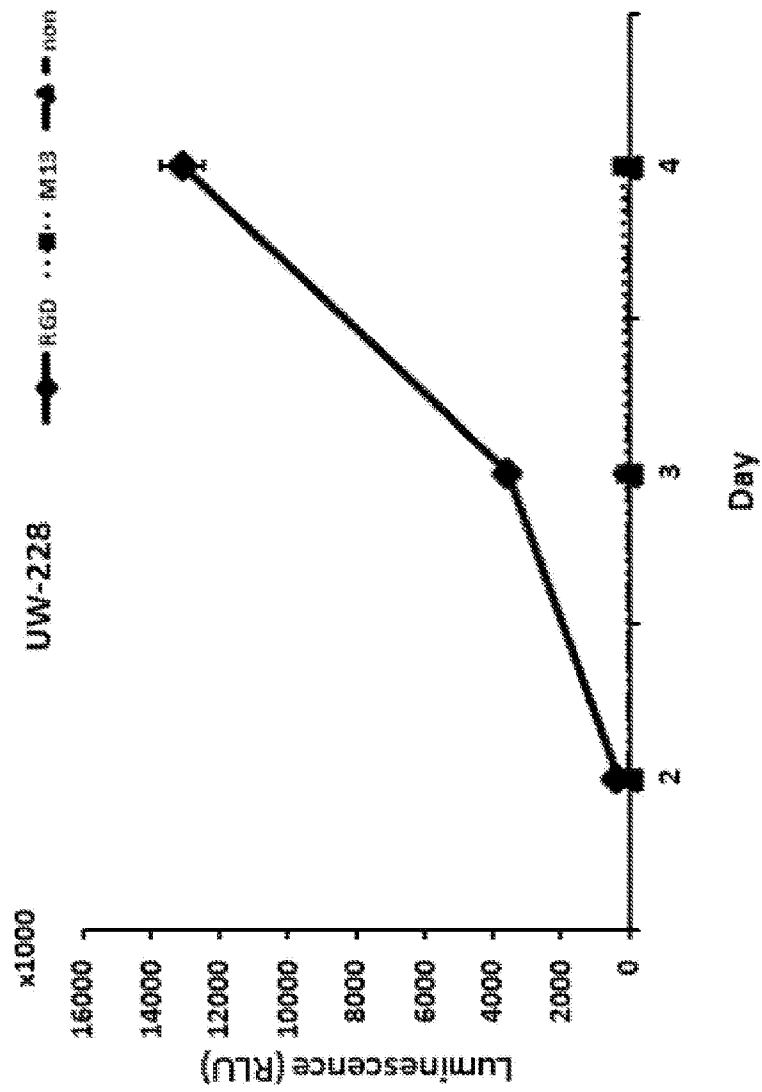
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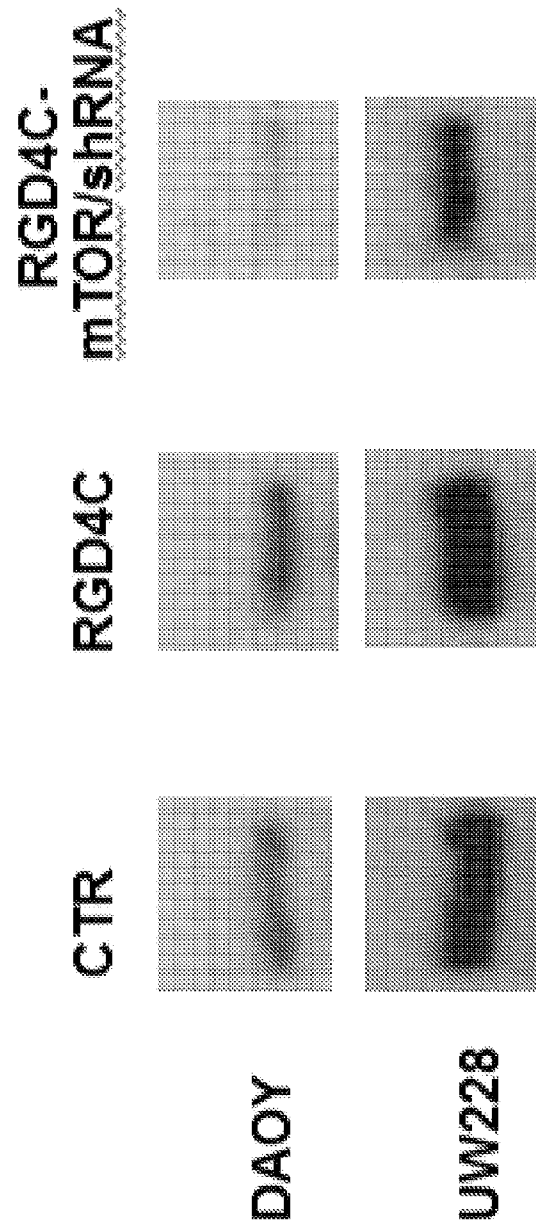
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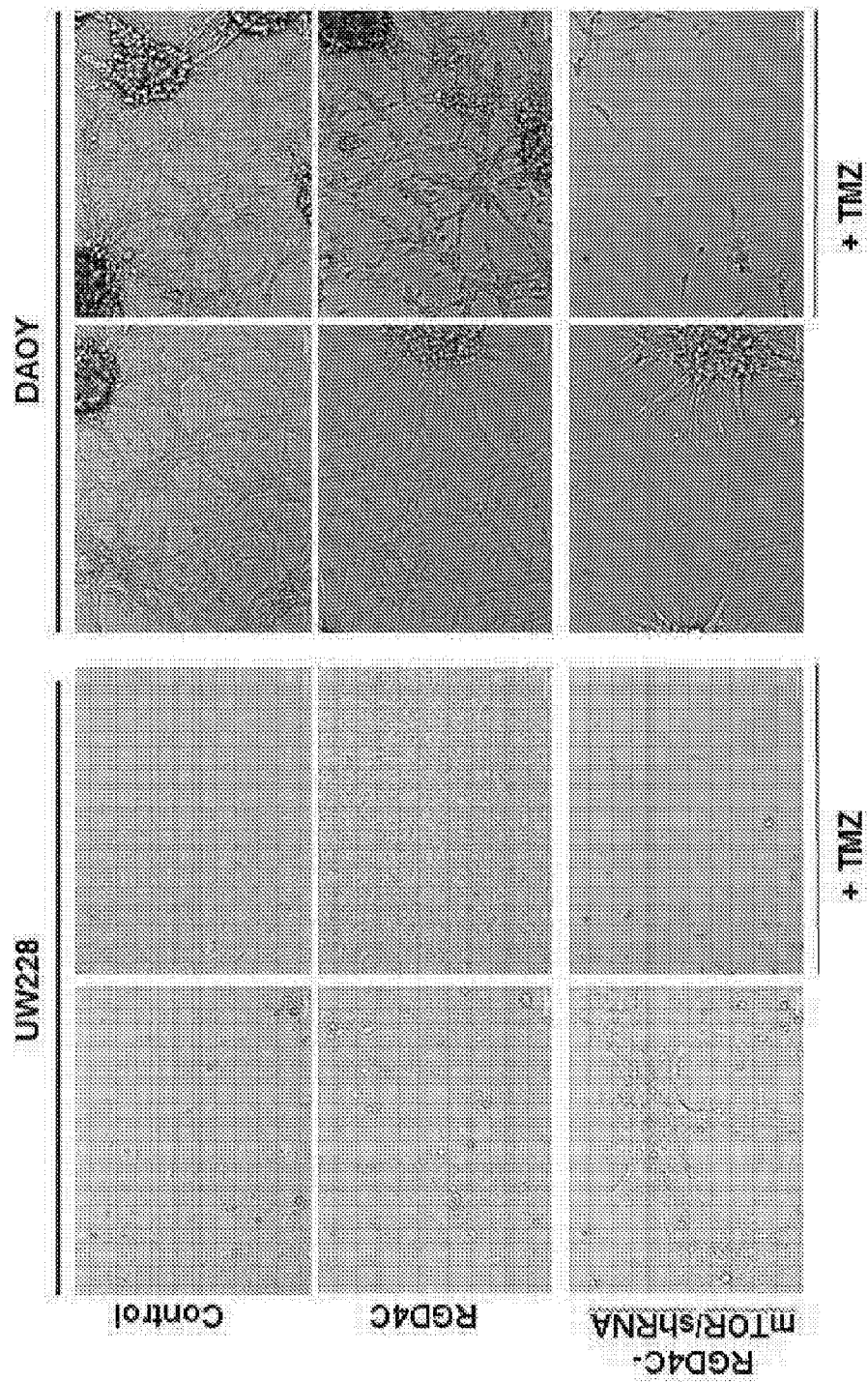
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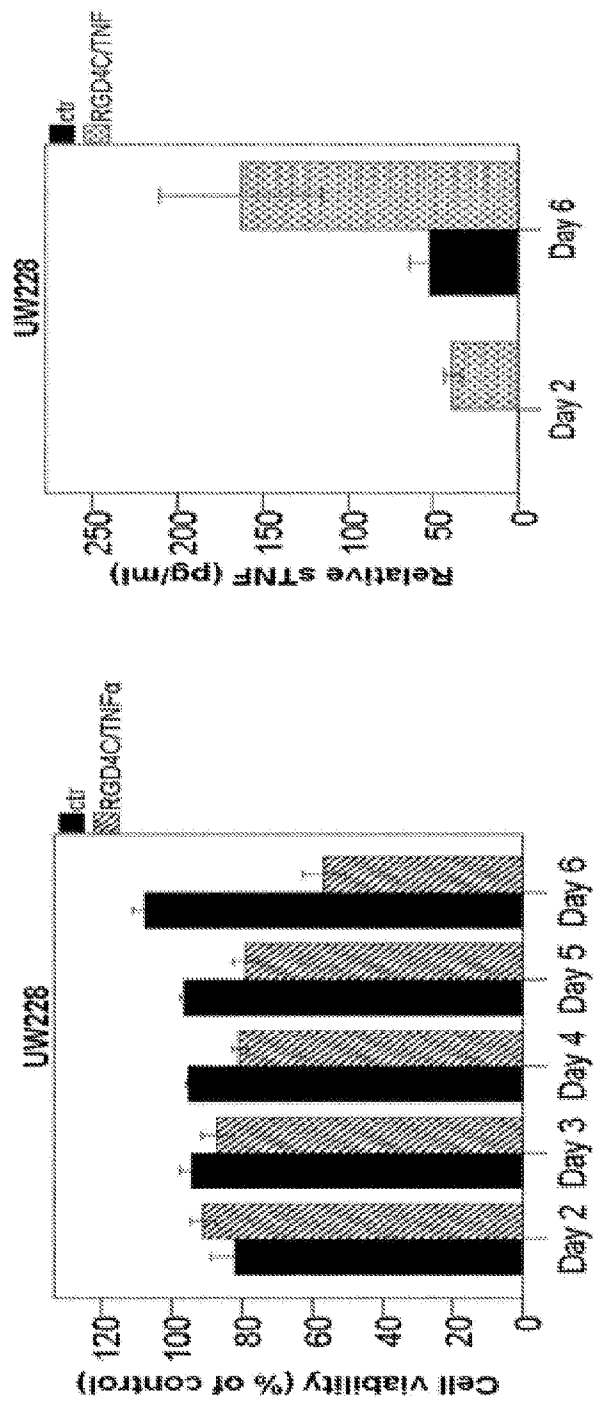
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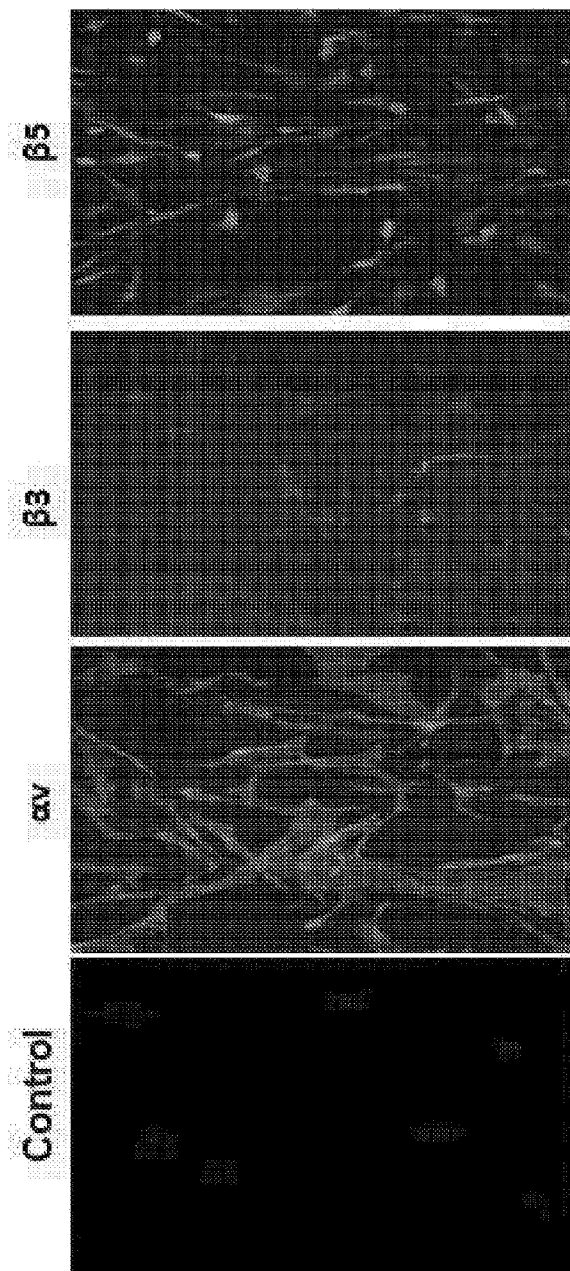
Figure 26

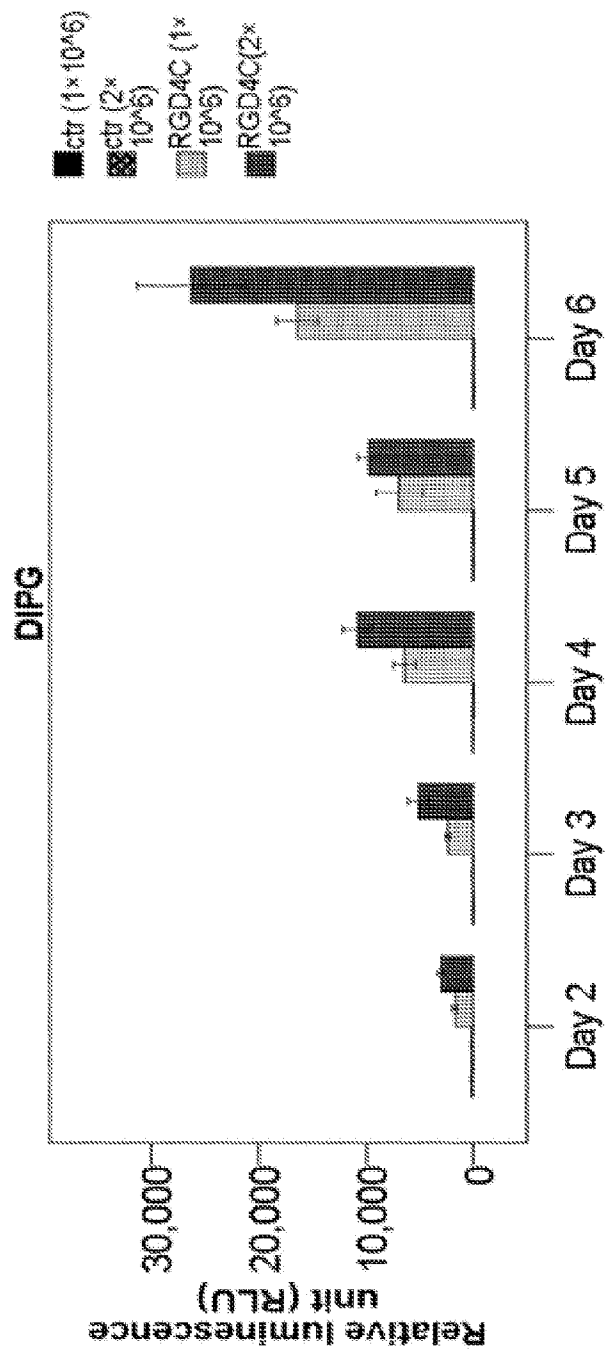
Figure 27

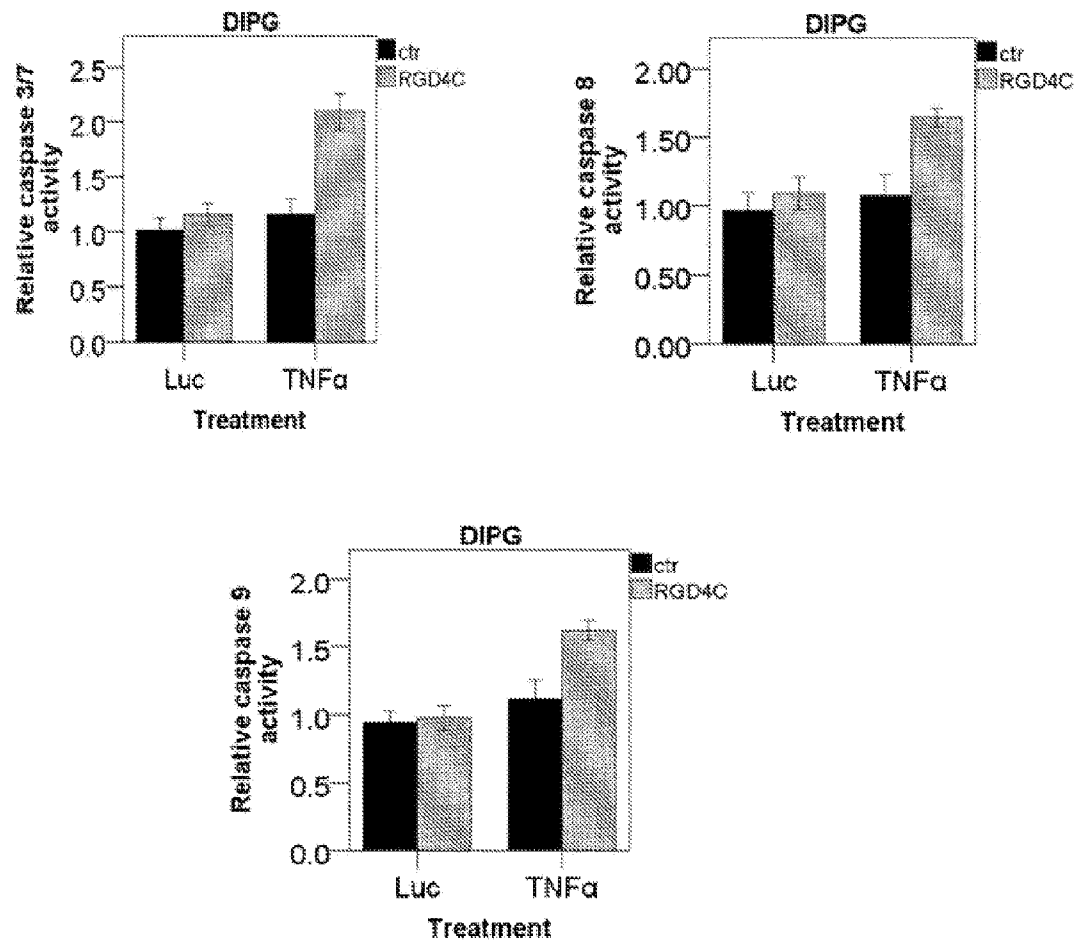
Figure 28

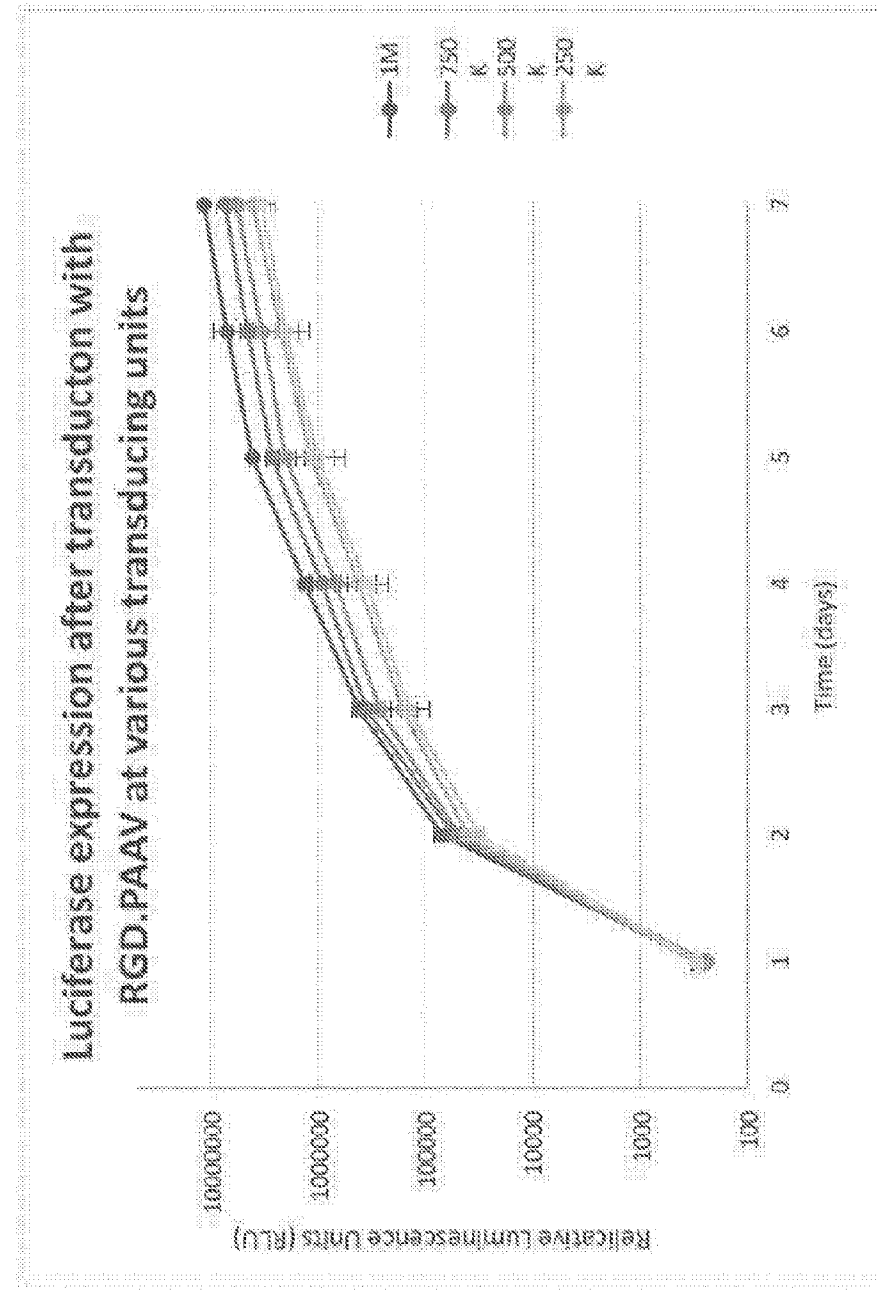
Figure 29

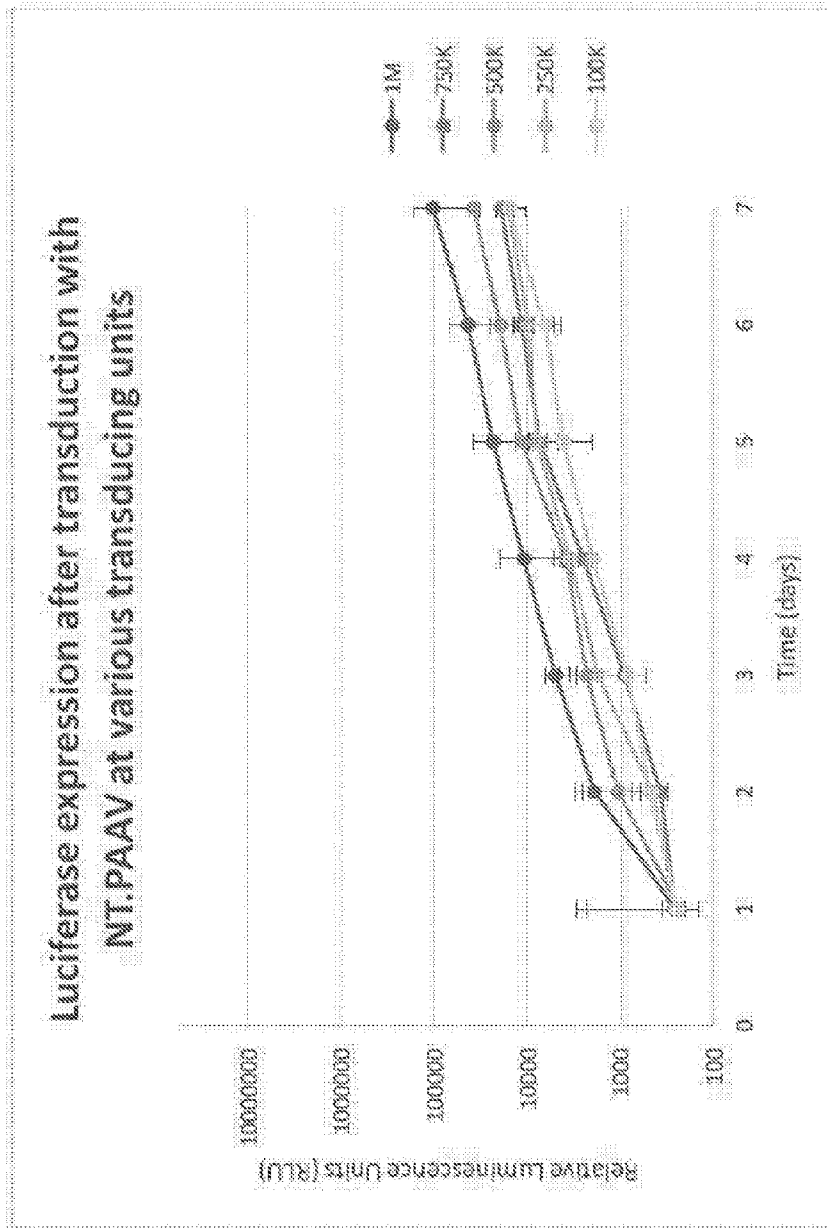
Figure 30

Figure 31