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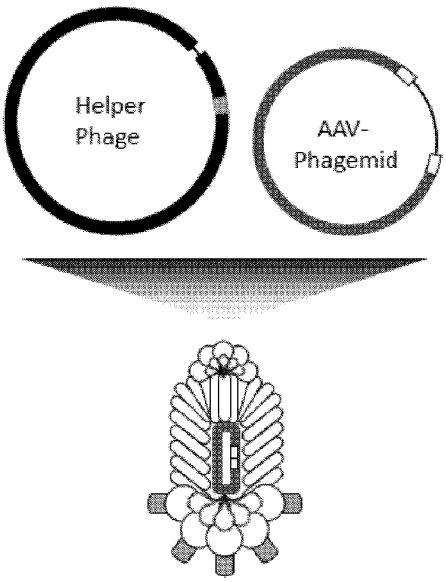
(54) Title: CANCER TREATMENT

Figure 2



Phagemid/Adeno-associated Virion (PAAV)

(57) Abstract: The present invention provides phagemid vectors and associated phagemid particles for cancer treatment, and in particular, to the use of novel phagemid particles and associated expression systems for the treatment, prevention, amelioration, or management of cancer. In particular, the invention relates to the use of phagemid particles and expression systems for the delivery of transgenes encoding cytokines, for the treatment, prevention, amelioration, or management of cancer. The invention also extends to the use of phagemid particles and expression systems for the delivery of transgenes, and for the combination of such treatment with the use of adoptively transferred T cells, for the treatment, prevention, amelioration, or management of cancer.

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[Continued on next page]

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CANCER TREATMENT

The present invention relates to cancer treatment, and in particular, to the use of novel phagemid particles and associated expression systems for the treatment, prevention, amelioration, or management of cancer. In particular, the invention relates to the use of phagemid particles and expression systems for the delivery of transgenes encoding cytokines, for the treatment, prevention, amelioration, or management of cancer. The invention also extends to the use of phagemid particles and expression systems for the delivery of transgenes, and for the combination of such treatment with the use of adoptively transferred T cells, for the treatment, prevention, amelioration, or management of cancer.

Paediatric high-grade gliomas are a heterogeneous group of tumours that accounts for 15%–20% of all paediatric central nervous system (CNS) tumours in children. Tumours can originate from any site within the CNS. When arising from the brainstem, specifically the pons, they are referred to as DIPG. Diagnosis is based on imaging and histological appearance. Despite many clinical trials, prognosis remains poor with 2-year survival rates being less than 10%, making it one of the major causes of brain cancer-related deaths in children. Given its location, the tumour is inoperable and conventional fractionated radiation remains the mainstay treatment to provide temporary benefit, with no other treatment showing any efficacy over conventional radiotherapy. Poor response to conventional treatment in DIPG therefore warrants innovative treatment approaches.

In recent years, efforts have been focused on researching gene therapy as a biological mechanism-based technique to specifically target tumour cells and cause anti-tumour activity. Gene therapy has traditionally been conceived for the treatment of congenital diseases, but has increasingly been used in cancer treatment to improve efficiencies of co-administered treatments such as chemotherapy and radiotherapy, and directly induce death in tumour cells. Considering the many side effects that arise from conventional cancer treatments lacking discrimination between healthy and tumour tissue, delivery of therapeutic genes via targeted vectors offer more specificity and safety.

Various cytokine genes are suitable for use in cancer immune-gene therapy such as interleukins (IL-4, IL-12 and IL-15) and the Tumour Necrosis factor Alpha (TNFα) and TNF-related apoptosis-inducing ligand (TRAIL). TRAIL, a member of the TNF family,

induces apoptosis in various cancer cell lines, with high tolerance and minimal toxicity to normal tissue. Both local and systemic injection of TRAIL protein exerts antitumor effects on human tumour xenografts in mice. However, the rapid clearance of TRAIL following systemic administration and large dose required to achieve tumour regression have limited the TRAIL effectiveness in patients. The presently disclosed particles are ideal and cost-effective for the efficient delivery and sustained expression of the TRAIL gene in cancer. IL-4 was efficacious in the induction of antitumour effect, in mouse models of cancer. Moreover, a phase I clinical trial was initiated to determine the safety and tolerability of IL-4 in recurrent human malignant glioma when injected intratumorally by convection enhanced delivery (CED). No histological evidence of neurotoxicity to normal brain was identified in any patient and no drug-related systemic toxicity was evident in any treated patients. Six of nine patients showed glioma necrosis, and one remained disease free for >18 months after the procedure. Yet, again, the large protein dose and cost required to achieve efficacy, and the invasiveness of intracranial delivery places important limitations.

During the past two decades, IL-12 has emerged as one of the most potent cytokines in mediating antitumor activity in a variety of preclinical models. Through pleiotropic effects on different immune cells that form the tumour microenvironment, IL-12 establishes a link between innate and adaptive immunity that involves different immune effector cells and cytokines depending on the type of tumour or the affected tissue. Although IL-12 has no direct effect on tumour cells, it improves activation of cytotoxic T and NK effector cells which mediates tumour lysis. IL-12, moreover, improves the Th1 cell response, induces a panel of cytokines including IFN-ɣ, and exhibits antiangiogenesis. In mice, local intratumoral adenoviral vector delivery of IL-12 gene was completed safely, significantly prolonged animal survival and induced dramatic regression of tumour size.

Recently, intracranial injection of recombinant rAAV vector encoding the IL-12 was used for brain tumour gene therapy in a rat model, and resulted in antitumour effects associated with increased induction of activated microglia cells. Finally, The TNFα displays powerful anti-tumour cell effects, directly by apoptosis or indirectly by immunomodulatory activity; it also targets and destroys tumour neovascularization.

Interleukin 15 participates in the development of important immune antitumor mechanisms. It activates CD8+ T cells, natural killer (NK) cells, NK T cells, and can

promote the formation of antitumor antibodies. IL-15 can also protect T effector cells from the action of T regulatory cells and reverse tolerance to tumour associated antigens.

The advantages of IL-15 in tumour immunotherapy result from its unique ability to activate important mechanisms of antitumor immunity, including development and activity of both NK cells and CD8+ T cells, and promoting a persistent immune response through its action on memory T cells. What is more, IL-15 is less toxic and less effective in inducing Treg cell activity, as compared with IL-2, and in certain circumstances it can even protect human effector T cells from the action of Treg cells. IL-15 is at the top of the National Cancer Institute's list of agents with the greatest potential use in tumor immunotherapy, and the first clinical study of recombinant human IL-15 in adults with refractory metastatic melanoma and metastatic renal cell cancer is currently recruiting patients (http://clinicaltrials.gov/ct2/show/ NCT01021059).

Survival of colon carcinoma-bearing mice has been significantly improved by treatment with IL-15 and was further improved by programmed death ligand1 (PD-L1) blockade. Still, the greatest therapeutic effects have been achieved with combination modality treatment based on application of IL- 15 and blockade of both PD-L1 and cytotoxic T-lymphocyte antigen 4 (CTLA-4).

Unfortunately, in spite of high expectations, while IL-15 shows efficacy in treatment of metastatic malignancy its lack of in vivo biochemical stability is a key limiting issue. The systemic rhIL-15 cytokine in clinical trials has a very short plasma half-life (<1 h) and rapid renal clearance, which easily results in impedance of efficacy. Furthermore, systemic administration of rhIL15 has the potential to cause toxic side effects, including the induction of autoimmunity.

Delivery of IL-15 directly into the tumour instead of systemic administration would be ideal to decrease toxicity and increase efficacy. However, direct injection into the tumour mass or incorporation of IL-15 as a transgene in adoptive cell transfer is difficult to implement. The presence of multiple metastatic sites or excessive growth and potential leukemic transformation of transduced cells may in fact limit therapeutic relevance.

Another solution to lengthen the time window of IL-15 bioavailability was found in gene therapy approaches. The advantages of gene therapy include loco-regional production, ability to generate fusion constructs and versatility for combination strategies. Unfortunately, systemic delivery using eukaryotic viruses has had limited success due to undesired uptake by the liver and reticulo endothelial system, insertional mutagenesis, immunogenicity arising from reactions with the complement system or pre-existing antibodies, and broad tropism for mammalian cells . Viral tropism may be modified by the addition of tissue-specific ligands to viral capsid proteins to mediate a ligand-receptor interaction on the target tissue. However, addition of these ligands to eukaryotic viruses can alter the structure of the viral capsid, which can reduce efficacy and diminish targeting properties of the peptides themselves.

Thus, while the cytokines described above are suitable for use in cancer immune-therapy, the majority of clinical trials involving cytokines fail to show sustained antitumor responses because of the lack of tumour selectivity resulting in systemic toxicity.

There is, therefore, a need for improved methods for delivery of cytokines in immune-oncotherapy.

In a first aspect, there is provided a recombinant phagemid particle for expressing a transgene in a target tumour cell transduced with the particle, for use in a method for treating, preventing, or ameliorating cancer, wherein the phagemid particle comprises at least one transgene expression cassette comprising a nucleic acid sequence encoding one or more cytokine, and comprises a genome which lacks at least 50% of its bacteriophage genome, and wherein the method comprises delivering the nucleic acid sequence to at least adjacent to the tumour cell, such that one or more cytokine is expressed.

In a preferred embodiment, the transgene expression cassette may encode a cytokine which may have the effect of apoptosis induction in the tumour cell, alternation of the tumour cell to promote endogenous anti-tumour responses, alternation of the tumour cell to facilitate other therapies, or alternation of the tumour microenvironment to facilitate therapy.

In a particular preferred embodiment, the cytokine may be IL-4, IL-12, IL-15, TNFα, TRAIL, IFN-γ, or any combination thereof. Preferably, the cytokine is IL-15, Preferably, the cytokine is IL-4. Preferably, the cytokine is IL-12. Preferably, the cytokine is TRAIL. Preferably, the cytokine is IFN-γ. In one preferred embodiment, the cytokine is not TNFα.

However, in another preferred embodiment, the cytokine is TNFα. Preferably, the cytokine is a hybrid TNFα comprising a non-endogenous signal peptide configured to increase expression and/or secretion of TNFα. Preferably, the signal peptide is a cytokine signal peptide other than that of TNFα. For example, the signal peptide is preferably the IL-2 signal peptide.

Thus, in one embodiment the transmembrane domain of TNFα is replaced with a cytokine signal peptide other than that of TNFα, preferably the IL-2 signal peptide. In another embodiment, a signal peptide different to any other cytokine of the invention, preferably the IL-2 signal peptide, is combined with any other cytokine of the invention, such that the expression and/or secretion of the resulting hybrid cytokine is increased. In particular, the hybrid cytokine may be any one of a hybrid IL-4, IL-12, IL-15, TRAIL or IFN-γ.

The hybrid IL-2-TNFα sequence relates to replacement of the transmembrane domain of TNFα, leaving the sequence encoding the secreted form of TNFα, with the signal peptide of IL-2, such that a hybrid TNFα produced that displays greater expression and/or secretion.

The skilled person would understand that "signal peptide sequence" can relate to an N-terminal sequence that functions to direct the translocation of a protein to the cellular membrane, and regulates secretion of the protein.

In particular, the skilled person would understand that "non-endogenous signal peptide sequence" can relate to a cytokine signal peptide, such as an interleukin, that is different to that of the cytokine being expressed.

The skilled person would understand that "hybrid cytokine" can relate to a cytokine that comprises a non-endogenous signal peptide sequence.

In one embodiment, the full length TNFα comprising the transmembrane domain has the amino acid sequence provided herein as SEQ ID No: 12, as follows:

5′
MSTESMIRDVELAEEALPKKTGGPQGSRRCLFLSLFSFLIVAGATTLFCLLHFGVIGPQR
EEFPRDLSLISPLAQAVRSSSRTPSDKPVAHVVANPQAEGQLQWLNRRANALLANGVELR
DNQLVVPSEGLYLIYSQVLFKGQGCPSTHVLLTHTISRIAVSYQTKVNLLSAIKSPCQRETPEG
AEAKPWYEPIYLGGVFQLEKGDRLSAEINRPDYLDFAESGQVYFGIIAL 3′

[SEQ ID No: 12]

The nucleic acid sequence encoding full length TNFα comprising the transmembrane domain may be represented herein, as follows:

5′
ATGAGCACTGAAAGCATGATCCGGGACGTGGAGCTGGCCGAGGAGGCGCTCCCCAAGAAGACAG
GGGGGCCCCAGGGCTCCAGGCGGTGCTTGTTCCTCAGCCTCTTCTCCTTCCTGATCGTGGCAGG
CGCCACCACGCTCTTCTGCCTGCTGCACTTTGGAGTGATCGGCCCCCAGAGGGAAGAGTTCCCC
AGGGACCTCTCTCTAATCAGCCCTCTGGCCCAGGCAGTCAGATCATCTTCTCGAACCCCGAGTG
ACAAGCCTGTAGCCCATGTTGTAGCAAACCCTCAAGCTGAGGGGCAGCTCCAGTGGCTGAACCG
CCGGGCCAATGCCCTCCTGGCCAATGGCGTGGAGCTGAGAGATAACCAGCTGGTGGTGCCATCA
GAGGGCCTGTACCTCATCTACTCCCAGGTCCTCTTCAAGGGCCAAGGCTGCCCCTCCACCCATG
TGCTCCTCACCCACACCATCAGCCGCATCGCCGTCTCCTACCAGACCAAGGTCAACCTCCTCTC
TGCCATCAAGAGCCCCTGCCAGAGGGAGACCCCAGAGGGGGCTGAGGCCAAGCCCTGGTATGAG
CCCATCTATCTGGGAGGGGTCTTCCAGCTGGAGAAGGGTGACCGACTCAGCGCTGAGATCAATC
GGCCCGACTATCTCGACTTTGCCGAGTCTGGGCAGGTCTACTTTGGGATCATTGCCCTGTGA
3′

[SEQ ID No: 13]

In one embodiment, the secreted form of TNFα has the amino acid sequence provided herein as SEQ ID No: 18, as follows:

VRSSSRTPSDKPVAHVVANPQAEGQLQWLNRRANALLANGVELRDNQLVVPSEGLYLIYS
QVLFKGQGCPSTHVLLTHTISRIAVSYQTKVNLLSAIKSPCQRETPEGAEAKPWYEPIYL

GGVFQLEKGDRLSAEINRPDYLDFAESGQVYFGIIAL

[SEQ ID No: 18]

In one embodiment, the nucleic acid sequence encoding the secreted form of TNFα may be represented herein, as follows:

5′
GTCAGATCATCTTCTCGAACCCCGAGTGACAAGCCTGTAGCCCATGTTGTAGCAAACCCTCAAG
CTGAGGGGCAGCTCCAGTGGCTGAACCGCCGGGCCAATGCCCTCCTGGCCAATGGCGTGGAGCT
GAGAGATAACCAGCTGGTGGTGCCATCAGAGGGCCTGTACCTCATCTACTCCCAGGTCCTCTTC
AAGGGCCAAGGCTGCCCCTCCACCCATGTGCTCCTCACCCACACCATCAGCCGCATCGCCGTCT
CCTACCAGACCAAGGTCAACCTCCTCTCTGCCATCAAGAGCCCCTGCCAGAGGGAGACCCCAGA
GGGGGCTGAGGCCAAGCCCTGGTATGAGCCCATCTATCTGGGAGGGGTCTTCCAGCTGGAGAAG
GGTGACCGACTCAGCGCTGAGATCAATCGGCCCGACTATCTCGACTTTGCCGAGTCTGGGCAGG
TCTACTTTGGGATCATTGCCCTGTGA 3′

[SEQ ID No: 19]

In one embodiment, the IL-2 signal peptide has the amino acid sequence provided herein as SEQ ID No: 20, as follows:

MYRMQLLSCIALSLALVTNS

[SEQ ID No: 20]

In one embodiment, the nucleic acid sequence encoding the IL-2 signal peptide may be represented herein as SEQ ID No: 21, as follows:

5′ ATGTACAGAATGCAACTCCTGTCTTGTATTGCACTAAGTCTCGCACTTGTCACAAACAGT 3′

[SEQ ID No: 21]

Accordingly, in one embodiment, the hybrid IL-2- TNFα has the amino acid sequence provided herein as SEQ ID No 22, as follows:

MYRMQLLSCIALSLALVTNSESVRSSSRTPSDKPVAHVVANPQAEGQLQWLNRRANALLA

NGVELRDNQLVVPSEGLYLIYSQVLFKGQGCPSTHVLLTHTISRIAVSYQTKVNLLSAIK
SPCQRETPEGAEAKPWYEPIYLGGVFQLEKGDRLSAEINRPDYLDFAESGQVYFGIIAL

[SEQ ID No: 22]

Accordingly, in one embodiment, the nucleic acid sequence encoding hybrid IL-2-TNFα may be represented herein as SEQ ID No: 23, as follows:

5′
ATGTACAGAATGCAACTCCTGTCTTGTATTGCACTAAGTCTCGCACTTGTCACAAACAGTGAAT
TCGTCAGATCATCTTCTCGAACCCCGAGTGACAAGCCTGTAGCCCATGTTGTAGCAAACCCTCA
AGCTGAGGGGCAGCTCCAGTGGCTGAACCGCCGGGCCAATGCCCTCCTGGCCAATGGCGTGGAG
CTGAGAGATAACCAGCTGGTGGTGCCATCAGAGGGCCTGTACCTCATCTACTCCCAGGTCCTCT
TCAAGGGCCAAGGCTGCCCCTCCACCCATGTGCTCCTCACCCACACCATCAGCCGCATCGCCGT
CTCCTACCAGACCAAGGTCAACCTCCTCTCTGCCATCAAGAGCCCCTGCCAGAGGGAGACCCCA
GAGGGGGCTGAGGCCAAGCCCTGGTATGAGCCCATCTATCTGGGAGGGGTCTTCCAGCTGGAGA
AGGGTGACCGACTCAGCGCTGAGATCAATCGGCCCGACTATCTCGACTTTGCCGAGTCTGGGCA
GGTCTACTTTGGGATCATTGCCCTGTGA 3′

[SEQ ID No: 23]

Thus, in one embodiment, the one or more cytokine comprises an amino acid sequence of any one of SEQ ID Nos: 12, 18, 20 and 22 or a fragment or variant thereof.

Preferably, the one or more cytokine comprises an amino acid sequence as substantially set out in SEQ ID No: 22 or a fragment or variant thereof.

Thus, in one embodiment, the nucleic acid sequence encoding the one or more cytokine comprises any one of SEQ ID Nos: 13, 19, 21, and 23 or a fragment or variant thereof.

Preferably, the nucleic acid sequence encoding the one or more cytokine comprises SEQ ID No: 23 or a fragment or variant thereof.

In a second aspect, there is provided a method of treating, preventing, or ameliorating cancer in a subject, the method comprising administering, to a subject in need of such

treatment, a therapeutically effective amount of the recombinant phagemid particle according to the first aspect.

The method of the second aspect may be used for the management of cancer in a subject.

In an embodiment, the recombinant phagemid particle is for use in treating, preventing or ameliorating paediatric brain tumours. In other embodiments, the recombinant phagemid particle is for use in treating, preventing or ameliorating diffuse intrinsic pontine glioma (DIPG) or medulloblastoma.

In a third aspect, there is provided a system for producing a recombinant phagemid particle from a prokaryotic host, the system comprising:-

(i) a first vector configured to persist inside a prokaryotic host, and comprising at least one transgene expression cassette comprising a nucleic acid sequence encoding one or more cytokine, and a packaging signal for enabling replication of the vector into single-stranded DNA; and

(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.

In a fourth aspect, there is provided a method for producing a recombinant phagemid particle from a prokaryotic host, the method comprising:-

(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 comprising a nucleic acid sequence encoding one or more cytokine, and a packaging signal for enabling replication of the vector into single-stranded DNA;

(ii) introducing, into the host, a helper phage comprising nucleic acid encoding bacteriophage structural proteins; and

(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.

In a fifth aspect, there is provided a method for producing a recombinant phagemid particle from a prokaryotic host, the method comprising:-

(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 comprising a nucleic acid sequence encoding one or more cytokine, 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

(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.

In a sixth aspect of the invention, there is provided a pharmaceutical composition comprising the recombinant phagemid viral particle produced by the system according to the third aspect, or produced by the methods of the fourth or fifth aspect, and a pharmaceutically acceptable vehicle.

The invention also provides, in a seventh aspect, a process for making the pharmaceutical composition according to the sixth aspect, the process comprising contacting a therapeutically effective amount of the recombinant phagemid particle produced by the system according to the third aspect, or produced by the methods of the fourth or fifth aspect, and a pharmaceutically acceptable vehicle.

In another aspect of the invention there is provided a recombinant phagemid particle for expressing a transgene in a target tumour cell transduced with the particle, wherein the phagemid particle comprises at least one transgene expression cassette comprising a nucleic acid sequence encoding one or more cytokine, and comprises a genome which lacks at least 50% of its bacteriophage genome, and wherein, in use, the particle is configured to deliver the nucleic acid sequence to at least adjacent to the tumour cell, such that one or more cytokine is expressed, and wherein the cytokine is any one of IL-4, IL-12, IL-15, TRAIL, IFN-γ, hybrid TNFα or any combination thereof.

Preferably, the cytokine is IL-15, Preferably, the cytokine is IL-4. Preferably, the cytokine is IL-12. Preferably, the cytokine is TRAIL. Preferably, the cytokine is IFN-γ.

Preferably, the cytokine is a hybrid TNFα comprising a non-endogenous signal peptide configured to increase expression and/or secretion of TNFα. Preferably, the signal

peptide is a cytokine signal peptide other than that of TNFα. For example, the signal peptide is preferably the IL-2 signal peptide.

In another embodiment, a signal peptide different to any other cytokine of the invention, preferably the IL-2 signal peptide, is combined with any other cytokine of the invention, such that the expression and/or secretion of the resulting hybrid cytokine is increased. In particular, the hybrid cytokine may be any one of a hybrid IL-4, IL-12, IL-15, TRAIL or IFN-γ.

In a preferred embodiment, the transgene expression cassette may encode a cytokine which may have the effect of apoptosis induction in the tumour cell, alternation of the tumour cell to promote endogenous anti-tumour responses, alternation of the tumour cell to facilitate other therapies, or alternation of the tumour microenvironment to facilitate therapy

In a further aspect, there is provided a recombinant phagemid particle for expressing a transgene in a target tumour cell transduced with the particle, wherein the phagemid particle comprises at least one transgene expression cassette comprising a nucleic acid sequence encoding one or more cytokine, and comprises a genome which lacks at least 50% of its bacteriophage genome, and wherein, in use, the particle is configured to deliver the nucleic acid sequence to at least adjacent to the tumour cell, such that one or more cytokine is expressed, and wherein the cytokine is any one of IL-4, IL-12, IL-15, TRAIL, IFN-γ, hybrid TNFα or any combination thereof, for use in therapy or diagnosis.

Preferably, the cytokine is IL-15, Preferably, the cytokine is IL-4. Preferably, the cytokine is IL-12. Preferably, the cytokine is TRAIL. Preferably, the cytokine is IFN-γ.

Preferably, the cytokine is a hybrid TNFα comprising a non-endogenous signal peptide configured to increase expression and/or secretion of TNFα. Preferably, the signal peptide is a cytokine signal peptide other than that of TNFα. For example, the signal peptide is preferably the IL-2 signal peptide.

In another embodiment, a signal peptide different to any other cytokine of the invention, preferably the IL-2 signal peptide, is combined with any other cytokine of the invention, such that the expression and/or secretion of the resulting hybrid

cytokine is increased. In particular, the hybrid cytokine may be any one of a hybrid IL-4, IL-12, IL-15, TRAIL or IFN-γ.

There is, also a need for improved methods for treating cancer, for use with adoptive transfer therapy, such as CAR T cell therapy. The inventors have shown in the Examples that it is possible to use the particles described herein for decorating or labelling tumour cells with antigens which are recognisable by CAR T cells, and the like.

Thus, according to an eighth aspect of the invention, there is provided a recombinant phagemid particle for expressing at least one antigen in a target tumour cell transduced with the particle, the phagemid particle comprising at least one transgene expression cassette comprising a nucleic acid sequence encoding one or more antigen that is recognised by one or more adoptively transferred T cell, and comprising a genome which lacks at least 50% of its bacteriophage genome, and wherein, in use, the particle is configured to deliver the nucleic acid sequence to at least adjacent to the target tumour cell, such that the one or more antigen is expressed, and recognised by one or more adoptively transferred T cell.

In a ninth aspect, there is provided the recombinant phagemid particle according to the eighth aspect, for use in therapy or diagnosis.

In a tenth aspect, there is provided the recombinant phagemid particle according to the eighth aspect, for use in treating, preventing or ameliorating cancer.

Advantageously, using the particles of the present invention to target the delivery and expression of cytokines and antigens to tumour cells results in greater tumour killing efficacies, and reduced off target effects that are associated with conventional therapies. The inventors have developed novel vectors and systems (a so-called “hybrid phagemid viral vector system”) that are particularly advantageous for the delivery of antigens or cytokines to tumour cells. There is provided a so-called 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”. Provided herein is a bacteriophage guided cytokine therapy. Also provided herein is a bacteriophage-guided CAR T cell therapy.

Unlike the prior art AAVP genome (*Nature protocols* **2**, 523-531 (2007); *Cell* **125**, 385-398 (2006)), which consists of a rAAV cassette inserted into the filamentous phage genome, the PAAV genome of the invention does not contain any structural bacteriophage (phage) genes, and so a prokaryotic helper phage virus is required to facilitate vector assembly in the host. This is advantageous as AAVP still has certain inherent limitations of bacteriophage and thus leaves room for significant improvement of AAVP or phage vectors in general. 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 AAVP 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.

Advantageously, re-engineering hybrid viral vectors (e.g. AAV or lentivirus) into the phagemid particle according to the eighth or any above aspect, 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.

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).

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 virus (e.g. rAAV or lentivirus) 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. Preferably, therefore, the particle comprises multiple transgene cassettes.

In an embodiment, the recombinant phagemid particle is for use in treating a subject that has not been exposed to the delivered one or more antigen, for example by prior vaccination.

In one embodiment, the phagemid particle is preferably configured to deliver the transgene expression cassette to a cell that is adjacent to the target tumour cell, such that the nucleic acid sequence is expressed, thereby producing the or each antigen or cytokine, the antigen can then associate with, or become attached to, the target the tumour cell.

However, in a preferred embodiment, the phagemid particle is configured to deliver the transgene expression cassette to the target tumour cell. Preferably, the or each antigen is a peptide or protein which is expressed on the cell surface of the target tumour cell. Preferably, the or each antigen is a peptide or protein that, when expressed by the tumour cell, would be accessible to a CAR T cell. The peptide or protein may be such that, when expressed by the tumour cell, it is present as a folded peptide protein at or on the cell-surface.

In an eleventh aspect of the invention, there is provided a recombinant phagemid particle for expressing at least one antigen in a target tumour cell transduced with the particle, the phagemid particle comprising at least one transgene expression cassette comprising a nucleic acid sequence encoding one or more cytokine, and comprising a genome which lacks at least 50% of its bacteriophage genome, and wherein, in use, the

particle is configured to deliver the nucleic acid sequence to at least adjacent to the target tumour cell, such that the one or more cytokine is expressed.

In a twelfth aspect, there is provided the recombinant phagemid particle according to the eleventh aspect, for use in therapy or diagnosis.

In a thirteenth aspect, there is provided the recombinant phagemid particle according to the eleventh aspect, for use in treating, preventing or ameliorating cancer.

In a fourteenth aspect, there is provided a method of treating, preventing, or ameliorating cancer in a subject, the method comprising administering, to a subject in need of such treatment, a therapeutically effective amount of the recombinant phagemid particle according to the eleventh aspect.

In a fifteenth aspect, 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 eleventh aspect from a prokaryotic host.

In a sixteenth aspect, there is provided a recombinant phagemid viral particle according to the eleventh aspect, produced by the system according to the third aspect, produced by the methods of the fourth or fifth aspect, or produced according to the use of the fifteenth aspect, wherein the recombinant phagemid particle is for production of a recombinant viral vector comprising or derived from the viral genome within the genome of the phagemid particle, wherein the recombinant viral vector is used for delivering the nucleic acid sequence encoding one or more antigen, to at least adjacent to the tumour cell, such that one or more cytokine is expressed.

In a seventeenth aspect, there is provided a recombinant vector comprising rAAV, rep-cap, adenohelper genes, and a nucleic acid sequence encoding one or more antigen or cytokine, for use in the treatment, prevention, or amelioration of cancer.

In an eighteenth aspect, there is provided a recombinant phagemid particle comprising the vector of the seventeenth aspect, for use in a method for the treatment, prevention, or amelioration of cancer.

The or each antigen may be a known target for existing CAR T cells suitable for use in humans. For example, the or each antigen may be selected from a group consisting of: MUC1; PSMA; CD19; CD20; estrogen-related receptor beta type 2 (ErRB2); or any combination thereof. The or each antigen may be MUC1 or PSMA.

In one embodiment, a nucleic acid sequence encoding the suitable antigen (e.g. MUC1.CD28.IL4) may be represented herein, as follows:

ATGGCTCTCCCAGTGACTGCCCTACTGCTTCCCCTAGCGCTTCTCCTGCATGCAACCGCCCCTCCAGCCC
ACGGAGTGACCAGCGCCCCTGACACCCGGCCTGCTCCTGGAAGCACAGCTCCACCTGCCCACGGCGTTAC
CTCTGCACCAGATACTAGGCCTGCTCCAGGCTCCATCGAGGTGATGTACCCCCCCCCCTACCTGGACAAC
GAGAAGAGCAACGGCACCATCATCCACGTGAAGGGCAAGCACCTGTGCCCCAGCCCCCTGTTCCCCGGCC
CCAGCAAGCCCTTCTGGGTGCTGGTGGTGGTGGGCGGCGTGCTGGCCTGCTACAGCCTGCTGGTGACCGT
GGCCTTCATCATCTTCTGGGTGCGGAGCAAGAGGAGAAAGCGCAGCGGTTCCGGCGAGGGCCGGGGCAGC
CTGCTGACCTGCGGCGACGTGGAGGAGAACCCCGGCCCTATGGGCCTGACCAGCCAGCTTCTGCCCCCCC
TGTTCTTCCTGCTGGCCTGCGCCGGCAACTTCGTGCACGGCCACAAGTGCGACATCACCCTGCAGGAGAT
CATCAAGACCCTGAACAGCCTGACCGAGCAGAAGACCCTGTGCACCGAGCTGACCGTGACCGACATCTTC
GCCGCCAGCAAGAACACCACCGAGAAGGAGACCTTCTGCCGGGCCGCCACCGTGCTGCGGCAGTTCTACA
GCCACCACGAGAAGGACACCCGGTGCCTGGGCGCCACCGCCCAGCAGTTCCACCGGCACAAGCAACTGAT
CCGGTTCCTGAAGCGGCTGGACCGGAACCTGTGGGGCCTGGCCGGCCTGAACAGTTGCCCCGTGAAGGAG
GCCAACCAGAGCACCCTGGAGAACTTCCTGGAGCGGCTGAAGACCATCATGCGGGAGAAGTACAGCAAGT
GCAGCAGCTAG

[SEQ ID NO:14]

In another embodiment, a nucleic acid sequence encoding the antigen (e.g. MUC1.GPI.IL4) may be represented herein, as follows:

ATGGCTCTCCCAGTGACTGCCCTACTGCTTCCCCTAGCGCTTCTCCTGCATGCAACCGCCCCTCCAGCCC
ACGGAGTGACCAGCGCCCCTGACACCCGGCCTGCTCCTGGAAGCACAGCTCCACCTGCCCACGGCGTTAC
CTCTGCACCAGATACTAGGCCTGCTCCAGGCTCCCCCAACAAGGGCAGCGGCACAACCAGCGGAACCACC
AGGCTGTTGAGCGGCCACACCTGCTTCACCCTGACAGGCCTGCTGGGCACCCTGGTGACAATGGGCCTGC
TGACCAGGAGAAAGCGCAGCGGTTCCGGCGAGGGCCGGGGCAGCCTGCTGACCTGCGGCGACGTGGAGGA
GAACCCCGGCCCTATGGGCCTGACCAGCCAGCTTCTGCCCCCCCTGTTCTTCCTGCTGGCCTGCGCCGGC
AACTTCGTGCACGGCCACAAGTGCGACATCACCCTGCAGGAGATCATCAAGACCCTGAACAGCCTGACCG
AGCAGAAGACCCTGTGCACCGAGCTGACCGTGACCGACATCTTCGCCGCCAGCAAGAACACCACCGAGAA
GGAGACCTTCTGCCGGGCCGCCACCGTGCTGCGGCAGTTCTACAGCCACCACGAGAAGGACACCCGGTGC
CTGGGCGCCACCGCCCAGCAGTTCCACCGGCACAAGCAACTGATCCGGTTCCTGAAGCGGCTGGACCGGA

ACCTGTGGGGCCTGGCCGGCCTGAACAGTTGCCCCGTGAAGGAGGCCAACCAGAGCACCCTGGAGAACTT
CCTGGAGCGGCTGAAGACCATCATGCGGGAGAAGTACAGCAAGTGCAGCAGCTAG

[SEQ ID NO:15]

In yet another embodiment, a nucleic acid sequence encoding the antigen (e.g. PSMA) may be represented herein, as follows:

ATGTGGAACCTGCTGCACGAGACTGACAGCGCCGTGGCAACCGCACGGAGACCCCGGTGGCTGTGCGCTG
GCGCACTGGTGCTGGCCGGCGGGTTCTTTCTGCTGGGGTTCCTGTTTGGATGGTTTATCAAAAGCTCCAA
CGAGGCCACCAATATTACACCTAAGCACAATATGAAAGCATTCCTGGATGAACTGAAGGCCGAGAACATC
AAGAAATTCCTGTACAACTTTACTCAGATTCCACATCTGGCTGGCACCGAGCAGAACTTTCAGCTGGCAA
AACAGATCCAGAGCCAGTGGAAGGAATTCGGGCTGGACTCCGTGGAGCTGGCCCACTACGATGTCCTGCT
GAGTTATCCAAATAAGACACATCCCAACTATATCTCAATCATTAACGAAGACGGAAATGAGATTTTCAAC
ACTTCACTGTTTGAACCCCCTCCACCCGGCTACGAGAACGTGAGCGACATCGTCCCTCCATTCTCAGCCT
TTAGCCCACAGGGAATGCCTGAGGGGGATCTGGTGTACGTCAATTATGCTCGCACCGAAGACTTCTTTAA
GCTGGAGCGAGATATGAAAATCAACTGTAGCGGCAAGATCGTGATTGCCAGATACGGCAAAGTGTTTCGC
GGGAATAAGGTCAAAAACGCTCAGCTGGCCGGGGCTAAGGGAGTGATTCTGTACTCTGACCCCGCTGATT
ATTTCGCACCTGGAGTGAAGAGTTATCCAGACGGATGGAATCTGCCAGGAGGAGGAGTGCAGCGAGGAAA
CATCCTGAACCTGAATGGGGCCGGAGATCCTCTGACCCCAGGATACCCCGCCAACGAATACGCTTATAGG
CGAGGAATTGCAGAGGCAGTGGGACTGCCTTCCATCCCAGTCCACCCCATTGGCTACTATGACGCCCAGA
AGCTGCTGGAGAAAATGGGAGGCTCTGCTCCCCCTGATTCTAGTTGGAGAGGCAGTCTGAAGGTGCCTTA
CAATGTCGGCCCAGGGTTCACAGGGAACTTTTCAACTCAGAAGGTGAAAATGCACATCCATAGCACTAAT
GAAGTGACCAGGATCTATAACGTCATTGGAACTCTGCGAGGCGCCGTGGAGCCTGACAGATACGTCATTC
TGGGGGGACACCGCGACTCCTGGGTGTTTGGCGGGATCGATCCACAGTCTGGCGCCGCTGTGGTCCATGA
AATTGTGCGGTCTTTCGGCACACTGAAGAAAGAGGGGTGGAGACCCCGACGGACTATCCTGTTTGCAAGT
TGGGATGCCGAGGAATTCGGCCTGCTGGGGAGTACAGAATGGGCCGAGGAAAATTCACGGCTGCTGCAGG
AGAGAGGGGTGGCTTACATCAATGCAGACTCAAGCATTGAAGGAAACTATACACTGCGGGTGGATTGCAC
TCCCCTGATGTACAGCCTGGTCCACAACCTGACCAAGGAGCTGAAATCCCCTGACGAGGGATTCGAAGGC
AAAAGCCTGTATGAATCCTGGACAAAGAAAAGTCCATCACCCGAGTTTAGCGGAATGCCTCGAATCTCTA
AGCTGGGAAGTGGCAATGATTTCGAAGTGTTCTTTCAGAGACTGGGGATTGCCTCCGGAAGAGCTAGGTA
CACCAAAAATTGGGAGACAAACAAGTTCTCCGGCTACCCACTGTATCACAGCGTGTACGAGACTTATGAA
CTGGTCGAGAAATTCTACGACCCCATGTTTAAGTATCATCTGACCGTGGCACAGGTCAGGGGAGGCATGG
TGTTTGAGCTGGCCAATTCCATCGTCCTGCCATTCGACTGTAGAGATTATGCTGTGGTCCTGAGGAAGTA
CGCAGACAAAATCTATAGCATTTCCATGAAACATCCCCAGGAGATGAAGACCTACTCTGTGAGTTTCGAT
TCCCTGTTTTCTGCCGTCAAAAACTTCACAGAAATCGCTAGTAAGTTTTCAGAGCGCCTGCAGGACTTCG
ATAAGTCTAATCCCATTGTGCTGAGGATGATGAACGACCAGCTGATGTTCCTGGAACGCGCCTTTATCGA
CCCTCTGGGGCTGCCTGATCGCCCCTTCTACCGACACGTGATCTACGCACCTTCCTCTCATAACAAGTAC
GCCGGAGAGTCTTTTCCAGGCATCTATGACGCTCTGTTCGATATTGAATCAAAGGTCGATCCCAGCAAAG

CATGGGGCGAGGTCAAGAGACAGATCTACGTGGCAGCCTTCACCGTCCAGGCTGCAGCCGAAACACTGAG
CGAGGTGGCCTGA

[SEQ ID NO:16]

Thus, preferably the transgene comprises a nucleic acid sequence substantially as set out in any one of SEQ ID No: 14-16, or a fragment or variant thereof.

In yet another embodiment, the antigen may comprise or be derived from an amino acid sequence (e.g. MUC1, Genbank accession number: P15941), substantially as set out in SEQ ID No:17, or a fragment or variant thereof:

mtpgtqspff llllltvltv vtgsghasst pggeketsat qrssvpsste knavsmtssv
lsshspgsgs sttqgqdvtl apatepasgs aatwgqdvts vpvtrpalgs ttppahdvts
apdnkpapgs tappahgvts apdtrpapgs tappahgvts apdtrpapgs tappahgvts
apdtrpapgs tappahgvts apdtrpapgs tappahgvts apdtrpapgs tappahgvts
apdtrpapgs tappahgvts apdtrpapgs tappahgvts apdtrpapgs tappahgvts
apdtrpapgs tappahgvts apdtrpapgs tappahgvts apdtrpapgs tappahgvts
apdtrpapgs tappahgvts apdtrpapgs tappahgvts apdtrpapgs tappahgvts
apdtrpapgs tappahgvts apdtrpapgs tappahgvts apdtrpapgs tappahgvts
apdtrpapgs tappahgvts apdtrpapgs tappahgvts apdtrpapgs tappahgvts
apdtrpapgs tappahgvts apdtrpapgs tappahgvts apdtrpapgs tappahgvts
apdtrpapgs tappahgvts apdtrpapgs tappahgvts apdtrpapgs tappahgvts
apdtrpapgs tappahgvts apdtrpapgs tappahgvts apdtrpapgs tappahgvts
apdtrpapgs tappahgvts apdtrpapgs tappahgvts apdtrpapgs tappahgvts
apdtrpapgs tappahgvts apdtrpapgs tappahgvts apdnrpalgs tappvhnvts
asgsasgsas tlvhngtsar atttpaskst pfsipshhsd tpttlashst ktdassthhs
svppltssnh stspqlstgv sffflsfhis nlqfnssled pstdyyqelq rdisemflqi
ykqggflgls nikfrpgsvv vqltlafreg tinvhdvetq fnqykteaas rynltisdvs
vsdvpfpfsa qsgagvpgwg iallvlvcvl valaivylia lavcqcrrkn ygqldifpar
dtyhpmseyp tyhthgryvp psstdrspye kvsagnggss lsytnpavaa tsanl

[SEQ ID NO:17]

In some embodiments, one antigen type or species, or cytokine is delivered to at least adjacent to the target tumour cell. In other embodiments, two or more antigen types or species, or cytokine are delivered to at least adjacent to the tumour cell. The two or more antigens or cytokines may be delivered by the same recombinant phagemid

particle, or they may be delivered separately by two or more recombinant phagemid particles.

In one embodiment, the or each antigen may be a self-antigen to which a CAR T cell has been developed. A self antigen may be considered to be an antigen expressed by non-tumour tissues of the subject, and such antigens have been utilised as targets for CAR T cells and are suitable for use with the present invention.

In a preferred embodiment, however, the or each antigen may be a wholly non-self antigen to which a CAR T cell has been developed. A non-self antigen may include neo-antigens that are newly expressed by tumours, and these neo-antigens have also been utilised as targets for CAR T cells and are also suitable for use with the present invention. A non-self antigen may include foreign antigens, not expressed by any tissues of the subject, to which a CAR T cell has been developed. Such foreign antigens may be advantageous because the therapy may result in fewer "off-target" effects caused by targeting of non-tumour tissues expressing the target antigen.

In an embodiment, the or each antigen may be derived from the dengue virus or derived from yellow fever virus.

In a preferred embodiment, the recombinant phagemid particle is for use in a method that further comprises the use of adoptively transferred T cells. The adoptively transferred T cells may be specific for the or each antigen introduced by the recombinant phagemid particle into the target tumour cell. Preferably, more than one type of T cell is adoptively transferred. Preferably, the more than one type of T cell is specific for the same antigen or for different antigens.

Preferably, the adoptively transferred T cell which recognises the one or more antigen expressed at least adjacent to the target tumour cell is selected from a group consisting of a chimeric antigen receptor (CAR) T cell; a T cell receptor (TCR) transgenic T cell; and a tumour infiltrating lymphocyte (TIL). The TCR transgenic T cell may be specific for an epitope associated with the or each introduced antigen. The TIL may originate from a tissue known to contain lymphocytes with a specificity to a known antigen.

Most preferably, however, the adoptively transferred T cell is a chimeric antigen receptor (CAR) T cell. The CAR T cell may be specific for the or each antigen introduced by the recombinant phagemid particle into the tumour cell.

In a most preferred embodiment, the recombinant phagemid particle is for use in a method that comprises the specific targeting of tumour cells by the recombinant phagemid particle and subsequent delivery to a tumour cell of one or more sequence encoding one or more antigen or cytokine for expression by the tumour cell, and in relation to the antigen, the transfer of CAR T cells with a specificity for the one or more delivered antigen, and subsequent cytotoxic activity against the tumour cells for the treatment, prevention, or amelioration of cancer.

In an embodiment, the CAR T cell may be a dual specificity CAR T cell, which may have specificity for one or more antigen delivered by one or more recombinant phagemid particle. For instance, a single type or species of recombinant phagemid particle may deliver two or more antigens for recognition by a single type of dual-specificity CAR T cell.

Preferably, the phagemid particle comprises a virion. 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.

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:

ACGCGCCCTGTAGCGGCGCATTAAGCGCGGCGGGTGTGGTGGTTACGCGCAGCGTGACCGCTACACTTGC
CAGCGCCCTAGCGCCCGCTCCTTTCGCTTTCTTCCCTTCCTTTCTCGCCACGTTCGCCGGCTTTCCCCGT
CAAGCTCTAAATCGGGGGCTCCCTTTAGGGTTCCGATTTAGTGCTTTACGGCACCTCGACCCCAAAAAAC
TTGATTTGGGTGATGGTTCACGTAGTGGGCCATCGCCCTGATAGACGGTTTTTCGCCCTTTGACGTTGGA
GTCCACGTTCTTTAATAGTGGACTCTTGTTCCAAACTGGAACAACACTCAACCCTATCTCGGGCTATTCT

TTTGATTTATAAGGGATTTTGCCGATTTCGGCCTATTGGTTAAAAAATGAGCTGATTTAACAAAAATTTA
ACGCGAATTTTAACAAAATATTAACGTTTACAATTT

[SEQ ID NO:1]

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:

TTGAGATCCTTTTTTTCTGCGCGTAATCTGCTGCTTGCAAACAAAAAAACCACCGCTACCAGCGGTGGTT
TGTTTGCCGGATCAAGAGCTACCAACTCTTTTTCCGAAGGTAACTGGCTTCAGCAGAGCGCAGATACCAA
ATACTGTCCTTCTAGTGTAGCCGTAGTTAGGCCACCACTTCAAGAACTCTGTAGCACCGCCTACATACCT
CGCTCTGCTAATCCTGTTACCAGTGGCTGCTGCCAGTGGCGATAAGTCGTGTCTTACCGGGTTGGACTCA
AGACGATAGTTACCGGATAAGGCGCAGCGGTCGGGCTGAACGGGGGGTTCGTGCACACAGCCCAGCTTGG
AGCGAACGACCTACACCGAACTGAGATACCTACAGCGTGAGCTATGAGAAAGCGCCACGCTTCCCGAAGG
GAGAAAGGCGGACAGGTATCCGGTAAGCGGCAGGGTCGGAACAGGAGAGCGCACGAGGGAGCTTCCAGGG
GGAAACGCCTGGTATCTTTATAGTCCTGTCGGGTTTCGCCACCTCTGACTTGAGCGTCGATTTTTGTGAT
GCTCGTCAGGGGGGCGGAGCCTATGGAAA

[SEQ ID NO:2]

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.

In another embodiment, preferably the phagemid particle may be used as a recombinant vector for the delivery of the transgene to a tissue specific target (e.g. tumour tissue), irrespective of whether the vector is administered systemically or locally to a subject *in 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 embodiment, comprise a lentivirus transgene expression cassette. The at least one transgene expression cassette is preferably an adeno-associated virus (AAV) transgene expression cassette.

The transgene expression cassette may comprise any nucleic acid encoding the or each antigen or cytokine, which is expressed in the target tumour 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.

As shown in the illustrative Example 7, the phagemid may be used to deliver a gene to tumour cells. In this example, down-regulation of mTOR expression in tumour cells (e.g. medulloblastoma cells) may be achieved with treatment with RGD4C-phagemid carrying a sequence encoding the mTOR/shRNA (RGD4C-mTOR/ shRNA). Example 7 provides another illustrative example of the use of the phagemid to deliver genes to tumour cells. As shown in Example 7, RGD4C-phagemid can successfully deliver TNFα to DIPG (diffuse intrinsic pontine glioma) in a selective manner, resulting in apoptosis induction. Therefore, the RGD4C-phagemid has therapeutic potential for use in targeted therapy against DIPG using cytokines

However, it will be appreciated that the type of tumour cell, which is targeted by the recombinant phagemid particle depends on the type of cell-targeting ligand, which may be expressed on the surface of the particle. Cell-targeting ligands are discussed below.

The transgene expression cassette may comprise one or more functional elements required for expression of the nucleic acid in the target tumour 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
GCGTTACATAACTTACGGTAAATGGCCCGCCTGGCTGACCGCCCAACGACCCCCGCCCATTGACGTCAAT
AATGACGTATGTTCCCATAGTAACGTCAATAGGGACTTTCCATTGACGTCAATGGGTGGAGTATTTACGG
TAAACTGCCCACTTGGCAGTACATCAAGTGTATCATATGCCAAGTACGCCCCCTATTGACGTCAATGACG

GTAAATGGCCCGCCTGGCATTATGCCCAGTACATGACCTTATGGGACTTTCCTACTTGGCAGTACATCTA
CGTATTAGTCATCGCTATTACCATGGTGATGCGGTTTTGGCAGTACATCAATGGGCGTGGATAGCGGTTT
GACTCACGGGGATTTCCAAGTCTCCACCCCATTGACGTCAATGGGAGTTTGTTTTGCACCAAAATCAACG
GGACTTTCCAAAATGTCGTAACAACTCCGCCCCATTGACGCAAATGGGCGGTAGGCGTGTACGGTGGGAG
GTCTATATAAGCAGAGCTCGTTTAGTGAACCGTCAGATCGCCTGGAGACGCCATCCACGCTGTTTTGACC
TCCATAGAAGACACCGGGACCGATCCAGCCTCC

[SEQ ID NO:3]

In another preferred embodiment, the transgene expression cassette comprises a promoter which is only active in the target tumour cell. The promoter may therefore be tumour-activated and/or temozolomide-induced.

In an embodiment, the promoter may be multidrug resistant promoter (MDR), which is highly active in cancer cells. This promoter can be activated by cancer drugs to produce the P-gp and ABC transporter to efflux the drug out from the cell. This promoter may be used for gene therapy in combination with cancer drugs.

In another embodiment, the promoter may be human telomerase reverse transcriptase (hTERT) promoter. This promoter is exclusively active in tumour cells, but not in normal proliferating cells. Hence, this promoter can be used as a tumour-specific promoter.

In 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
CCGAGGGACAGGCAGCTGCTCAACCAATAGGACCAGCTCTCAGGGCGGATGCTGCCTCTCATTGGCGGCC
GTTAAGAATGACCAGTAGCCAATGAGTCGGCTGGGGGGCGCGTACCAGTGACGTGAGTTGCGGAGGAGGC
CGCTTCGAATCGGCAGCGGCCAGCTTGGTGGCATGAACCAACCAGCGGCCTCCAACGAGTAGCGAGTTCA
CCAATCGGAGGCCTCCACGACGGGGCTGCGGGGAGGATATATAAGCCGAGTCGGCGACCGGCGCGCTCGA
TACTGGCTGTGACTACACTGACTTGGAC

[SEQ ID NO: 8]

Preferably, the transgene expression cassette comprises nucleic acid for encoding a polyA tail attachable to the or each expressed antigen or cytokine. 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:

ACGGGTGGCATCCCTGTGACCCCTCCCCAGTGCCTCTCCTGGCCCTGGAAGTTGCCACTCCAGTGCCCAC
CAGCCTTGTCCTAATAAAATTAAGTTGCATCATTTTGTCTGACTAGGTGTCCTTCTATAATATTATGGGG
TGGAGGGGGGTGGTATGGAGCAAGGGGCAAGTTGGGAAGACAACCTGTAGGGCCTGCGGGGTCTATTGGG
AACCAAGCTGGAGTGCAGTGGCACAATCTTGGCTCACTGCAATCTCCGCCTCCTGGGTTCAAGCGATTCT
CCTGCCTCAGCCTCCCGAGTTGTTGGGATTCCAGGCATGCATGACCAGGCTCAGCTAATTTTTGTTTTTT
TGGTAGAGACGGGGTTTCACCATATTGGCCAGGCTGGTCTCCAACTCCTAATCTCAGGTGATCTACCCAC
CTTGGCCTCCCAAATTGCTGGGATTACAGGCGTGAACCACTGCTCCCTTCCCTGTCCTT

[SEQ ID NO:4]

Preferably, the transgene expression cassette comprises left and/or right Inverted Terminal Repeat sequences (ITRs). An ITR can be specific to an AAV or Long Terminal Repeats Sequences (LTRs) specific to a lentivirus 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:

CCTGCAGGCAGCTGCGCGCTCGCTCGCTCACTGAGGCCGCCCGGGCGTCGGGCGACCTTTGGTCGCCCGG
CCTCAGTGAGCGAGCGAGCGCGCAGAGAGGGAGTGGCCAACTCCATCACTAGGGGTTCCT

[SEQ ID NO:5]

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
AAAGGTCGCCCGACGCCCGGGCTTTGCCCGGGCGGCCTCAGTGAGCGAGCGAGCGCGCAGCTGCCTGCAG
G

[SEQ ID NO:6]

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.

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 tumour 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.

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.

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.

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.

Preferably, the phagemid particle comprises a genome which has a relative genome size of less than 14 Kb. The relative genome size may be less than about 13 Kb, less than about 12 Kb, less than about 11 Kb, less than about 10Kb, less than about 9 Kb, less than about 8 Kb, or less than about 7 Kb. The relative genome size may be between 3 Kb and 9 Kb, 4 Kb and 8 Kb, 5 Kb and 7 Kb, or preferably approximately 6 Kb.

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.

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.

Accordingly, given that the genome of the recombinant phagemid particle of the eighth or any above aspect 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 eight or any above aspect, involving the use of a separate so-called “helper virus” vector. In effect, therefore, the particle of the eighth or any above aspect is a hybrid phagemid vector, which includes components of a phagemid and a eukaryotic virus.

Hence, in a nineteenth aspect, there is provided a system for producing a recombinant phagemid particle from a prokaryotic host, the system comprising:-

(i) a first vector configured to persist inside a prokaryotic host, and comprising at least one transgene expression cassette comprising a nucleic acid sequence encoding one or more cytokine, or one or more antigen that is recognised by one or more adoptively

transferred T cell, and a packaging signal for enabling replication of the vector into single-stranded DNA; and

(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.

Advantageously, separating the reproductive elements of the phagemid particle into the first "therapeutic" vector carrying the transgene encoding the or each antigen recognised by the or each adoptively transferred T cell or cytokine, and the second separate "helper" vector carrying the viral structural genes substantially decreases the genome/vector size, and thereby significantly increases transgene capacity. The phagemid particle is used therapeutically (e.g. in CAR-T cell therapy) and the increased transgene capacity 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.

The novelty of the system of the nineteenth aspect is its ability to package the genome of eukaryotic viruses (such as AAV or lentivirus), 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 nineteenth aspect (i.e. PAAV) is a chimera between prokaryotic viral phenotypes and a eukaryotic viral genotype.

The antigen may be any as disclosed herein. In a preferred embodiment, the or each antigen is a peptide or protein which is expressed on the cell surface of the target tumour cell. Preferably, the or each antigen is a peptide or protein that, when expressed by the tumour cell, would be accessible to a CAR T cell. The peptide or protein may be such that, when expressed by the tumour cell, it is present as a folded peptide protein at or on the cell-surface. The or each antigen may be a known target for existing CAR T cells suitable for use in humans. For example, the or each antigen may be selected from a group consisting of: MUC1; PSMA; CD19; CD20; estrogen-related receptor beta type 2 (ErRB2); or any combination thereof. In an embodiment, the or each antigen may be dengue virus or yellow fever vaccine antigens.

Preferably, the system of the nineteenth aspect is used to produce the recombinant phagemid particle of the eighth aspect. 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.

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.

The 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 comprise a lentivirus transgene expression cassette or a AAV transgene expression cassette. An AAV transgene expression cassette is preferred.

One preferred example 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.

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 eighth aspect) is desired.

In one example, therefore, the second vector may comprise a first nucleic acid sequence encoding a pIII minor coat protein that is configured to display a cell-targeting ligand for enabling delivery of the recombinant phagemid particle to the 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).

In another example, 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 and/or have inherent biological/chemical functionality *in vivo* or *in vitro*. For example, immune stimulation *in vivo* via antigen display, wherein the peptide displayed on the pVIII coat protein may be processed through the exogenous pathway and presented on the MHC II of the target cell, thus presenting another target for adoptively transferred T cells. Alternatively, the at least one pVIII major coat protein might have binding functionality, for instance to nanoparticles (e.g. gold) *in vitro* via displaying a gold-binding peptide.

The first vector may be a member of the Retroviridae family, or of the Orthoretrovirinae Sub-family. The first vector may be a member of the Lentivirus genus. Preferably, the first vector is a member of the Parvoviridae family or sub-family. Preferably, the first vector is a member of the Dependoparvovirus, or adeno-associated virus species.

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 eighth or any above aspect. 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 used in the eighth or any above aspect.

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, wherein "N" is a transgene.:

CCTGCAGGCAGCTGCGCGCTCGCTCGCTCACTGAGGCCGCCCGGGCGTCGGGCGACCTTTGGTCGCCCGG
CCTCAGTGAGCGAGCGAGCGCGCAGAGAGGGAGTGGCCAACTCCATCACTAGGGGTTCCTGCGGCCGCNA
GGAACCCCTAGTGATGGAGTTGGCCACTCCCTCTCTGCGCGCTCGCTCGCTCACTGAGGCCGGGCGACCA
AAGGTCGCCCGACGCCCGGGCTTTGCCCGGGCGGCCTCAGTGAGCGAGCGAGCGCGCAGCTGCCTGCAGG
GGCGCCTGATGCGGTATTTTCTCCTTACGCATCTGTGCGGTATTTCACACCGCATACGTCAAAGCAACCA
TAGTACGCGCCCTGTAGCGGCGCATTAAGCGCGGCGGGTGTGGTGGTTACGCGCAGCGTGACCGCTACAC
TTGCCAGCGCCCTAGCGCCCGCTCCTTTCGCTTTCTTCCCTTCCTTTCTCGCCACGTTCGCCGGCTTTCC
CCGTCAAGCTCTAAATCGGGGGCTCCCTTTAGGGTTCCGATTTAGTGCTTTACGGCACCTCGACCCCAAA
AAACTTGATTTGGGTGATGGTTCACGTAGTGGGCCATCGCCCTGATAGACGGTTTTTCGCCCTTTGACGT
TGGAGTCCACGTTCTTTAATAGTGGACTCTTGTTCCAAACTGGAACAACACTCAACCCTATCTCGGGCTA
TTCTTTTGATTTATAAGGGATTTTGCCGATTTCGGCCTATTGGTTAAAAAATGAGCTGATTTAACAAAAA
TTTAACGCGAATTTTAACAAAATATTAACGTTTACAATTTTATGGTGCACTCTCAGTACAATCTGCTCTG
ATGCCGCATAGTTAAGCCAGCCCCGACACCCGCCAACACCCGCTGACGCGCCCTGACGGGCTTGTCTGCT
CCCGGCATCCGCTTACAGACAAGCTGTGACCGTCTCCGGGAGCTGCATGTGTCAGAGGTTTTCACCGTCA
TCACCGAAACGCGCGAGACGAAAGGGCCTCGTGATACGCCTATTTTTATAGGTTAATGTCATGATAATAA
TGGTTTCTTAGACGTCAGGTGGCACTTTTCGGGGAAATGTGCGCGGAACCCCTATTTGTTTATTTTTCTA
AATACATTCAAATATGTATCCGCTCATGAGACAATAACCCTGATAAATGCTTCAATAATATTGAAAAAGG
AAGAGTATGAGTATTCAACATTTCCGTGTCGCCCTTATTCCCTTTTTTGCGGCATTTTGCCTTCCTGTTT
TTGCTCACCCAGAAACGCTGGTGAAAGTAAAAGATGCTGAAGATCAGTTGGGTGCACGAGTGGGTTACAT
CGAACTGGATCTCAACAGCGGTAAGATCCTTGAGAGTTTTCGCCCCGAAGAACGTTTTCCAATGATGAGC
ACTTTTAAAGTTCTGCTATGTGGCGCGGTATTATCCCGTATTGACGCCGGGCAAGAGCAACTCGGTCGCC
GCATACACTATTCTCAGAATGACTTGGTTGAGTACTCACCAGTCACAGAAAAGCATCTTACGGATGGCAT
GACAGTAAGAGAATTATGCAGTGCTGCCATAACCATGAGTGATAACACTGCGGCCAACTTACTTCTGACA
ACGATCGGAGGACCGAAGGAGCTAACCGCTTTTTTGCACAACATGGGGGATCATGTAACTCGCCTTGATC
GTTGGGAACCGGAGCTGAATGAAGCCATACCAAACGACGAGCGTGACACCACGATGCCTGTAGCAATGGC
AACAACGTTGCGCAAACTATTAACTGGCGAACTACTTACTCTAGCTTCCCGGCAACAATTAATAGACTGG
ATGGAGGCGGATAAAGTTGCAGGACCACTTCTGCGCTCGGCCCTTCCGGCTGGCTGGTTTATTGCTGATA
AATCTGGAGCCGGTGAGCGTGGGTCTCGCGGTATCATTGCAGCACTGGGGCCAGATGGTAAGCCCTCCCG
TATCGTAGTTATCTACACGACGGGGAGTCAGGCAACTATGGATGAACGAAATAGACAGATCGCTGAGATA
GGTGCCTCACTGATTAAGCATTGGTAACTGTCAGACCAAGTTTACTCATATATACTTTAGATTGATTTAA
AACTTCATTTTTAATTTAAAAGGATCTAGGTGAAGATCCTTTTTGATAATCTCATGACCAAAATCCCTTA
ACGTGAGTTTTCGTTCCACTGAGCGTCAGACCCCGTAGAAAAGATCAAAGGATCTTCTTGAGATCCTTTT
TTTCTGCGCGTAATCTGCTGCTTGCAAACAAAAAAACCACCGCTACCAGCGGTGGTTTGTTTGCCGGATC
AAGAGCTACCAACTCTTTTTCCGAAGGTAACTGGCTTCAGCAGAGCGCAGATACCAAATACTGTCCTTCT
AGTGTAGCCGTAGTTAGGCCACCACTTCAAGAACTCTGTAGCACCGCCTACATACCTCGCTCTGCTAATC
CTGTTACCAGTGGCTGCTGCCAGTGGCGATAAGTCGTGTCTTACCGGGTTGGACTCAAGACGATAGTTAC

CGGATAAGGCGCAGCGGTCGGGCTGAACGGGGGGTTCGTGCACACAGCCCAGCTTGGAGCGAACGACCTA
CACCGAACTGAGATACCTACAGCGTGAGCTATGAGAAAGCGCCACGCTTCCCGAAGGGAGAAAGGCGGAC
AGGTATCCGGTAAGCGGCAGGGTCGGAACAGGAGAGCGCACGAGGGAGCTTCCAGGGGGAAACGCCTGGT
ATCTTTATAGTCCTGTCGGGTTTCGCCACCTCTGACTTGAGCGTCGATTTTTGTGATGCTCGTCAGGGGG
GCGGAGCCTATGGAAAAACGCCAGCAACGCGGCCTTTTTACGGTTCCTGGCCTTTTGCTGGCCTTTTGCT
CACATGT

[SEQ ID No: 9]

In one preferred embodiment, the second vector (helper phage with RGD sequence) comprises a nucleic acid sequence substantially as set out in SEQ ID No: 10, or a fragment or variant thereof, wherein SEQ ID No: 10 is represented as follows:

AACGCTACTACTATTAGTAGAATTGATGCCACCTTTTCAGCTCGCGCCCCAAATGAAAATATAGCTAAAC
AGGTTATTGACCATTTGCGAAATGTATCTAATGGTCAAACTAAATCTACTCGTTCGCAGAATTGGGAATC
AACTGTTACATGGAATGAAACTTCCAGACACCGTACTTTAGTTGCATATTTAAAACATGTTGAGCTACAG
CACCAGATTCAGCAATTAAGCTCTAAGCCATCCGCAAAAATGACCTCTTATCAAAAGGAGCAATTAAAGG
TACTCTCTAATCCTGACCTGTTGGAGTTTGCTTCCGGTCTGGTTCGCTTTGAAGCTCGAATTAAAACGCG
ATATTTGAAGTCTTTCGGGCTTCCTCTTAATCTTTTTGATGCAATCCGCTTTGCTTCTGACTATAATAGT
CAGGGTAAAGACCTGATTTTTGATTTATGGTCATTCTCGTTTTCTGAACTGTTTAAAGCATTTGAGGGGG
ATTCAATGAATATTTATGACGATTCCGCAGTATTGGACGCTATCCAGTCTAAACATTTTACTATTACCCC
CTCTGGCAAAACTTCTTTTGCAAAAGCCTCTCGCTATTTTGGTTTTTATCGTCGTCTGGTAAACGAGGGT
TATGATAGTGTTGCTCTTACTATGCCTCGTAATTCCTTTTGGCGTTATGTATCTGCATTAGTTGAATGTG
GTATTCCTAAATCTCAACTGATGAATCTTTCTACCTGTAATAATGTTGTTCCGTTAGTTCGTTTTATTAA
CGTAGATTTTTCTTCCCAACGTCCTGACTGGTATAATGAGCCAGTTCTTAAAATCGCATAAGGTAATTCA
CAATGATTAAAGTTGAAATTAAACCATCTCAAGCCCAATTTACTACTCGTTCTGGTGTTTCTCGTCAGGG
CAAGCCTTATTCACTGAATGAGCAGCTTTGTTACGTTGATTTGGGTAATGAATATCCGGTTCTTGTCAAG
ATTACTCTTGATGAAGGTCAGCCAGCCTATGCGCCTGGTCTGTACACCGTTCATCTGTCCTCTTTCAAAG
TTGGTCAGTTCGGTTCCCTTATGATTGACCGTCTGCGCCTCGTTCCGGCTAAGTAACATGGAGCAGGTCG
CGGATTTCGACACAATTTATCAGGCGATGATACAAATCTCCGTTGTACTTTGTTTCGCGCTTGGTATAAT
CGCTGGGGGTCAAAGATGAGTGTTTTAGTGTATTCTTTCGCCTCTTTCGTTTTAGGTTGGTGCCTTCGTA
GTGGCATTACGTATTTTACCCGTTTAATGGAAACTTCCTCATGAAAAAGTCTTTAGTCCTCAAAGCCTCT
GTAGCCGTTGCTACCCTCGTTCCGATGCTGTCTTTCGCTGCTGAGGGTGACGATCCCGCAAAAGCGGCCT
TTAACTCCCTGCAAGCCTCAGCGACCGAATATATCGGTTATGCGTGGGCGATGGTTGTTGTCATTGTCGG
CGCAACTATCGGTATCAAGCTGTTTAAGAAATTCACCTCGAAAGCAAGCTGATAAACCGATACAATTAAA
GGCTCCTTTTGGAGCCTTTTTTTTTGGAGATTTTCAACGTGAAAAAATTATTATTCGCAATTCCTTTAGT
TGTTCCTTTCTATTCTCACTCCGCTTGTGATTGTAGGGGGGATTGTTTTTGTGAAACTGTTGAAAGTTGT
TTAGCAAAACCCCATACAGAAAATTCATTTACTAACGTCTGGAAAGACGACAAAACTTTAGATCGTTACG
CTAACTATGAGGGTTGTCTGTGGAATGCTACAGGCGTTGTAGTTTGTACTGGTGACGAAACTCAGTGTTA
CGGTACATGGGTTCCTATTGGGCTTGCTATCCCTGAAAATGAGGGTGGTGGCTCTGAGGGTGGCGGTTCT
GAGGGTGGCGGTTCTGAGGGTGGCGGTACTAAACCTCCTGAGTACGGTGATACACCTATTCCGGGCTATA
CTTATATCAACCCTCTCGACGGCACTTATCCGCCTGGTACTGAGCAAAACCCCGCTAATCCTAATCCTTC
TCTTGAGGAGTCTCAGCCTCTTAATACTTTCATGTTTCAGAATAATAGGTTCCGAAATAGGCAGGGGGCA
TTAACTGTTTATACGGGCACTGTTACTCAAGGCACTGACCCCGTTAAAACTTATTACCAGTACACTCCTG
TATCATCAAAAGCCATGTATGACGCTTACTGGAACGGTAAATTCAGAGACTGCGCTTTCCATTCTGGCTT
TAATGAGGATCCATTCGTTTGTGAATATCAAGGCCAATCGTCTGACCTGCCTCAACCTCCTGTCAATGCT
GGCGGCGGCTCTGGTGGTGGTTCTGGTGGCGGCTCTGAGGGTGGTGGCTCTGAGGGTGGCGGTTCTGAGG
GTGGCGGCTCTGAGGGAGGCGGTTCCGGTGGTGGCTCTGGTTCCGGTGATTTTGATTATGAAAAGATGGC
AAACGCTAATAAGGGGGCTATGACCGAAAATGCCGATGAAAACGCGCTACAGTCTGACGCTAAAGGCAAA
CTTGATTCTGTCGCTACTGATTACGGTGCTGCTATCGATGGTTTCATTGGTGACGTTTCCGGCCTTGCTA
ATGGTAATGGTGCTACTGGTGATTTTGCTGGCTCTAATTCCCAAATGGCTCAAGTCGGTGACGGTGATAA
TTCACCTTTAATGAATAATTTCCGTCAATATTTACCTTCCCTCCCTCAATCGGTTGAATGTCGCCCTTTT
GTCTTTAGCGCTGGTAAACCATATGAATTTTCTATTGATTGTGACAAAATAAACTTATTCCGTGGTGTCT
TTGCGTTTCTTTTATATGTTGCCACCTTTATGTATGTATTTTCTACGTTTGCTAACATACTGCGTAATAA
GGAGTCTTAATCATGCCAGTTCTTTTGGGTATTCCGTTATTATTGCGTTTCCTCGGTTTCCTTCTGGTAA
CTTTGTTCGGCTATCTGCTTACTTTTCTTAAAAAGGGCTTCGGTAAGATAGCTATTGCTATTTCATTGTT
TCTTGCTCTTATTATTGGGCTTAACTCAATTCTTGTGGGTTATCTCTCTGATATTAGCGCTCAATTACCC

TCTGACTTTGTTCAGGGTGTTCAGTTAATTCTCCCGTCTAATGCGCTTCCCTGTTTTTATGTTATTCTCT
CTGTAAAGGCTGCTATTTTCATTTTTGACGTTAAACAAAAAATCGTTTCTTATTTGGATTGGGATAAATA
ATATGGCTGTTTATTTTGTAACTGGCAAATTAGGCTCTGGAAAGACGCTCGTTAGCGTTGGTAAGATTCA
GGATAAAATTGTAGCTGGGTGCAAAATAGCAACTAATCTTGATTTAAGGCTTCAAAACCTCCCGCAAGTC
GGGAGGTTCGCTAAAACGCCTCGCGTTCTTAGAATACCGGATAAGCCTTCTATATCTGATTTGCTTGCTA
TTGGGCGCGGTAATGATTCCTACGATGAAAATAAAAACGGCTTGCTTGTTCTCGATGAGTGCGGTACTTG
GTTTAATACCCGTTCTTGGAATGATAAGGAAAGACAGCCGATTATTGATTGGTTTCTACATGCTCGTAAA
TTAGGATGGGATATTATTTTTCTTGTTCAGGACTTATCTATTGTTGATAAACAGGCGCGTTCTGCATTAG
CTGAACATGTTGTTTATTGTCGTCGTCTGGACAGAATTACTTTACCTTTTGTCGGTACTTTATATTCTCT
TATTACTGGCTCGAAAATGCCTCTGCCTAAATTACATGTTGGCGTTGTTAAATATGGCGATTCTCAATTA
AGCCCTACTGTTGAGCGTTGGCTTTATACTGGTAAGAATTTGTATAACGCATATGATACTAAACAGGCTT
TTTCTAGTAATTATGATTCCGGTGTTTATTCTTATTTAACGCCTTATTTATCACACGGTCGGTATTTCAA
ACCATTAAATTTAGGTCAGAAGATGAAATTAACTAAAATATATTTGAAAAAGTTTTCTCGCGTTCTTTGT
CTTGCGATTGGATTTGCATCAGCATTTACATATAGTTATATAACCCAACCTAAGCCGGAGGTTAAAAAGG
TAGTCTCTCAGACCTATGATTTTGATAAATTCACTATTGACTCTTCTCAGCGTCTTAATCTAAGCTATCG
CTATGTTTTCAAGGATTCTAAGGGAAAATTAATTAATAGCGACGATTTACAGAAGCAAGGTTATTCACTC
ACATATATTGATTTATGTACTGTTTCCATTAAAAAAGGTAATTCAAATGAAATTGTTAAATGTAATTAAT
TTTGTTTTCTTGATGTTTGTTTCATCATCTTCTTTTGCTCAGGTAATTGAAATGAATAATTCGCCTCTGC
GCGATTTTGTAACTTGGTATTCAAAGCAATCAGGCGAATCCGTTATTGTTTCTCCCGATGTAAAAGGTAC
TGTTACTGTATATTCATCTGACGTTAAACCTGAAAATCTACGCAATTTCTTTATTTCTGTTTTACGTGCT
AATAATTTTGATATGGTTGGTTCAATTCCTTCCATAATTCAGAAGTATAATCCAAACAATCAGGATTATA
TTGATGAATTGCCATCATCTGATAATCAGGAATATGATGATAATTCCGCTCCTTCTGGTGGTTTCTTTGT
TCCGCAAAATGATAATGTTACTCAAACTTTTAAAATTAATAACGTTCGGGCAAAGGATTTAATACGAGTT
GTCGAATTGTTTGTAAAGTCTAATACTTCTAAATCCTCAAATGTATTATCTATTGACGGCTCTAATCTAT
TAGTTGTTAGTGCACCTAAAGATATTTTAGATAACCTTCCTCAATTCCTTTCTACTGTTGATTTGCCAAC
TGACCAGATATTGATTGAGGGTTTGATATTTGAGGTTCAGCAAGGTGATGCTTTAGATTTTTCATTTGCT
GCTGGCTCTCAGCGTGGCACTGTTGCAGGCGGTGTTAATACTGACCGCCTCACCTCTGTTTTATCTTCTG
CTGGTGGTTCGTTCGGTATTTTTAATGGCGATGTTTTAGGGCTATCAGTTCGCGCATTAAAGACTAATAG
CCATTCAAAAATATTGTCTGTGCCACGTATTCTTACGCTTTCAGGTCAGAAGGGTTCTATCTCTGTTGGC
CAGAATGTCCCTTTTATTACTGGTCGTGTGACTGGTGAATCTGCCAATGTAAATAATCCATTTCAGACGA
TTGAGCGTCAAAATGTAGGTATTTCCATGAGCGTTTTTCCTGTTGCAATGGCTGGCGGTAATATTGTTCT
GGATATTACCAGCAAGGCCGATAGTTTGAGTTCTTCTACTCAGGCAAGTGATGTTATTACTAATCAAAGA
AGTATTGCTACAACGGTTAATTTGCGTGATGGACAGACTCTTTTACTCGGTGGCCTCACTGATTATAAAA
ACACTTCTCAAGATTCTGGCGTACCGTTCCTGTCTAAAATCCCTTTAATCGGCCTCCTGTTTAGCTCCCG
CTCTGATTCCAACGAGGAAAGCACGTTATACGTGCTCGTCAAAGCAACCATAGTACGCGCCCTGTAGCGG
CGCATTAAGCGCGGCGGGTGTGGTGGTTACGCGCAGCGTGACCGCTACACTTGCCAGCGCCCTAGCGCCC
GCTCCTTTCGCTTTCTTCCCTTCCTTTCTCGCCACGTTCGCCGGCTTTCCCCGTCAAGCTCTAAATCGGG
GGCTCCCTTTAGGGTTCCGATTTAGTGCTTTACGGCACCTCGACCCCAAAAAACTTGATTTGGGTGATGG
TTCACGTAGTGGGCCATCGCCCTGATAGACGGTTTTTCGCCCTTTGACGTTGGAGTCCACGTTCTTTAAT
AGTGGACTCTTGTTCCAAACTGGAACAACACTCAACCCTATCTCGGGACGGATCGCTTCATGTGGCAGGA
GAAAAAAGGCTGCACCGGTGCGTCAGCAGAATATGTGATACAGGATATATTCCGCTTCCTCGCTCACTGA
CTCGCTACGCTCGGTCGTTCGACTGCGGCGAGCGGAAATGGCTTACGAACGGGGCGGAGATTTCCTGGAA
GATGCCAGGAAGATACTTAACAGGGAAGTGAGAGGGCCGCGGCAAAGCCGTTTTTCCATAGGCTCCGCCC
CCCTGACAAGCATCACGAAATCTGACGCTCAAATCAGTGGTGGCGAAACCCGACAGGACTATAAAGATAC
CAGGCGTTTCCCCCTGGCGGCTCCCTCGTGCGCTCTCCTGTTCCTGCCTTTCGGTTTACCGGTGTCATTC
CGCTGTTATGGCCGCGTTTGTCTCATTCCACGCCTGACACTCAGTTCCGGGTAGGCAGTTCGCTCCAAGC
TGGACTGTATGCACGAACCCCCCGTTCAGTCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTC
CAACCCGGAAAGACATGCAAAAGCACCACTGGCAGCAGCCACTGGTAATTGATTTAGAGGAGTTAGTCTT
GAAGTCATGCGCCGGTTAAGGCTAAACTGAAAGGACAAGTTTTGGTGACTGCGCTCCTCCAAGCCAGTTA
CCTCGGTTCAAAGAGTTGGTAGCTCAGAGAACCTTCGAAAAACCGCCCTGCAAGGCGGTTTTTTCGTTTT
CAGAGCAAGAGATTACGCGCAGACCAAAACGATCTCAAGAAGATCATCTTATTAAGGGGTCTGACGCTCA
GTGGAACGAAAACTCACGTTAAGGGATTTTGGTCATGAGATTATCAAAAAGGATCTTCACCTAGATCCTT
TTAAATTAAAAATGAAGTTTTAAATCAATCTAAAGTATATATGAGTAAACTTGGTCTGACAGTTACCAAT
GCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTCGTTCATCCATAGTTGCCTGACTCCCCGT
CGTGTAGATAACTACGATACGGGAGGGCTTACCATCTGGCCCCAGTGCTGCAATGATACCGCGAGACCCA
CGCTCACCGGCTCCAGATTTATCAGCAATAAACCAGCCAGCCGATTCGAGCTCGCCCCGGGGATCGACCA
GTTGGTGATTTTGAACTTTTGCTTTGCCACGGAACGGTCTGCGTTGTCGGGAAGATGCGTGATCTGATCC
TTCAACTCAGCAAAAGTTCGATTTATTCAACAAAGCCGCCGTCCCGTCAAGTCAGCGTAATGCTCTGCCA
GTGTTACAACCAATTAACCAATTCTGATTAGAAAAACTCATCGAGCATCAAATGAAACTGCAATTTATTC

ATATCAGGATTATCAATACCATATTTTTGAAAAAGCCGTTTCTGTAATGAAGGAGAAAACTCACCGAGGC
AGTTCCATAGGATGGCAAGATCCTGGTATCGGTCTGCGATTCCGACTCGTCCAACATCAATACAACCTAT
TAATTTCCCCTCGTCAAAAATAAGGTTATCAAGTGAGAAATCACCATGAGTGACGACTGAATCCGGTGAG
AATGGCAAAAGCTTATGCATTTCTTTCCAGACTTGTTCAACAGGCCAGCCATTACGCTCGTCATCAAAAT
CACTCGCATCAACCAAACCGTTATTCATTCGTGATTGCGCCTGAGCGAGACGAAATACGCGATCGCTGTT
AAAAGGACAATTACAAACAGGAATCGAATGCAACCGGCGCAGGAACACTGCCAGCGCATCAACAATATTT
TCACCTGAATCAGGATATTCTTCTAATACCTGGAATGCTGTTTTCCCGGGGATCGCAGTGGTGAGTAACC
ATGCATCATCAGGAGTACGGATAAAATGCTTGATGGTCGGAAGAGGCATAAATTCCGTCAGCCAGTTTAG
TCTGACCATCTCATCTGTAACATCATTGGCAACGCTACCTTTGCCATGTTTCAGAAACAACTCTGGCGCA
TCGGGCTTCCCATACAATCGATAGATTGTCGCACCTGATTGCCCGACATTATCGCGAGCCCATTTATACC
CATATAAATCAGCATCCATGTTGGAATTTAATCGCGGCCTCGAGCAAGACGTTTCCCGTTGAATATGGCT
CATAACACCCCTTGTATTACTGTTTATGTAAGCAGACAGTTTTATTGTTCATGATGATATATTTTTATCT
TGTGCAATGTAACATCAGAGATTTTGAGACACAACGTGGCTTTCCCCCCCCCCCCTGCAGGTCTCGGGC
TATTCTTTTGATTTATAAGGGATTTTGCCGATTTCGGCCTATTGGTTAAAAAATGAGCTGATTTAACAAA
AATTTAACGCGAATTTTAACAAAATATTAACGTTTACAATTTAAATATTTGCTTATACAATCTTCCTGTT
TTTGGGGCTTTTCTGATTATCAACCGGGGTACATATGATTGACATGCTAGTTTTACGATTACCGTTCATC
GATTCTCTTGTTTGCTCCAGACTCTCAGGCAATGACCTGATAGCCTTTGTAGACCTCTCAAAAATAGCTA
CCCTCTCCGGCATGAATTTATCAGCTAGAACGGTTGAATATCATATTGATGGTGATTTGACTGTCTCCGG
CCTTTCTCACCCTTTTGAATCTTTACCTACACATTACTCAGGCATTGCATTTAAAATATATGAGGGTTCT
AAAAATTTTTATCCTTGCGTTGAAATAAAGGCTTCTCCCGCAAAAGTATTACAGGGTCATAATGTTTTTG
GTACAACCGATTTAGCTTTATGCTCTGAGGCTTTATTGCTTAATTTTGCTAATTCTTTGCCTTGCCTGTA
TGATTTATTGGATGTT

[SEQ ID No: 10]

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:

AACGCTACTACTATTAGTAGAATTGATGCCACCTTTTCAGCTCGCGCCCCAAATGAAAATATAGCTAAAC
AGGTTATTGACCATTTGCGAAATGTATCTAATGGTCAAACTAAATCTACTCGTTCGCAGAATTGGGAATC
AACTGTTACATGGAATGAAACTTCCAGACACCGTACTTTAGTTGCATATTTAAAACATGTTGAGCTACAG
CACCAGATTCAGCAATTAAGCTCTAAGCCATCCGCAAAAATGACCTCTTATCAAAAGGAGCAATTAAAGG
TACTCTCTAATCCTGACCTGTTGGAGTTTGCTTCCGGTCTGGTTCGCTTTGAAGCTCGAATTAAAACGCG
ATATTTGAAGTCTTTCGGGCTTCCTCTTAATCTTTTTGATGCAATCCGCTTTGCTTCTGACTATAATAGT
CAGGGTAAAGACCTGATTTTTGATTTATGGTCATTCTCGTTTTCTGAACTGTTTAAAGCATTTGAGGGGG
ATTCAATGAATATTTATGACGATTCCGCAGTATTGGACGCTATCCAGTCTAAACATTTTACTATTACCCC
CTCTGGCAAAACTTCTTTTGCAAAAGCCTCTCGCTATTTTGGTTTTTATCGTCGTCTGGTAAACGAGGGT
TATGATAGTGTTGCTCTTACTATGCCTCGTAATTCCTTTTGGCGTTATGTATCTGCATTAGTTGAATGTG
GTATTCCTAAATCTCAACTGATGAATCTTTCTACCTGTAATAATGTTGTTCCGTTAGTTCGTTTTATTAA
CGTAGATTTTTCTTCCCAACGTCCTGACTGGTATAATGAGCCAGTTCTTAAAATCGCATAAGGTAATTCA
CAATGATTAAAGTTGAAATTAAACCATCTCAAGCCCAATTTACTACTCGTTCTGGTGTTTCTCGTCAGGG
CAAGCCTTATTCACTGAATGAGCAGCTTTGTTACGTTGATTTGGGTAATGAATATCCGGTTCTTGTCAAG
ATTACTCTTGATGAAGGTCAGCCAGCCTATGCGCCTGGTCTGTACACCGTTCATCTGTCCTCTTTCAAAG
TTGGTCAGTTCGGTTCCCTTATGATTGACCGTCTGCGCCTCGTTCCGGCTAAGTAACATGGAGCAGGTCG
CGGATTTCGACACAATTTATCAGGCGATGATACAAATCTCCGTTGTACTTTGTTTCGCGCTTGGTATAAT
CGCTGGGGGTCAAAGATGAGTGTTTTAGTGTATTCTTTCGCCTCTTTCGTTTTAGGTTGGTGCCTTCGTA
GTGGCATTACGTATTTTACCCGTTTAATGGAAACTTCCTCATGAAAAAGTCTTTAGTCCTCAAAGCCTCT
GTAGCCGTTGCTACCCTCGTTCCGATGCTGTCTTTCGCTGCTGAGGGTGACGATCCCGCAAAAGCGGCCT
TTAACTCCCTGCAAGCCTCAGCGACCGAATATATCGGTTATGCGTGGGCGATGGTTGTTGTCATTGTCGG
CGCAACTATCGGTATCAAGCTGTTTAAGAAATTCACCTCGAAAGCAAGCTGATAAACCGATACAATTAAA
GGCTCCTTTTGGAGCCTTTTTTTTTGGAGATTTTCAACGTGAAAAAATTATTATTCGCAATTCCTTTAGT
TGTTCCTTTCTATTCTCACTCCGCTGAAACTGTTGAAAGTTGTTTAGCAAAACCCCATACAGAAAATTCA
TTTACTAACGTCTGGAAAGACGACAAAACTTTAGATCGTTACGCTAACTATGAGGGTTGTCTGTGGAATG
CTACAGGCGTTGTAGTTTGTACTGGTGACGAAACTCAGTGTTACGGTACATGGGTTCCTATTGGGCTTGC
TATCCCTGAAAATGAGGGTGGTGGCTCTGAGGGTGGCGGTTCTGAGGGTGGCGGTTCTGAGGGTGGCGGT
ACTAAACCTCCTGAGTACGGTGATACACCTATTCCGGGCTATACTTATATCAACCCTCTCGACGGCACTT
ATCCGCCTGGTACTGAGCAAAACCCCGCTAATCCTAATCCTTCTCTTGAGGAGTCTCAGCCTCTTAATAC

TTTCATGTTTCAGAATAATAGGTTCCGAAATAGGCAGGGGGCATTAACTGTTTATACGGGCACTGTTACT
CAAGGCACTGACCCCGTTAAAACTTATTACCAGTACACTCCTGTATCATCAAAAGCCATGTATGACGCTT
ACTGGAACGGTAAATTCAGAGACTGCGCTTTCCATTCTGGCTTTAATGAGGATCCATTCGTTTGTGAATA
TCAAGGCCAATCGTCTGACCTGCCTCAACCTCCTGTCAATGCTGGCGGCGGCTCTGGTGGTGGTTCTGGT
GGCGGCTCTGAGGGTGGTGGCTCTGAGGGTGGCGGTTCTGAGGGTGGCGGCTCTGAGGGAGGCGGTTCCG
GTGGTGGCTCTGGTTCCGGTGATTTTGATTATGAAAAGATGGCAAACGCTAATAAGGGGGCTATGACCGA
AAATGCCGATGAAAACGCGCTACAGTCTGACGCTAAAGGCAAACTTGATTCTGTCGCTACTGATTACGGT
GCTGCTATCGATGGTTTCATTGGTGACGTTTCCGGCCTTGCTAATGGTAATGGTGCTACTGGTGATTTTG
CTGGCTCTAATTCCCAAATGGCTCAAGTCGGTGACGGTGATAATTCACCTTTAATGAATAATTTCCGTCA
ATATTTACCTTCCCTCCCTCAATCGGTTGAATGTCGCCCTTTTGTCTTTAGCGCTGGTAAACCATATGAA
TTTTCTATTGATTGTGACAAAATAAACTTATTCCGTGGTGTCTTTGCGTTTCTTTTATATGTTGCCACCT
TTATGTATGTATTTTCTACGTTTGCTAACATACTGCGTAATAAGGAGTCTTAATCATGCCAGTTCTTTTG
GGTATTCCGTTATTATTGCGTTTCCTCGGTTTCCTTCTGGTAACTTTGTTCGGCTATCTGCTTACTTTTC
TTAAAAAGGGCTTCGGTAAGATAGCTATTGCTATTTCATTGTTTCTTGCTCTTATTATTGGGCTTAACTC
AATTCTTGTGGGTTATCTCTCTGATATTAGCGCTCAATTACCCTCTGACTTTGTTCAGGGTGTTCAGTTA
ATTCTCCCGTCTAATGCGCTTCCCTGTTTTTATGTTATTCTCTCTGTAAAGGCTGCTATTTTCATTTTTG
ACGTTAAACAAAAAATCGTTTCTTATTTGGATTGGGATAAATAATATGGCTGTTTATTTTGTAACTGGCA
AATTAGGCTCTGGAAAGACGCTCGTTAGCGTTGGTAAGATTCAGGATAAAATTGTAGCTGGGTGCAAAAT
AGCAACTAATCTTGATTTAAGGCTTCAAAACCTCCCGCAAGTCGGGAGGTTCGCTAAAACGCCTCGCGTT
CTTAGAATACCGGATAAGCCTTCTATATCTGATTTGCTTGCTATTGGGCGCGGTAATGATTCCTACGATG
AAAATAAAAACGGCTTGCTTGTTCTCGATGAGTGCGGTACTTGGTTTAATACCCGTTCTTGGAATGATAA
GGAAAGACAGCCGATTATTGATTGGTTTCTACATGCTCGTAAATTAGGATGGGATATTATTTTTCTTGTT
CAGGACTTATCTATTGTTGATAAACAGGCGCGTTCTGCATTAGCTGAACATGTTGTTTATTGTCGTCGTC
TGGACAGAATTACTTTACCTTTTGTCGGTACTTTATATTCTCTTATTACTGGCTCGAAAATGCCTCTGCC
TAAATTACATGTTGGCGTTGTTAAATATGGCGATTCTCAATTAAGCCCTACTGTTGAGCGTTGGCTTTAT
ACTGGTAAGAATTTGTATAACGCATATGATACTAAACAGGCTTTTTCTAGTAATTATGATTCCGGTGTTT
ATTCTTATTTAACGCCTTATTTATCACACGGTCGGTATTTCAAACCATTAAATTTAGGTCAGAAGATGAA
ATTAACTAAAATATATTTGAAAAAGTTTTCTCGCGTTCTTTGTCTTGCGATTGGATTTGCATCAGCATTT
ACATATAGTTATATAACCCAACCTAAGCCGGAGGTTAAAAAGGTAGTCTCTCAGACCTATGATTTTGATA
AATTCACTATTGACTCTTCTCAGCGTCTTAATCTAAGCTATCGCTATGTTTTCAAGGATTCTAAGGGAAA
ATTAATTAATAGCGACGATTTACAGAAGCAAGGTTATTCACTCACATATATTGATTTATGTACTGTTTCC
ATTAAAAAAGGTAATTCAAATGAAATTGTTAAATGTAATTAATTTTGTTTTCTTGATGTTTGTTTCATCA
TCTTCTTTTGCTCAGGTAATTGAAATGAATAATTCGCCTCTGCGCGATTTTGTAACTTGGTATTCAAAGC
AATCAGGCGAATCCGTTATTGTTTCTCCCGATGTAAAAGGTACTGTTACTGTATATTCATCTGACGTTAA
ACCTGAAAATCTACGCAATTTCTTTATTTCTGTTTTACGTGCTAATAATTTTGATATGGTTGGTTCAATT
CCTTCCATAATTCAGAAGTATAATCCAAACAATCAGGATTATATTGATGAATTGCCATCATCTGATAATC
AGGAATATGATGATAATTCCGCTCCTTCTGGTGGTTTCTTTGTTCCGCAAAATGATAATGTTACTCAAAC
TTTTAAAATTAATAACGTTCGGGCAAAGGATTTAATACGAGTTGTCGAATTGTTTGTAAAGTCTAATACT
TCTAAATCCTCAAATGTATTATCTATTGACGGCTCTAATCTATTAGTTGTTAGTGCACCTAAAGATATTT
TAGATAACCTTCCTCAATTCCTTTCTACTGTTGATTTGCCAACTGACCAGATATTGATTGAGGGTTTGAT
ATTTGAGGTTCAGCAAGGTGATGCTTTAGATTTTTCATTTGCTGCTGGCTCTCAGCGTGGCACTGTTGCA
GGCGGTGTTAATACTGACCGCCTCACCTCTGTTTTATCTTCTGCTGGTGGTTCGTTCGGTATTTTTAATG
GCGATGTTTTAGGGCTATCAGTTCGCGCATTAAAGACTAATAGCCATTCAAAAATATTGTCTGTGCCACG
TATTCTTACGCTTTCAGGTCAGAAGGGTTCTATCTCTGTTGGCCAGAATGTCCCTTTTATTACTGGTCGT
GTGACTGGTGAATCTGCCAATGTAAATAATCCATTTCAGACGATTGAGCGTCAAAATGTAGGTATTTCCA
TGAGCGTTTTTCCTGTTGCAATGGCTGGCGGTAATATTGTTCTGGATATTACCAGCAAGGCCGATAGTTT
GAGTTCTTCTACTCAGGCAAGTGATGTTATTACTAATCAAAGAAGTATTGCTACAACGGTTAATTTGCGT
GATGGACAGACTCTTTTACTCGGTGGCCTCACTGATTATAAAAACACTTCTCAAGATTCTGGCGTACCGT
TCCTGTCTAAAATCCCTTTAATCGGCCTCCTGTTTAGCTCCCGCTCTGATTCCAACGAGGAAAGCACGTT
ATACGTGCTCGTCAAAGCAACCATAGTACGCGCCCTGTAGCGGCGCATTAAGCGCGGCGGGTGTGGTGGT
TACGCGCAGCGTGACCGCTACACTTGCCAGCGCCCTAGCGCCCGCTCCTTTCGCTTTCTTCCCTTCCTTT
CTCGCCACGTTCGCCGGCTTTCCCCGTCAAGCTCTAAATCGGGGGCTCCCTTTAGGGTTCCGATTTAGTG
CTTTACGGCACCTCGACCCCAAAAAACTTGATTTGGGTGATGGTTCACGTAGTGGGCCATCGCCCTGATA
GACGGTTTTTCGCCCTTTGACGTTGGAGTCCACGTTCTTTAATAGTGGACTCTTGTTCCAAACTGGAACA
ACACTCAACCCTATCTCGGGACGGATCGCTTCATGTGGCAGGAGAAAAAAGGCTGCACCGGTGCGTCAGC
AGAATATGTGATACAGGATATATTCCGCTTCCTCGCTCACTGACTCGCTACGCTCGGTCGTTCGACTGCG
GCGAGCGGAAATGGCTTACGAACGGGGCGGAGATTTCCTGGAAGATGCCAGGAAGATACTTAACAGGGAA
GTGAGAGGGCCGCGGCAAAGCCGTTTTTCCATAGGCTCCGCCCCCCTGACAAGCATCACGAAATCTGACG
CTCAAATCAGTGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGCGGCTCCCTC

GTGCGCTCTCCTGTTCCTGCCTTTCGGTTTACCGGTGTCATTCCGCTGTTATGGCCGCGTTTGTCTCATT
CCACGCCTGACACTCAGTTCCGGGTAGGCAGTTCGCTCCAAGCTGGACTGTATGCACGAACCCCCCGTTC
AGTCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGGAAAGACATGCAAAAGCACC
ACTGGCAGCAGCCACTGGTAATTGATTTAGAGGAGTTAGTCTTGAAGTCATGCGCCGGTTAAGGCTAAAC
TGAAAGGACAAGTTTTGGTGACTGCGCTCCTCCAAGCCAGTTACCTCGGTTCAAAGAGTTGGTAGCTCAG
AGAACCTTCGAAAAACCGCCCTGCAAGGCGGTTTTTTCGTTTTCAGAGCAAGAGATTACGCGCAGACCAA
AACGATCTCAAGAAGATCATCTTATTAAGGGGTCTGACGCTCAGTGGAACGAAAACTCACGTTAAGGGAT
TTTGGTCATGAGATTATCAAAAAGGATCTTCACCTAGATCCTTTTAAATTAAAAATGAAGTTTTAAATCA
ATCTAAAGTATATATGAGTAAACTTGGTCTGACAGTTACCAATGCTTAATCAGTGAGGCACCTATCTCAG
CGATCTGTCTATTTCGTTCATCCATAGTTGCCTGACTCCCCGTCGTGTAGATAACTACGATACGGGAGGG
CTTACCATCTGGCCCCAGTGCTGCAATGATACCGCGAGACCCACGCTCACCGGCTCCAGATTTATCAGCA
ATAAACCAGCCAGCCGATTCGAGCTCGCCCCGGGGATCGACCAGTTGGTGATTTTGAACTTTTGCTTTGC
CACGGAACGGTCTGCGTTGTCGGGAAGATGCGTGATCTGATCCTTCAACTCAGCAAAAGTTCGATTTATT
CAACAAAGCCGCCGTCCCGTCAAGTCAGCGTAATGCTCTGCCAGTGTTACAACCAATTAACCAATTCTGA
TTAGAAAAACTCATCGAGCATCAAATGAAACTGCAATTTATTCATATCAGGATTATCAATACCATATTTT
TGAAAAAGCCGTTTCTGTAATGAAGGAGAAAACTCACCGAGGCAGTTCCATAGGATGGCAAGATCCTGGT
ATCGGTCTGCGATTCCGACTCGTCCAACATCAATACAACCTATTAATTTCCCCTCGTCAAAAATAAGGTT
ATCAAGTGAGAAATCACCATGAGTGACGACTGAATCCGGTGAGAATGGCAAAAGCTTATGCATTTCTTTC
CAGACTTGTTCAACAGGCCAGCCATTACGCTCGTCATCAAAATCACTCGCATCAACCAAACCGTTATTCA
TTCGTGATTGCGCCTGAGCGAGACGAAATACGCGATCGCTGTTAAAAGGACAATTACAAACAGGAATCGA
ATGCAACCGGCGCAGGAACACTGCCAGCGCATCAACAATATTTTCACCTGAATCAGGATATTCTTCTAAT
ACCTGGAATGCTGTTTTCCCGGGGATCGCAGTGGTGAGTAACCATGCATCATCAGGAGTACGGATAAAAT
GCTTGATGGTCGGAAGAGGCATAAATTCCGTCAGCCAGTTTAGTCTGACCATCTCATCTGTAACATCATT
GGCAACGCTACCTTTGCCATGTTTCAGAAACAACTCTGGCGCATCGGGCTTCCCATACAATCGATAGATT
GTCGCACCTGATTGCCCGACATTATCGCGAGCCCATTTATACCCATATAAATCAGCATCCATGTTGGAAT
TTAATCGCGGCCTCGAGCAAGACGTTTCCCGTTGAATATGGCTCATAACACCCCTTGTATTACTGTTTAT
GTAAGCAGACAGTTTTATTGTTCATGATGATATATTTTTATCTTGTGCAATGTAACATCAGAGATTTTGA
GACACAACGTGGCTTTCCCCCCCCCCCCTGCAGGTCTCGGGCTATTCTTTTGATTTATAAGGGATTTTG
CCGATTTCGGCCTATTGGTTAAAAAATGAGCTGATTTAACAAAAATTTAACGCGAATTTTAACAAAATAT
TAACGTTTACAATTTAAATATTTGCTTATACAATCTTCCTGTTTTTGGGGCTTTTCTGATTATCAACCGG
GGTACATATGATTGACATGCTAGTTTTACGATTACCGTTCATCGATTCTCTTGTTTGCTCCAGACTCTCA
GGCAATGACCTGATAGCCTTTGTAGACCTCTCAAAAATAGCTACCCTCTCCGGCATGAATTTATCAGCTA
GAACGGTTGAATATCATATTGATGGTGATTTGACTGTCTCCGGCCTTTCTCACCCTTTTGAATCTTTACC
TACACATTACTCAGGCATTGCATTTAAAATATATGAGGGTTCTAAAAATTTTTATCCTTGCGTTGAAATA
AAGGCTTCTCCCGCAAAAGTATTACAGGGTCATAATGTTTTTGGTACAACCGATTTAGCTTTATGCTCTG
AGGCTTTATTGCTTAATTTTGCTAATTCTTTGCCTTGCCTGTATGATTTATTGGATGTT

[SEQ ID No: 11]

The system according to the nineteenth aspect can be used to produce a particle for use in accordance with any use as disclosed herein. The particle may be for use in accordance with the second, ninth or tenth aspect. In an embodiment, the recombinant phagemid particle produced is for use in treating a subject that has not been exposed to the delivered one or more antigen, for example by prior vaccination.

In an embodiment, the recombinant phagemid particle produced by the system of the nineteenth aspect is for use in a method that further comprises the use of one or more adoptively transferred T cell. Preferably, the adoptively transferred T cell is selected from a group consisting of a chimeric antigen receptor (CAR) T cell; T cell receptor (TCR) transgenic T cell; and tumour infiltrating lymphocyte (TIL). The adoptively transferred T cells may be specific for the one or more antigen introduced by the

recombinant phagemid particle into the tumour cell. In a preferred embodiment, the adoptively transferred T cells are CAR T cells.

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.

Hence, in a twentieth aspect, there is provided a method for producing a recombinant phagemid particle from a prokaryotic host, the method comprising:-

(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 comprising a nucleic acid sequence encoding one or more cytokine, or one or more antigen that is recognised by one or more adoptively transferred T cell, and a packaging signal for enabling replication of the vector into single-stranded DNA;

(ii) introducing, into the host, a helper phage comprising nucleic acid encoding bacteriophage structural proteins; and

(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.

Advantageously, this method (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.

The antigen may be any as disclosed herein. In a preferred embodiment, the or each antigen is a peptide or protein which is expressed on the cell surface of the target tumour cell. Preferably, the or each antigen is a peptide or protein that, when expressed by the tumour cell, would be accessible to a CAR T cell. The peptide or protein may be such that, when expressed by the tumour cell, it is present as a folded peptide protein at or on the cell-surface. The or each antigen may be a known target for existing CAR T cells suitable for use in humans. For example, the or each antigen may be selected from a group consisting of: MUC1; PSMA; CD19; CD20; estrogen-related

receptor beta type 2 (ErRB2); or any combination thereof. In an embodiment, the or each antigen may be dengue virus or yellow fever vaccine antigens.

The cytokine may be any as disclosed herein. In particular the cytokine may be IL-4, IL-12, IL-15, TNFα, TRAIL, IFN-γ, or any combination thereof. Preferably, the cytokine is IL-15, Preferably, the cytokine is IL-4. Preferably, the cytokine is IL-12. Preferably, the cytokine is TRAIL. Preferably, the cytokine is IFN-γ.

Preferably, the cytokine is not TNFα. Preferably, the cytokine is TNFα. Preferably, the cytokine is a hybrid TNFα comprising a non-endogenous signal peptide configured to increase expression and/or secretion of TNFα. Preferably, the signal peptide is a cytokine signal peptide other than that of TNFα.

Preferably, the method of the twentieth aspect is used to produce the recombinant phagemid particle of the eighth aspect. 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.

The method according to the twentieth aspect can be used to produce a particle for use in accordance with any use as disclosed herein. The particle may be for use in accordance with the second, ninth or tenth aspect. In an embodiment, the recombinant phagemid particle produced is for use in treating a subject that has not been exposed to the delivered one or more antigen, for example by prior vaccination.

In an embodiment, the recombinant phagemid particle produced by the method of the twentieth aspect is for use in a method that further comprises the use of one or more adoptively transferred T cell. Preferably, the adoptively transferred T cell is selected from a group consisting of a chimeric antigen receptor (CAR) T cell; T cell receptor (TCR) transgenic T cell; and tumour infiltrating lymphocyte (TIL). The adoptively transferred T cells may be specific for the one or more antigen introduced by the recombinant phagemid particle into the tumour cell. In a preferred embodiment, the adoptively transferred T cells are CAR T cells.

In a twenty-first aspect, there is provided a method for producing a recombinant phagemid particle from a prokaryotic host, the method comprising:-

(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 comprising a nucleic acid sequence encoding one or more cytokine, or one or more antigen that is recognised by one or more adoptively transferred T cell, 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

(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.

Advantageously, this method (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.

The antigen may be any as disclosed herein. In a preferred embodiment, the or each antigen is a peptide or protein which is expressed on the cell surface of the target tumour cell. Preferably, the or each antigen is a peptide or protein that, when expressed by the tumour cell, would be accessible to a CAR T cell. The peptide or protein may be such that, when expressed by the tumour cell, it is present as a folded peptide protein at or on the cell-surface. The or each antigen may be a known target for existing CAR T cells suitable for use in humans. For example, the or each antigen may be selected from a group consisting of: MUC1; PSMA; CD19; CD20; estrogen-related receptor beta type 2 (ErRB2); or any combination thereof. In an embodiment, the or each antigen may be dengue virus or yellow fever vaccine antigens.

The cytokine may be any as disclosed herein. In particular the cytokine may be IL-4, IL-12, IL-15, TNFα, TRAIL, IFN-γ, or any combination thereof. Preferably, the cytokine is IL-15. Preferably, the cytokine is IL-4. Preferably, the cytokine is IL-12. Preferably, the cytokine is TRAIL. Preferably, the cytokine is IFN-γ.

Preferably, the cytokine is not TNFα. Preferably, the cytokine is TNFα. Preferably, the cytokine is a hybrid TNFα comprising a non-endogenous signal peptide configured to increase expression and/or secretion of TNFα. Preferably, the signal peptide is a cytokine signal peptide other than that of TNFα.

Preferably, the method of the twenty-first aspect is used to produce the recombinant phagemid particle of the eighth aspect. 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 of replication. Preferably, the origin of replication in the first vector comprises an F1 ori, more preferably from an F1 bacteriophage.

The method of the twenty-first aspect can be used to produce a recombinant phagemid particle for any use as disclosed herein. The particle may be for use in accordance with the second, ninth or tenth aspect. In an embodiment, the recombinant phagemid particle is for use in treating a subject that has not been exposed to the delivered one or more antigen, for example by prior vaccination.

In an embodiment, the recombinant phagemid particle produced by the method of the twenty-first aspect is for use in a method that further comprises the use of one or more adoptively transferred T cell. Preferably, the adoptively transferred T cells are selected from a group consisting of chimeric antigen receptor (CAR) T cells; T cell receptor (TCR) transgenic T cells; and tumour infiltrating lymphocytes (TILs). The adoptively transferred T cells may be specific for the one or more antigen introduced by the recombinant phagemid particle into the tumour cell. In a preferred embodiment, the adoptively transferred T cells are CAR T cells.

In an twenty-second aspect, 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 eighth aspect from a prokaryotic host.

There is provided a host cell comprising the first and/or second vector as defined in the twenty-first aspect.

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) Δ(mcrB-hsdSM)5, ($r_K^- m_K^-$)*, Plasmids: F' [*traD36 proAB$^+$ lacI^q lacZΔM15*]), (ii) DH5αF´IQ™ (Genotype: F-φ80lacZΔM15 Δ(lacZYA-argF) U169 recA1 endA1 hsdR17 (rk-, mk+) phoA supE44 λ- thi-1 gyrA96 relA1, Plasmids: F´ proAB+ lacIqZΔM15 zzf::Tn5 [KmR]; and (iii) XL1-Blue MRF´ (Genotype: Δ(mcrA)183 Δ(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac, Plasmids: F´ proAB lacIqZΔM15 Tn10 (Tetr).

The particle is used therapeutically or in diagnostic methods, preferably *in vivo*.

The invention may be used for the treatment of a wide variety of cancers 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 cancer, or after the development of a cancer, to ameliorate, manage, and/or treat it.

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 cancers depending on the nature of the particles and the displayed foreign proteins (if an antigen). It will be appreciated that cytokine may not be displayed on the tumour cell. The target cell in the gene therapy technique is preferably eukaryotic, and preferably mammalian.

The gene therapy technique is used to treat, prevent, ameliorate, or manage cancer. The tumour may be a liquid tumour, such as a blood malignancy or a blood-forming tissue, or a haematological malignancy. The tumour may be a solid tumour. Tumours may be in the brain, e.g. medulloblastoma, glioblastoma, or diffuse intrinsic pontine glioma (DIPG). The recombinant phagemid particle may be used in combination with conventional treatments, such as chemotherapeutic drugs (e.g. doxorubicin, temozolomide, lomustine, cisplatin, vincristine), radiation therapy, or other drugs/xenobiotic compound, including but not limited to inhibitors of histone deacetylases (HDAC inhibitors), proteasome inhibiting drugs (e.g. MG132, borzotemib, carfilzomib) and anticancer products from natural and dietary sources (e.g. genistein, Epigallocatechin gallate(EGCG), resveratrol).

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 , or proteins, or proteins with related biological activities. The subject may be mammalian, and is preferably human.

It will be appreciated that the recombinant phagemid particles and systems according to the invention (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, managing, or preventing cancer. For example, a combined therapeutic approach using the phagemid particles and systems of the invention with existing chemotherapeutics, such as Temozolamide, cisplatin, Doxorubicin, vincristine, or Genistein, is preferred.

In another preferred embodiment, therapy may comprise the combination of the recombinant phagemid particle and system of the invention 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.

The agents according to the invention (i.e. the recombinant phagemid particle used in the eighth or any above aspect, or produced by the system according to the nineteenth aspect) 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 invention should be one which is well-tolerated by the subject to whom it is given.

Medicaments comprising the agents according to the invention 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 of the invention 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.

Agents according to the invention 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 used according to the invention is required and which would normally require frequent administration (e.g. at least daily injection).

In a preferred embodiment, agents and compositions according to the invention 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 convention (convection enhanced delivery – relevant to local injections at disease site).

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.

Generally, a daily dose of between 0.01μg/kg of body weight and 500mg/kg of body weight of the agent according to the invention 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.

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 according to the invention to a patient without the need to administer repeated doses.

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 according to the invention and precise therapeutic regimes (such as daily doses of the agent and the frequency of administration).

Hence, in a twenty-third aspect of the invention, there is provided a pharmaceutical composition comprising the recombinant phagemid viral particle according to the eighth aspect, produced by the system according to the nineteenth aspect, produced by the methods of the twentieth or twenty-first aspect, or produced according to the use of the twenty-second aspect, and a pharmaceutically acceptable vehicle.

The invention also provides, in an twenty-fourth aspect, a process for making the pharmaceutical composition according to the twenty-third aspect, the process comprising contacting a therapeutically effective amount of the recombinant phagemid particle according to the eighth aspect, produced by the system according to the nineteenth aspect, produced by the methods of the twentieth or twenty-first aspect, or produced according to the use of the twenty-second aspect, and a pharmaceutically acceptable vehicle.

A “subject” may be a vertebrate, mammal, or domestic animal. Hence, agents, compositions and medicaments according to the invention 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.

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 tumour killing.

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.

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.

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 according to the invention. In tablets, the active agent (e.g. the particle or system of the invention) 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, polyvinylpyrrolidine, 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.

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 according to the invention 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.

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.

The recombinant phagemid particle, system and pharmaceutical compositions of the invention 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 according to the invention 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.

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. 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. In addition, lentiviral vectors 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.

The inventors have shown that in addition to exhibiting useful applications in gene therapy, the recombinant phagemid particle of the invention can also be used to produce recombinant viral vectors, such as AAV or lentivirus, in vitro or in vivo (including in situ). 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.

Thus, in a twenty-fifth aspect, there is provided a recombinant phagemid particle according to the eighth aspect, produced according to the system of the nineteenth aspect, produced by the methods of the twentieth or twenty-first aspect, or produced according to the use of the twenty-second aspect, wherein the recombinant phagemid particle is for production of a recombinant viral vector comprising or derived from the viral genome within the genome of the phagemid particle, wherein the recombinant viral vector is used for delivering the nucleic acid sequence encoding one or more antigen or cytokine, to at least adjacent to the tumour cell., when the sequence encodes one or more antigen, the one or more antigen is expressed, and recognisable by one or more adoptively transferred T cell.

A recombinant phagemid particle which can produce a recombinant viral vector which, in turn, encodes an antigen for recognition by an adoptively transferred T cell (such as a CAR T cell) can be advantageous. Figure 16 illustrates the delivery of a nucleic acid sequence to a malignant tumour; the production of the viral vector enables autoinfection of the tumour, such that the nucleic acid sequence is delivered throughout the tumour. This can be advantageous as this enables an antigen for recognition by an adoptively transferred T cell to be delivered throughout a tumour. This synergises with any subsequent adoptive transfer therapy, such as CAR T cell therapy, as it provides a suitable target for the CAR T cells. Furthermore, it may allow a lower concentration of the introduced vector (i.e. the recombinant phagemid particle) to be used.

In a preferred embodiment, the or each antigen is a peptide or protein which is expressed on the cell surface of the target tumour cell. Preferably, the or each antigen is a peptide or protein that, when expressed by the tumour cell, would be accessible to a CAR T cell. The peptide or protein may be such that, when expressed by the tumour cell, it is present as a folded peptide protein at or on the cell-surface. The or each antigen may be a known target for existing CAR T cells suitable for use in humans. For example, the or each antigen may be selected from a group consisting of: MUC1; PSMA; CD19; CD20; estrogen-related receptor beta type 2 (ErRB2); or any combination thereof. In an embodiment, the or each antigen may be dengue virus or yellow fever vaccine antigens.

The recombinant phagemid particle according to the twenty-fifth aspect can be for any use as disclosed herein. The particle may be for use in accordance with the second, ninth or tenth aspect. In an embodiment, the recombinant phagemid particle is for use in treating a subject that has not been exposed to the delivered one or more antigen, for example by prior vaccination.

In an embodiment, the recombinant phagemid particle of the twenty-fifth aspect is for use in a method that further comprises the use of one or more adoptively transferred T cell. Preferably, the adoptively transferred T cells are selected from a group consisting of chimeric antigen receptor (CAR) T cells; T cell receptor (TCR) transgenic T cells; and tumour infiltrating lymphocytes (TILs). The adoptively transferred T cells may be specific for the one or more antigen introduced by the recombinant phagemid particle into the tumour cell. In a preferred embodiment, the adoptively transferred T cells are CAR T cells.

There is also provided a method for producing recombinant viral vector, the method comprising introducing into, a eukaryotic host cell, the recombinant phagemid particle according to the eighth aspect, or the system according to the nineteenth aspect, and allowing the host cell to produce recombinant viral vector.

Preferably, the recombinant virus product is a recombinant mammalian virus, such as AAV or lentivirus. Preferably, the viral vector product is rAAV. Preferably, the phagemid viral particle according to the eighth aspect, or the system according to the nineteenth aspect 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).

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.

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

For example, in hybrid phagemid particle-guided rAAV production, the rAAV gene may be carried by the recombinant phagemid viral particle according to the eighth aspect, as shown in Figure 3, and the adenohelper and rep-cap genes may be carried on separate vectors, or be integrated into the eukaryotic host genome. For example, Figure 12 shows the adenohelper genes on one vector, and Figure 13 shows the rep-cap on a separate vector. Any combinations of the rAAV, rep-cap and adenohelper genes may be carried on one or more vectors, i.e. in cis or trans configurations. Alternatively, rep-cap or adenohelper 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.

In one preferred embodiment, rAAV, rep-cap and adenohelper 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.

Hence, in a twenty–sixth aspect, there is provided a recombinant vector comprising rAAV, rep-cap, adenohelper genes, and a nucleic acid sequence encoding one or more cytokine, or one or more antigen for recognition by one or more adoptively transferred T cell, for use in the treatment, prevention, or amelioration of cancer.

The recombinant vector can be for any use or method disclosed herein.

In a twenty –seventh aspect, there is provided a recombinant phagemid particle comprising the vector of the twelfth aspect, for use in a method for the treatment, prevention, or amelioration of cancer.

The recombinant phagemid particle can be for any use or method disclosed herein.

There is provided use of the vector according to the twenty –sixth aspect or the particle of the twenty-seventh aspect, to produce a recombinant AAV viral vector comprising or derived from the viral genome of the phagemid particle.

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 twenty – sixth aspect or the particle of the twenty-seventh aspect, and allowing the host cell to produce recombinant viral vector.

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.

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.

In a twenty–eighth aspect, there is provided a recombinant phagemid particle for expressing a transgene in a target tumour cell transduced with the particle, for use in a method for treating, preventing or ameliorating cancer, wherein the phagemid particle comprises at least one transgene expression cassette comprising a nucleic acid sequence encoding one or more cytokine, or antigen recognisable by one or more adoptively transferred T cell, and comprises a genome which lacks at least 50% of its bacteriophage genome, and wherein the method comprises delivering the nucleic acid sequence to at least adjacent to the tumour cell, when the nucleic encodes an one or more antigen, the one or more antigen is expressed, and recognisable by one or more adoptively transferred T cell.

It will be appreciated that the invention 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.

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.

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.

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 (v) 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.

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.

Preferably, calculation of percentage identities between two amino acid/polynucleotide/polypeptide sequences is then calculated from such an alignment as (N/T)*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)*100.

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 3x sodium chloride/sodium citrate (SSC) at approximately 45°C followed by at least one wash in 0.2x 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.

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.

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. For the avoidance of doubt, reference to cytokine may preferably relate to IL-4, IL-12, IL-15,

TNFα, TRAIL, IFN-γ, or any combination thereof. Preferably, the cytokine is IL-15, Preferably, the cytokine is IL-4. Preferably, the cytokine is IL-12. Preferably, the cytokine is TRAIL. Preferably, the cytokine is IFN-γ.

However, in another preferred embodiment, the cytokine is TNFα. Preferably, the cytokine is a hybrid TNFα comprising a non-endogenous signal peptide configured to increase expression and/or secretion of TNFα. Preferably, the signal peptide is a cytokine signal peptide other than that of TNFα. For example, the signal peptide is preferably the IL-2 signal peptide.

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 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 f1 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, and as shown in SEQ ID Nos 26 and 27;

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 μg/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 a sequence encoding 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 a sequence encoding shRNA for mTOR in medulloblastoma. Medulloblastoma cells (UW228 and DAOY) were transduced with RGD4C-phagemid (RGD4C) or RGD4C-phagemid carrying a sequence encoding mTOR/shRNA (RGD4C-mTOR/shRNA). Untreated cells were also used as controls. At day 7 post vector treatment, temozolmide (TMZ, 100 uM) 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 TNFα phagemid vectors. UW228 cells were treated with RGD4C-phagemid-TNFa (RGD4C/TNFα) and non-targeted (ctr). **A)**Cell viability, using MTT assay, following expression of TNFα . **B)** Expression of TNFα in the medium of vector-treated cells, measured using human TNFα ELISA Max. Error bars: mean ± 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 UW228, DAOY, and DIPG cells by RGD4C-phagemid/AAV. Increasing vector dose 1 x 10^6 or 2 x 10^6 TU/cell of RGD4C-phagemid-Luc (RGD4C) carrying the reporter Luc (luciferase) gene was used to treat the cells. Luc expression was measured daily. Non-targeted vector lacking RGD4C (ctr) was used as negative control for targeting. Error bars: mean ± SEM. (A) shows treatment of DIPG cells, (B) shows treatment of UW228, (C) shows treatment of DAOY cells;

Figure 28 shows treatment with RGD4C-phagemid-TNFα of UW288, DAOY, or DIPG cells. DIPG were transduced with 2 x 10^6 TU/cell RGD4C-phagemid-TNFα (RGD4C) and non-targeted vector as negative control (ctr). UW288 or DAOY cells were transduced with 1×10^6 TU/cell with or without DEAE dextran. Apoptotic activity was measured at day 9 post-vector treatment by measuring the percentage of viable cells or using caspase-Glo assay (caspase 3/7, caspase 8, and caspase9). Error bars: mean ± 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 transducing units of RGD.PAAV;

Figure 30 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;

Figure 31 shows schematic diagrams of embodiments of the expression plasmid constructs for bacteriophage-guided CAR T cell therapy; 31a represents MUC1-CD28.IL4 expression plasmid driven by CMV promoter, 31b represents MUC1-GPI.IL4 expression plasmid driven by CMV promoter, 31c represents PSMA expression plasmid driven by CMV promoter, 31d represents MUC1-CD28.IL4 expression plasmid driven by Grp78 promoter, 31e represents MUC1-GPI.IL4 expression plasmid driven by Grp78 promoter and 31f represents PSMA expression plasmid driven by Grp78 promoter.

Figure 32 shows MUC-1.CD28, MUC-1.GPI and PSMA antigen expression on day 6 post-transduction. HEK 293 cells were transduced by either 10^6 TU/cell of RGD targeted PAAV (RGD) or non-targeted PAAV (NT). Untreated HEK 293 cells (Ctrl) and, targeted PAAV transduced-HEK 293 cells with only 488 secondary antibody staining

(Ant.488) were shown as control. **A** represents the MUC-1 or PSMA expression of HEK 293 cells transduced by CMV promoter-driven PAAV vector (construct in figure 31a, 31b and 31c). DEAE-dextran was added. **B** represents the MUC-1 or PSMA expression of HEK 293 cells transduced by CMV promoter-driven PAAV vector (construct in figure 31a, 31b and 31c) without DEAE dextran added. **C** represents the MUC-1 expression of HEK 293 cells transduced by Grp78 promoter-driven PAAV vector (construct in figure 31d and 31e) without DEAE dextran added.;

Figure 33 shows schematic diagrams of the expression plasmid constructs with puromycin resistance gene for stable cell line selection bacteriophage-guided CAR T cell therapy therapy; 33a represents MUC1-CD28.IL4 expression plasmid driven by CMV promoter, 33b represents MUC1-GPI.IL4 expression plasmid driven by CMV promoter, 33c represents PSMA expression plasmid driven by CMV promoter, 33d represents MUC1-CD28.IL4 expression plasmid driven by Grp78 promoter, 33e represents MUC1-GPI.IL4 expression plasmid driven by Grp78 promoter and 33f represents PSMA expression plasmid driven by Grp78 promoter;

Figure 34 shows MUC-1.CD28, MUC-1.GPI and PSMA antigen expression of cancer cells on day 6 post-transduction. Cancer cells were transduced by either 106 TU/cell of RGD targeted PAAV (RGD) or non-targeted PAAV (M13). Untreated cells without any antibody staining (CTRL) were shown as control while untreated cells were stained with primary and secondary antibody (Stain) to checked internal expression of the antigen by FACS. A represents the MUC-1 or PSMA expression of A549 cells transduced by CMV promoter-driven PAAV vector. B represents the MUC-1 or PSMA expression of Suit2 cells transduced by CMV promoter-driven PAAV vector. C represents the MUC-1 expression of UW 228 cells transduced by CMV promoter-driven PAAV vector;

Figure 35 shows MUC-1.CD28, MUC-1.GPI and PSMA antigen expression in stable cancer cells. RGD4C-PAAV-Stably transduced cancer cells expressing MUC1 or PSMA antigen (Stable cell) were selected with puromycin antibiotic, then used to perform FACS analyses for MUC-1 or PSMA expression. Untreated cells without any antibody staining (CTRL) were shown as control while untreated cells were stained with primary and secondary antibody (Stain) to checked internal expression of the antigen. A represents the MUC-1 or PSMA expression of A549 cells. B represents the MUC-1 or PSMA expression of Suit2 cells. C represents the MUC-1 expression of UW 228 cells;

Figure 36 shows treatment and comparison of DIPG cell killing in vitro between PAAV carrying either the transmembrane tmTNFα or secreted sTNFα. DIPG cells were treated with either RGD4C-PAAV-tmTNFα or RGD4C-PAAV-sTNFα (RGD4C and non-targeted M13 [ctr]) and cell viability was measured at day 7 post-vector treatment. The

RGD4C-PAAV-sTNFα particle carrying the secreted sTNFα was more potent in inducing DIPG cell killing than the RGD4C-PAAV-tmTNFα, even in a transient transduction context where only a small population of cells are transduced by the vector;

Figure 37 shows schematic diagrams of embodiments of the plasmid constructs used by the presently disclosed methods; 37a represents PAAV-CMV-IRES GFP plasmid, 37b represents PAAV.Grp78.IRES.GFP;

Figure 38 shows expression of TNFα after PAAV-tm.TNFα transduction. (A) UW228 and Daoy were seeded in 96-well plate and transduced with 1×10^6 TU/cell with DEAE dextran, the supernatant was collected at day 6 and TNFα in the supernatant was determined by ELISA. . Data are represented as mean±SEM.

Figure 39 shows the effect of TNFα and cisplatin combination treatment in UW228 cells. Cell viability was measured at different time points using sulphorodamine B assay. Stably transduced UW228 were treated with 1μM and 5μM cisplatin chemotherapy 48 hrs after seeding the cells. . Data are represented as mean±SEM. ***P≤0.001. The figure shows that a combination with cisplatin (cis) chemotherapy increases tmTNFα cytokine gene therapy against medulloblastoma;

Figure 40 shows expression of TNF after PAAV-sTNF transduction. DIPG were seeded in 96-well plate and transduced with 2×10^6 TU/cell with DEAE dextran, the supernatant was collected at day 3 and TNFα in the supernatant was determined by ELISA;

Figure 41 shows the generation of PAAV-CMV-tmTNFα;

Figure 42 shows the generation of PAAV-sTNFα;

Figure 43 shows a targeted PAAV vector, showing RGD-4C ligand displayed on pIII coat proteins of the M13 filamentous phage. The hybrid genome shows important gene fragments that are necessary for expression of the desired gene;

Figure 44 shows a schematic for producing RGD pVIII helper viruses;

Figure 45 shows a schematic for producing RGD pVIII PAAV-GFP and RGD pVIII PAAV-lucia, which are used in in vitro transduction experiments to assess efficiency of vector and level of gene expression;

Figure 46 shows a schematic for producing PAAV-hTRAIL (image from SnapGene);

Figure 47 shows fluorescent microscopic images of DIPG cells incubated with a primary anti-phage antibody and Alexa Fluor-488 labelled secondary antibody (green) to assess integrin (αv/β3/β5) expression. Images of control cells with no antibodies or secondary antibodies only were taken to account for background fluorescence. Nuclei were stained with DAPI (blue);

Figure 48 shows theorised constructs of RGD-4C ligands as displayed on pIII (left) or pVIII (right) coat proteins of the M13 filamentous phage;

Figure 49 shows fluorescent microscopic images of HEK293T cells incubated with un-targeted, RGD pIII PAAV-GFP or RGD pVIII PAAV-GFP vectors at 0.1m TU, 0.5m TU and 1m TU at day 6 post-transduction. GFP expression is highest in RGD pIII PAAV-GFP at all Tus;

Figure 50 shows RLU of HEK293T cells incubated with un-targeted, RGD pIII PAAV-lucia or RGD pVIII PAAV-lucia vectors at 0.1m TU, 0.5m TU and 1m TU at Day 6 post-transduction. RLU is highest in RGD pIII PAAV-lucia at all TUs. Error bars are +/- 1 standard error;

Figure 51 shows RLU of HEK293T cells incubated with vectors at Day 4 post-transduction. RLU is highest in H5W RGD pIII PAAV-lucia at all TUs. Error bars are +/- 1 standard error. (Sajee Waramit, unpublished data);

Figure 52 shows RLU of DIPG cells incubated with un-targeted, RGD pIII PAAV-lucia or H5W RGD pIII PAAV-lucia vectors at 1m TU and 2m TU at Day 3 post-transduction. RLU is highest in H5W RGD pIII PAAV-lucia at all TUs. Error bars are +/- 1 standard error;

Figure 53 Microscopic images of DIPG cells transfected with PAAV-hTRAIL and control PAAV-GFP plasmids at 0.2ng, 0.4ng and 0.6ng DNA. Images were taken at 18 hours post-transfection. Cell viability is lower in cells transfected with PAAV-hTRAIL at all DNA concentrations;

Figure 54 shows a Bar graph showing IL-12 concentration of media collected on day 6 post transduction with PAAV-CMV-IL-12 normalised to 1µg of protein. Controls of transduction with a targeted and untargeted empty vector with a CMV promoter and mock transduction are shown. The outer selection bar designates analysis of IL-12 production data of all vector titres and controls by 2-way ANOVA. The inner selection bars designate comparison between vectors and controls at each titre by unpaired t-test. The experiment was performed in triplicate;

Figure 55 shows a line graph showing mouse IL-12 concentration of media sampled at various days post transduction with PAAV-CMV-mIL-12. Controls of transduction with a targeted and untargeted empty vector with a CMV promoter and mock transduction are shown. The outer selection bar designates analysis of mouse IL-12 production data from all sampled days for vectors and controls by 2-way ANOVA. The asterisks over specific data points designate comparison between vectors and controls at each specified day by unpaired t-test;

Figure 56 shows a bar graph showing the mean tumour size in mm3 of B16-F10 murine melanoma tumours in C57BL/6 mice over seven days after treatment with RGD-4C-targeted PAAV-CMV-mIL-12 (n=4) with RGD-4C-targeted PAAV-CMV with no transgene (n=4) and no treatment controls (n=4). Three doses of 5×10^{10} TU vector were administered intravenously on days 0, 2 and 5. Data were analysed by 2-way ANOVA with a Tukey's multiple comparisons test;

Figure 57 shows the IL2 signal sequence;

Figure 58 shows the Il-2/TNFα construct;

Figure 59 shows cell killing efficiency of RGD4C-sTNFα and RGD4C-tmTNFα in DIPG. DIPG cells were transduced with PAAV targeted (RGD4C) or non-targeted (M13) carrying either secreted or transmembrane TNFα (sTNFα) transgene. The cells were seeded in 96-well plate. Two day later, the cells were transduced with 2× 〚10〛^6 TU/cell with 40ng/μg of protein DEAE dextran. The viability was measured with sulforhodamine B (SRB) assay. Styatistical significance was determined by student's t-test Data are represented as mean±SEM. *P≤0.05, **P≤0.01;

Figure 60 shows expression of TNFα after transduction with PAAV-sTNFα and PAAV-tmTNFα. DIPG cells were seeded in 6-well plate and transduced with RGD4C and M13 carrying either secreted or transmembrane form of TNFα transgene. A) RNA was extracted and expression of TNFα was determined by qRT-PCR. B) The supernatant was collected and TNFα in the supernatant was determined by ELISA. Data are represented as mean±SEM. **P≤0.01 ***P≤0.001 .Statistical significance was determined by student's t-test;

Background

The development of gene delivery technologies 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.

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.

As described in the Examples below, the inventors have:-

1. Designed and constructed a hybrid Phagemid - AAV Vector (PAAV) particle expression system;
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:
    a. Binding to the cell surface,
    b. Internalisation of the vector from the cell surface,
    c. Recombinant transgene expression.
3. Determined whether the hybrid phagemid PAAV vector system is capable of producing rAAV from a mammalian producer cell-line.
4. Demonstrated that the system can be used in CAR-T therapy for cancer treatment.

5. Demonstrated that the system can be used to deliver the cytokines IL-12, TRAIL and hybrid TNFα to target cells for cancer treatment.
6. Designed and constructed a phagemid particle comprising a hybrid TNFα construct.
7. Demonstrated that a hybrid TNFα constructs show increased expression, secretion and cell killing efficiency when compared to full length TNFα.

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

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

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.

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).

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.

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. 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 allow concatamer formation of AAV and subsequently enable AAV transgene cassettes to be stably expressed over a long period of time.

The phagemid 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

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.

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 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 tumour cells and angiogenic tumour-associated endothelial cells that express $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrin receptors. Thus, referring to Figure 8, the genome of the helper phage comprises the RGD4C peptide (CDCRGDCFC – SEQ ID No: 7) targeting these $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins.

Once the PAAV 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

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:

- 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.

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

With reference to Figure 9:

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. Incubate culture at 37° in shaker (250 RPM).
3. Once OD_{600} is in the range of 0.5 to 0.8 (log phase), add at least 1×10^{10} transducing units of helper phage (M13KO7) to culture.
4. Invert to mix. Incubate at 37° for 30 minutes.
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. Incubate overnight in an orbital shaker at 37°, 250rpm for 16-20 hours.
7. Purify phagemid (PAAV) particles from culture supernatant.

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

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

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. Pick a colony and inoculate 5mL 2xYT (100µg/mL Ampicillin + 50µg/mL Kanamycin) supplemented with 1% glucose.
3. Incubate in an orbital shaker at 37°, 250rpm for 4 hours
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. Incubate overnight in an orbital shaker at 37°, 250rpm for 16-20 hours
6. Purify phagemid particles from culture supernatant

PAAV Phagemid Particle Purification

1. Transfer the warm overnight culture to centrifuge bottles and pellet the bacteria by centrifugation at 3300G, 4° for 30 minutes.
2. Discard the pellet and transfer supernatant to a clean centrifuge bottle.
3. Add 30% volume of supernatant in each bottle with ice-cold 20% PEG-8000/2.5M NaCl and swirl to mix.
4. Incubate on ice for 4-24 hours
5. Precipitate phagemid particles by centrifugation at 10000G, 4° for 30 minutes. Discard the supernatant.
6. Dry the phagemid particle pellet by centrifugation at 10000G, 4° for 1 minute.
7. Remove remaining supernatant with PEG/NaCl
8. Resuspend the phagemid particle pellet in 0.5-2mL PBS
9. Filter the resuspended phagemid particle preparation using a 0.45 micron filter.
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

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.

As an example, the PAAV 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 one or more antigen for recognition by adoptively transferred T cells, such as CAR T cells. 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 (PAAV) for in vitro AAV production

In addition to gene therapy, the PAAV 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.

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.

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.

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.

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

Notes:

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:

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. 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 1million transducing units per cell.
3. Add an equal volume of serum-free DMEM (supplemented with 20mM GlutaMax) to the transduction mixture made in step 3.
4. Invert to mix. Incubate at room temperature for 15 minutes.
5. Wash the HEK293 cells plated in step 1 with PBS, repeat 3 times.
6. Add the transduction mixture and swirl gently to distribute the mixture evenly.
7. Incubate at 37°, 5% CO2 in a cell culture incubator for 72 hours
    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).

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:

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. Collect the cells and media by aspiration and trituration and transfer to a 50mL centrifuge tube.
3. Pellet the cells by centrifugation at 1500RPM, 5 minutes, Room temperature.
    a. Optional: collect the supernatant for further AAV purification.
4. Resuspend the cell pellet in 2-5mL serum-free DMEM.
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. Centrifuge the cell lysate at 10000G, 10 minutes at Room temperature.
    a. Aliquot the supernatant for quantification/further purification/concentration.
    b. Discard the pellet (debris).

<u>Example 5 – Use of Phagemid - AAV Vector (PAAV) for in situ AAV production</u>

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

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 therapeutic 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

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.

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

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 PAAV1-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

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).

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. PAAVP (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

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

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).

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.

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.

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 a sequence encoding the mTOR/shRNA (RGD4C-mTOR/shRNA)). These data demonstrate that the RGD4C-phagemid can be successfully used to deliver shRNA in tumour cells to knock down expression of the therapeutic target mTOR in a selective and efficient way.

Figure 24 shows combination treatment of temozolomide (TMZ) and RGD4C-phagemid carrying a sequence encoding 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 tumour cell eradication. Therefore, targeted knockdown of mTOR expression by the RGD4C-phagemid is an efficient strategy to use in combination with temozolomide against chemoresistant tumour cells, such as medulloblastoma.

Figure 25 shows treatment of medulloblastoma cells with TNFα vectors. Therefore, RGD4C/TNFα has therapeutic potential for use in targeted tumour 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 tumours, DIPG.

Figure 27 shows selective and dose dependent delivery of gene expression to UW288, DAOY, or 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. These data also indicate that RGD4C-PAAV shows efficient gene transfer to medulloblastoma *in vitro* that increased over time. There was no non-specific uptake in the cells transduced with the control (non-targeted PAAV-Luc). The transduction efficiency was enhanced with the cationic polymer DEAE dextran for medulloblastoma cell lines.

Figure 28 shows treatment with RGD4C-phagemid-TNFα. These data demonstrate that RGD4C-phagemid can successfully deliver TNFα to DIPG in a selective manner, resulting in apoptosis induction. Therefore, RGD4C-phagemid-TNFα has therapeutic potential for use in targeted therapy against DIPG. Figure 28 also shows that medulloblastoma is a good candidate for treatment with RGD4C-phagemid-TNFα, as the treatment resulted in tumour cell killing when tested with either of the cell lines UW288 or DAOY. For instance, UW288 showed about 60% cell death on day 6 relative to the control. Tumour cell killing was further enhanced with the cationic polymer DEAE dextran.

Example 8 – Luciferase expression of RGD4C-phagemid

Protocol:

HEK cells were plated in a 48-well plate in complete media (DMEM, 10% FBS, 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).

Figure 29 shows luciferase expression after transduction with RGD.PAAV at various concentrations of transducing units. The graph demonstrates a dose-dependent exponential relationship between time and expression of luciferase after incubation with hybrid phage/phagemid vectors at various concentrations. The figure demonstrates 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:

293AAV cells were seeded on 24-well plates in complete media (DMEM +10%FBS, 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.

Referring to Figure 30, 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.

Example 10 – Transduction of tumour cells by PAAV to express either MUC1 or PSMA antigen on their cell surface, such that they can be targeted by specific CAR T cells.

The conventional treatment for cancer currently consists of one or more of the following three options: surgery, chemotherapy and radiotherapy. Although the disease

can sometimes be cured by these interventions, in many cases the cancer cells are not completely eliminated, and so the recurrence rate is high. To make matters worse, chemotherapy and radiotherapy are associated with unpleasant side effects. As a result, there is a great interest in the development of alternative approaches for cancer treatment. One of the most promising of these new therapeutic techniques is cancer immunotherapy, which aims to harness the power and specificity of patients' own immune system to eliminate cancer cells. Cancer immunotherapy is an evolving avenue of treatment. Two main strategies involve active immunotherapy targeting tumour-associated antigens (TAAs) and passive immunotherapies that enhance existing anti-tumour responses.

Examples of cancers include paediatric brain tumours, such as medulloblastoma and Diffuse Intrinsic Pontine Glioma (DIPG). Medulloblastoma is the most common brain tumour and originates in the cerebellum with a five years survival rate following the current therapeutic strategy that consists of surgical resection, radiotherapy, and chemotherapy (Rudin et al., 2009). However, the survivors often have long term endocrinological and neurocognitive side effects. Therefore, development of novel therapeutic approaches that are non-invasive, tumour specific, safer, cost-effective and efficient is urgently needed to avoid the long-term side-effects from current treatment. On the other hand, diffuse intrinsic pontine glioma (DIPG) is the most aggressive brain tumour that arises exclusively in children with poor survival of only 6-10% beyond two years. Due to its diffuse nature, there is no effective therapeutic strategy for this type of cancer (Jansen et al., 2012, Mueller and Chang, 2009).

Due to the immune system's unique properties and its central and universal role in the organism, immunotherapy possesses the great potential to treat cancer and offers long-term protection while potentially providing fewer side effects than other treatments. One particular approach, adoptive cell therapy (ACT), involves the transfer of immune cells that have anti-tumour activity. These cells can be T cells that already exist in a tumour, known as tumour-infiltrating lymphocytes (TIL), some of which will be specific for TAAs. These cells can be isolated from excised tumour tissue, cultivated, activated and expanded *ex vivo*, then re-infused into patients. Other types of cells that are useful for ACT include genetically engineered T cells that express either a modified T cell receptor (TCR) or a chimeric antigen receptor (CAR). These artificial receptors specifically direct the T cells to target antigens expressed by tumour cells (Blankenstein T, *et al.* The determinants of tumour immunogenicity. Nat Rev Cancer. 2012;12(4):307-

13.; Sharpe M and Mount N. Genetically modified T cells in cancer therapy: opportunities and challenges. Dis Model Mech. 2015;8(4):337-50.).

CAR proteins are expressed on the surface of T cells, and contain extracellular binding domains which bind strongly to specific tumour antigens, a hinge region linking between extracellular domains and transmembrane domains, a transmembrane domain, and intracellular signalling domains (also called co-stimulatory domains) such as CD28 and OX40 (a tumour necrosis factor receptor). Co-stimulatory signals mediated by those domains enable efficiency and prolong the anti-tumour activity of the T cells (Sharpe M and Mount N. Genetically modified T cells in cancer therapy: opportunities and challenges. Dis Model Mech. 2015;8(4):337-50; Till BG, *et al.* CD20-specific adoptive immunotherapy for lymphoma using a chimeric antigen receptor with both CD28 and 4-1BB domains: pilot clinical trial results. Blood. 2012;119(17):3940-50; Koehler H, *et al.* CD28 co-stimulation overcomes transforming growth factor-beta-mediated repression of proliferation of redirected human CD4+ and CD8+ T cells in an antitumor cell attack. Cancer Res. 2007;67(5):2265-73). CAR T cells can be designed to recognise broad types of antigen, which may be expressed on tumour's cell surface. Potential target antigens include proteins, carbohydrates, and glycolipids. CAR T cells do not need the antigen to be processed and presented by MHC, unlike conventional T cells and transgenic TCR T cells. Therefore, the same CAR-based strategy can be applied in all patients expressing the same tumour antigen regardless of the patient's MHC haplotype (Sharpe M and Mount N. Genetically modified T cells in cancer therapy: opportunities and challenges. Dis Model Mech. 2015;8(4):337-50, Haji-Fatahaliha M, *et al.* CAR-modified T-cell therapy for cancer: an updated review. Artif Cells Nanomed Biotechnol. 2015:1-11).

Despite having many advantages, CAR T cell therapy has some limitations. For instance, the tumour may not express a suitable target antigen. This may occur for a variety of reasons such as the neo-antigens being unknown, expressed at inappropriate levels, expressed only on a sub-population of tumour cells, also expressed on non-tumour tissues, not expressed in a manner that is suitable for the targeting of CAR T cells to the tissue, or if expression of the antigen by the tumour is likely to be reduced or lost during treatment.

For example, it has been reported that some CAR T cell therapies have unwanted toxicity in both animal models and in clinical trials. The problem can occur when the

antigen recognised by the CAR T cell is not merely expressed on tumour cells but also presented on normal cells leading to damage of healthy tissue (Palmer DC, *et al.* Effective tumour treatment targeting a melanoma/melanocyte-associated antigen triggers severe ocular autoimmunity (Proc Natl Acad Sci U S A. 2008;105(23):8061-6; Morgan RA, *et al.* Case report of a serious adverse event following the administration of T cells transduced with a chimeric antigen receptor recognizing ERBB2. Mol Ther. 2010;18(4):843-51; Lamers CHJ, *et al.* Treatment of Metastatic Renal Cell Carcinoma With Autologous T-Lymphocytes Genetically Retargeted Against Carbonic Anhydrase IX: First Clinical Experience. Journal of Clinical Oncology. 2006;24(13):e20-e2; Grupp SA, *et al.* Chimeric antigen receptor-modified T cells for acute lymphoid leukemia. N Engl J Med. 2013;368(16):1509-18.). Therefore, the availability and selection of the target antigen are challenging. Ideally, the antigen presented exclusively by the tumour cells or alternatively by normal cells that is not essential for survival (Rosenberg SA, Restifo NP. Adoptive cell transfer as personalized immunotherapy for human cancer. Science. 2015;348(6230):62-8). At present, many CAR T cells have successfully been developed against various tumour antigens, such as mucin 1 (MUC1) for the treatment of prostate cancer and breast cancer (Sanchez C, *et al.* Combining T-cell immunotherapy and anti-androgen therapy for prostate cancer. Prostate Cancer Prostatic Dis. 2013;16(2):123-31, S1; Wilkie S, *et al.* Retargeting of Human T Cells to Tumor-Associated MUC1: The Evolution of a Chimeric Antigen Receptor. The Journal of Immunology. 2008;180(7):4901-9), prostate-specific membrane antigen (PSMA) for the treatment of prostate cancer(Maher J, *et al.* Human T-lymphocyte cytotoxicity and proliferation directed by a single chimeric TCR[zeta] /CD28 receptor. Nat Biotech. 2002;20(1):70-5.), CD19 and CD20 for the treatment of B-cell malignancies (Brentjens RJ, *et al.* Safety and persistence of adoptively transferred autologous CD19-targeted T cells in patients with relapsed or chemotherapy refractory B-cell leukemias. Blood. 2011;118(18):4817-28.) and estrogen-related receptor beta type 2 (ErRB2) for the treatment of prostate and breast cancer (Pinthus JH, *et al.* Immuno-Gene Therapy of Established Prostate Tumors Using Chimeric Receptor-redirected Human Lymphocytes. Cancer Research. 2003;63(10):2470-6.). Furthermore, to pursue a higher selectivity on tumour cell, T cells have been developed through several strategies including dual-CARs T cells that are modified to express two CARs targeting specifically two different antigens expressed on the same tumour cell (Kloss CC, *et al.* Combinatorial antigen recognition with balanced signalling promotes selective tumor eradication by engineered T cells. Nat Biotechnol. 2013;31(1):71-5; Wilkie S, *et al.* Dual targeting of ErbB2 and MUC1 in breast cancer using chimeric antigen receptors

engineered to provide complementary signalling. J Clin Immunol. 2012;32(5):1059-70.).

Molecular cloning and genetic engineering

Each PAAV-CMV-CD28-IL4, PAAV-CMV-GPI-IL4 and PAAV-CMV-PSMA (Fig. 32f) were constructed by combining PAAV-CMV-GFP plasmid (Fig. 3)with either pUC57-CD28-IL4 plasmid, pUC57-GPI-IL4 or pUC57-PSMA plasmid while PAAV-Grp78-GFP plasmid was combined with either pUC57-CD28-IL4 plasmid, pUC57-GPI-IL4 or pUC57-PSMA plasmid to construct PAAV-Grp78-CD28-IL4, PAAV-Grp78-GPI-IL4 and PAAV-Grp78-PSMA.

The new plasmids; PAAV.CMV.MUC1.CD28.IL4, PAAV.CMV.MUC1.GPI.IL4, PAAV.CMV.PSMA, PAAV.Grp78.MUC1.CD28.IL4, PAAV.Grp78.MUC1.GPI.IL4 and PAAV.Grp78.PSMA, were conducted by restriction enzyme digestion and ligation, transformed into TG1 competent E. coli and plated on 2xYT top agar with ampicillin. All constructs were validated firstly by restriction digestion and gel electrophoresis, and secondly by DNA sequencing (MRC CSC Genomics Core Laboratory, UK).

Figure 31 shows schematic diagrams of embodiments of the expression plasmid constructs for bacteriophage-guided CAR T cell therapy; 32a represents MUC1-CD28.IL4 expression plasmid driven by CMV promoter, 32b represents MUC1-GPI.IL4 expression plasmid driven by CMV promoter, 32c represents PSMA expression plasmid driven by CMV promoter, 32d represents MUC1-CD28.IL4 expression plasmid driven by Grp78 promoter, 32e represents MUC1-GPI.IL4 expression plasmid driven by Grp78 promoter and 32f represents PSMA expression plasmid driven by Grp78 promoter.

CD28, GPI and PSMA antigen expression

HEK 293 cells at approximately 60% confluent in 12-well plate were incubated 24 hours with each vector (1,000,000 TU/cell) in complete media consisting of Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), L-Glutamine (2mM, Sigma), penicillin (100 units/ml, Sigma), and streptomycin (100 mg/ml, Sigma). Untreated cells were used as a negative control. Next day, all vectors was removed from the culture and the cells were maintained in a humidified incubator at 37 °C in 5% CO2 as monolayers in complete medium. The medium was renewed every two days.

At day 6 post-transduction, the cells were harvested with cell dissociation buffer (Invitrogen), washed in washing buffer (PBS containing 2%FBS and 0.1%NaN3) and incubated in Clear Back (human Fc receptor blocking agent, MBL) for 20 minutes at room temperature. The cells were subsequently incubated with either HMFG2 antibody provided by Dr. John Maher (King's College London, UK) or PSMA antibody (MBL) diluted in washing buffer at 4 °C overnight. Next day, the cells were washed in washing buffer and incubated for 30 minutes at room temperature with anti-mouse IgG Alexa fluor 488 conjugated secondary antibodies (Invitrogen). The cells were finally washed in washing buffer and subjected to FACs calibur Flow cytometer (BD Biosciences). The mean fluorescence intensity was measured for at least 20,000 gated cells per triplicate well. Results were analyzed using Flowjo (TreeStar) software.

MUC-1.CD28, MUC-1.GPI and PSMA antigen expression on day 6 post-transduction

HEK 293 cells were transduced by either 10^6 TU/cell of RGD targeted PAAV (RGD) or non-targeted PAAV (NT). Figure 33 shows the resultant data. Untreated HEK 293 cells (Ctrl) and, targeted PAAV transduced-HEK 293 cells with only 488 secondary antibody staining (Ant.488) were shown as control. (A) represents the MUC-1 or PSMA expression of HEK 293 cells transduced by CMV promoter-driven PAAV vector. DEAE-dextran was added. (B) represents the MUC-1 or PSMA expression of HEK 293 cells transduced by CMV promoter-driven PAAV vector without DEAE dextran added. (C) represents the MUC-1 expression of HEK 293 cells transduced by Grp78 promoter-driven PAAV vector without DEAE dextran added.

Stable cell line selection

To select the stable cell line expressing MUC-1.CD28, MUC-1.GPI or PSMA antigen on their cell surfaces', puromycin resistant sequence was inserted in PAAV-CMV-CD28-IL4, PAAV-CMV-GPI-IL4, PAAV-CMV-PSMA, PAAV-Grp78-CD28-IL4, PAAV-Grp78-GPI-IL4 and PAAV-Grp78-PSMA plasmids.

Figure 34 shows schematic diagrams of the expression plasmid constructs with puromycin resistant gene for stable cell line selection bacteriophage-guided CAR T cell therapy therapy; 34a represents MUC1-CD28.IL4 expression plasmid driven by CMV promoter, 34b represents MUC1-GPI.IL4 expression plasmid driven by CMV promoter, 34c represents PSMA expression plasmid driven by CMV promoter, 34d represents MUC1-CD28.IL4 expression plasmid driven by Grp78 promoter, 34e represents MUC1-

GPI.IL4 expression plasmid driven by Grp78 promoter and 34f represents PSMA expression plasmid driven by Grp78 promoter.

The new plasmid was conducted by restriction enzyme digestion and ligation, transformed into TG1 competent E. coli and plated on 2xYT top agar with ampicillin. All construct was validated firstly by restriction digestion and gel electrophoresis, and secondly by DNA sequencing (MRC CSC Genomics Core Laboratory, UK)

Results

PAAV vectors encoding either MUC1 or PSMA transgene were produced using the helper phage system. These transgene products are subjected to display on tumour cell surface after being transduced by PAAV. MUC1 and PSMA antigens are specifically recognised by MUC1-CAR T cells and PSMA-CAR T cells.

Presented herein are data showing that the phagemid particles of the invention can be used to transduce cancer cells, and that the cancer cells subsequently stably display the delivered antigens in a manner suitable for use as the target of an adoptively transferred T cell.

Discussion

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 DEAE-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.

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

Hybrid phagemid vectors that are highly efficient at gene transfer to mammalian cells are described. These phagemid/AAV (PAAV) vectors have very large cloning capacities and are targeted to mammalian cells, meaning transfection reagents are not required. This platform allows the production of vectors that are suitable for therapeutic gene therapy. Evidence is provided that this platform can deliver genes, including antigens suitable for targeting by adoptive T cell transfer therapy or CAR T cell therapy, to tumour cells.

Example 11 - Superior phagemid/AAV hybrid vector for guided delivery of TRAIL gene to paediatric DIPG cells.

The use of cytokines in gene therapy was investigated as they serve diverse functions as differentiation, proliferation, activation or induction of cell death by apoptosis. The tumour necrosis factor (TNF) superfamily is one such group of molecules that are of interest because of their ability to induce death of tumour cells. Members of the TNF superfamily including Fas ligand (FasL), CD95 ligand (CD95L) and TNFα have been identified as important therapeutic agents for cancer biological therapy. Their administration can induce apoptosis in different cancer cells but also cause severe toxicity to liver, preventing their application in the clinic.

Given the dilemma of systemic toxicity, another member of the TNF superfamily, TRAIL, is rising as a promising cancer therapeutic agent. Preclinical and early clinical trials using recombinant TRAIL and antibodies against TRAIL receptors have shown that TRAIL has preferential toxicity toward tumour cells with generally little or no toxicity to normal tissues while retaining its anti-tumour properties. TRAIL is constitutively present in many tissues at the level of mRNA, most predominately in

spleen, lung and prostate, and is expressed mainly by cells of the immune system such as natural killer (NK) cells and macrophages.

TRAIL is synthesized as a Type II transmembrane protein that can also be proteolytically cleaved by a cysteine protease to generate a secretedform7. The membrane-bound conformation appears to be more potent, as TRAIL is biologically active as a homotrimer and this specific conformation presumably facilitates cross-linking of ligand-receptor complexes, thereby increasing signalling strength. The secreted form is less potent, but effects can be enhanced by engineering an extracellular domain fused to motifs such as a leucine zipper, which helps with stabilsation and formation of homotrimers.

Like other TNF superfamily members, TRAIL induces apoptosis through interacting with cross-linked receptor molecules on the surface of the target cells5,10. There are 5 receptors that have been identified: TRAIL R-1 and R-2 are death receptors that contain a cytoplasmic sequence death domain (DD) which triggers apoptosis, while TRAIL R-3, R-4 and osteoprotegrin are decoy receptors that prevent apoptosis5,6,10.

When homotrimer TRAIL binds to death receptors, the receptors form a trimer and recruit adaptor protein Fas-associated death domain (FADD). FADD recruits initiator caspases 8 or 10, forming the death inducing signal complex (DISC), where initiator caspases are auto-activated by proteolysis. Activated caspase 8 or 10 then cleave the effector caspase 3, causing cleavage of death substrates and cell death. If TRAIL binds to the decoy receptors instead, FADD is not recruited and apoptosis is not triggered. Even in cells resistant to TRAIL-induced apoptosis, TRAIL can induce necroptosis11. The TRAIL-receptor system can induce direct killing of tumour-supportive immune cells and its expression on NK cells is an important mechanism used by the immune system to kill cancer cells.

Current clinical trials to deliver agonists of those in the TNF superfamily, including recombinant TRAIL or agonist antibodies against TRAIL receptors, have unfortunately failed to produce a clinical benefit in cancer patients, partly due to insufficient agonistic activity and short half-life of the drugs. Additionally, often these drugs are limited in their efficacy from design due to concerns that systemic delivery of stronger agents can induce lethal adverse effects. In order to ensure the delivery of TRAIL at optimal

concentrations to produce clinical results, a suitable vector is required to selectively target and transport TRAIL to cancer cells.

Using phage display-based technology, viral vectors that display ligands that target and bind receptors selectively expressed in tumour tissues can be used to deliver TRAIL. Most research has focused on the use of eukaryotic viruses such as retrovirus and adenovirus as vectors as they provide superior transgene delivery. However, they have had limited success in systemic gene therapy due to their wide tropism for mammalian cell-membrane receptors, leading to undesired uptake by the liver, reticuloendothelial system and unwanted tissues, as well as immunogenicity. In contrast, prokaryotic viruses are advantageous, as they do not require ablation of native tropism for use in mammalian cells, are cost effective and readily produced in high titres. As they lack tropism, they are inherently poor vehicles for mammalian cell transduction13. However, by altering their coat proteins to display selective ligand peptide motifs, phages can be internalised into cells.

Effective administration of treatment solely to the tumour for prolonged effects without systemic toxicity can be achieved using vectors based on bacterial viruses, bacteriophage or phage. These bacteriophage-based vectors can be engineered to display selective ligand peptide motifs on its coat proteins to allow viral binding to targeted cells and subsequent internalisation for ligand-directed delivery of genes19. By combining the favourable biological attributes of eukaryotic and prokaryotic viruses, a chimeric virus vector was constructed comprising of recombinant adeno-associated virus (AAV) and M13-derived filamentous phage, named AAV/Phage or AAVP13. The pIII coat protein of the phage was engineered to display the double-cyclic peptide CDCRGDCFC (RGD-4C, SEQ ID NO: 7), which binds to specific αv integrin receptors (αvβ3 or αvβ5) that are over-expressed in both tumour and supporting angiogenic vaculature. This allows for superior ligand-directed delivery and cellular transduction of therapeutic transgenes as a targeted platform and these functional attributes have been confirmed in preclinical models of several cancers including prostate, breast cancer and soft-tissue sarcomas.

The inventors have improved known vector platforms by using the phagemid system to produce the next-generation vector known as Phagemid-AAV (PAAV) (Figure 43). PAAV vector is a chimeric virus – a M13 filamentous phage containing a hybrid genome constructed using DNA sequences from the AAV serotype 2. The gene of interest is

regulated by the constitutively active cytomegalovirus, CMV promoter and flanked by full-length inverted terminal repeats (ITRs). The phagemid contains an f1 origin of replication, which is used for single stranded replication and packaging into phage particles; as well as an origin of replication for double stranded replication once it enters the target cell. It can be selected with ampicillin during cloning and production of virus vector.

In this hybrid vector model, most of the phage genome is removed which allows for longer DNA sequences to be accommodated, but necessitates the use of helper viruses to provide the capsid and other phage components. RGD-4C peptide motif is first displayed on the pIII coat protein of the M13 bacteriophage to produce a targeted backbone helper virus. The helper virus is then used to transduce TG1 E.coli bacteria containing the engineered phagemid, and selection of the targeted vectors containing phagemid can be conducted using selection pressure with antibiotics. The new model was able to accommodate longer DNA sequences, had higher transduction efficiency and could be produced at higher titre over the original AAVP.

Without wishing to be bound to any particular theory, further improvements to transduction efficiency can be achieved via two methods. First, by engineering the RGD-4C peptide to display on pVIII instead of pIII coat protein. pVIII, being the major coat protein, is expressed in up to 2700 copies while pIII is only expressed up to 5 copies. A greater number of RGD-4C ligands that are available for targeting and binding integrins are therefore thought to be have higher efficiency at binding and transducing cells.

Without wishing to be bound to any particular theory, another strategy to enhance efficiency is by displaying the histidine rich H5W ligand, an endosomal escape peptide on the recombinant pVIII coat proteins. Intracellular barriers such as endosomes can limit the rate of gene expression even if the efficiency of internalisation is high, by trapping vectors and preventing it from exerting its therapeutic effects. The histidine side chain can form zwitterions, allowing it to act as a proton sponge and buffer the low pH in the endosome following ligand-directed endocytosis of the phage vector. When protons enter the endosome, water is drawn into the endosome via a vacuolar membrane proton pump, causing pores to form on the endosome and thereby releasing the phage vector.

The aim of this study was to develop a superior PAAV vector with optimal transduction efficiency of DIPG cells, by altering the peptides displayed on the vector's coat proteins. Next, the most suitable PAAV vector was used to deliver the TRAIL transgene to assess the effectiveness of TRAIL as a therapeutic gene in the treatment of DIPG.

Without being bound to any particular theory, the inventors hypothesised that:

1. Specificity of targeting and efficacy of genetic transfer can be enhanced by displaying the RGD-4C ligand on pVIII coat proteins or H5W peptide on the recombinant pVIII coat proteins
2. TRAIL is an effective therapeutic gene to specifically induce DIPG cell death in in vitro experiments and in an in vivo orthotopic DIPG animal model

Aims of study

1. Design and produce a viral vector for specific targeting of DIPG cells with optimal expression of the desired gene.
2. Assess and investigate the killing potential of therapeutic gene TRAIL against DIPG
3. To assess the specificity of targeting TRAIL expression to DIPG in vivo and investigate the therapeutic efficacy following intravenous administration of vector to immunodeficient mice with established orthotopic human DIPG.

Protocol

Cell culture

HEK293T cell lines were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% foetal bovine serum (FBS) and 1% penicillin and streptomycin. DIPG tumour cell lines were obtained from Hospital Saint Joan de Déu Barcelona. They were maintained in Tumour Stem Medium (TSM) Base supplemented with 10% FBS. TSM is made according the recipe provided (Table XXX).

Integrin staining

DIPG cells were seeded on poly-D-lysine coated coverslips in 24-well plates and grown until 60-70% confluent. 5 wells were used to stain for: control, secondary antibody only, αv, β3 and β5 integrins. Cells were washed and fixed in 4% formaldehyde for 10 minutes at room temperature (RT). Cells were washed three times in PBS and incubated with 50mM ammonium chloride for 5 minutes. Cell were washed again with PBS for three times and blocked for 30 minutes in 2% bovine serum album (BSA-PBS).

Subsequently, cells were incubated with primary antibodies anti-αv (1:100), anti-β3 (1:50) and anti-β5 integrins (1:100) diluted in 1% BSA-PBS for one day and kept in a moist chamber at 4°C. Following which, cells were washed with three times with 1% BSA-PBS and incubated for 1 hour with secondary Alex Fluor 488-conjugated antibodies (1:750) and 4',6-diamidino-2-phenylindole (DAPI, 1:2000). Coverslips were washed three times with 1% BSA-PBS, three times with PBS and once with sterile water. After air-drying, coverslips were mounted in Mowiol mounting medium. Cells were viewed and images were taken with a fluorescence microscope.

Production, purification and titration of helper phage with RGD-4C ligand displayed on pVIII coat proteins (Figure 44)

Wild-type M13 phage DNA was modified to express RGD-4C on pVIII coat proteins by polymerase chain reaction (PCR). The primers used in the PCR were designed by a colleague (Sajee Waramit) prior to the start of this project. PCR product was ligated using T4 DNA ligase (NEB) and transformed into DH5α bacteria for cloning. Plasmids are extracted by Miniprep (QIAGEN), validated by restriction digestion and gel electrophoresis and then sent for sequencing to ensure integrity of DNA sequences (MRC CSC Genomics Core). The selected clone was then transformed into TG1 competent E.coli (Zymo research) and plated on 2xYT (Sigma) agar with 50μg/ml kanamycin overnight in an incubator at 37°C.

After colonies form, a single colony is selected and grown in 1L of 2xYT media with kanamycin in a shaking incubator (32°C, 220 rpm) for 18 hours. The overnight culture was centrifuged at 6000g for 30 minutes at 4°C. Phage supernatant was collected and 20%v/v PEG/NaCl (20% PEG 6000, 2.5M NaCl) was added and mixed thoroughly. After incubation in the cold room overnight, the supernatant was centrifuged again at 10000g for 30 minutes at 4°C. Phage pellet was re-suspended in 16mL phosphate buffer saline (PBS) and 20%v/v PEG/NaCl and kept in the cold room overnight. The final phage suspension was centrifuged at 10,000g for 30 minutes at 4°C and the supernatant discarded. The purified phage pellet was re-suspended in 1-2 mL of PBS (depending on the pellet size) and sterile-filtered through a 0.45μm filter cartridge.

Titration was carried out using serial dilutions of 10-fold increments. 5μL of each dilution of phage was used to infect 500μL of naïve TG1 E.coli that has been grown to log phase (OD600=0.45-0.50) by incubating them for 30 minutes at 37°C. Following incubation, 100μL of the bacteria was plated on 2xYT agar with kanamycin overnight in

an incubator at 37°C. Colonies were counted on the following morning after 18 hours of incubation and virus titre estimated and expressed as bacterial transducing units (TU).

Production, purification and titration of RGD pVIII vectors containing reporter genes (Green Fluorescent Protein [GFP] and lucia luciferase [lucia]) (Figure 45)

To assess efficiency in transduction of DIPG cells, vectors were constructed containing phagemids with the reporter genes green fluorescent protein (GFP) and lucia (InvivoGen). GFP expression can be detected via fluorescent microscopy, while lucia is a secreted luciferase that can be quantified with its substrate Quanti-Luc by measuring Relative Luciferase Units (RLUs). The following protocol uses PAAV-GFP plasmid or PAAV-lucia plasmids.

Plasmids were first transformed into TG1 competent E.coli (Zymo research) and plated on 2xYT (Sigma) agar with 50μg/ml ampicillin overnight in an incubator at 37°C. After colonies form, a single colony is selected and grown in 2xYT media with ampicillin in a shaking incubator (32°C, 220 rpm). 100mL of the bacteria is grown to log phase (OD600=0.45-0.50), following which 15-20μL of the RGD pVIII helper phage is added and incubated in a shaking incubator (32°C, 150 rpm). The bacteria are then grown in 1L of 2xYT media containing kanamycin and ampicillin in a shaking incubator (32°C, 150 rpm) for 18 hours. Purification was carried out as per previous investigation. Titration was carried out by growing bacteria on 2xYT with ampicillin only and on another plate with kanamycin only, in order to estimate titre of reporter gene vector and helper phage respectively.

Production, purification and titration of H5W peptide RGD pIII vectors containing reporter gene (lucia)

H5W RGD pIII helper phage was produced by a colleague (Sajee Waramit) prior to the start of this project. Production was largely similar to previous stated protocol, with the exception that 1mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was also added to select for H5W RGD pIII vectors. Purification and titration of vectors were carried out as per previous investigation.

Transduction of vector into cells

Cells used in transduction (HEK293T or DIPG) were seeded in plates until 60-70% confluent. The media is then removed and cells were incubated overnight with vectors diluted in OptiMEM, the amount of which is derived based on the virus titre obtained

from titration. The transduction media is then removed and replaced with complete media. For cells transduced with GFP, at indicated time points the cell is viewed under fluorescence and images taken to look for cells expressing GFP. For cells transduced with lucia, at indicated time points 10µl of cell media and 25µl of Quanti-Luc (InvivoGen), the substrate for the lucia, were mixed in a white polystyrene 96-well plate then incubated for 5 minutes at RT. Luciferase expression was quantified using a Promega Glomax microplate reader.

Assess and investigate the killing potential of therapeutic gene TRAIL against DIPG

Production of PAAV-human TRAIL (hTRAIL) plasmid for transfection (Figure 46)
The GFP sequence in a PAAV-GFP plasmid was replaced by the hTRAIL gene sequence by PCR. hTRAIL gene was amplified and designed to contain restriction sites for EcoRI and SalI at the ends by cloning pUNO1-hTRAIL (InvivoGen) using specific primers (Table 1). Molecular cloning, plasmid extraction and DNA sequence validation were carried out as per previous investigation. The selected clone was then transformed into TG1 bacteria for amplification, then extracted via Maxiprep (QIAGEN).

Table 1: Primer sequences (Invitrogen) used to produce hTRAIL fragment with restrictions sites (EcoRI/SalI) using pUNO1-hTRAIL and PCR.

Forward primer 5' – 3'
GAG TGA ATT CGC TGT GAC CGG CGC CTA C – SEQ ID NO: 24
Reverse primer 5' – 3'
GCT CGT CGA CTC ATG TCT GGC CAG CTA GCT TAG CC – SEQ ID NO: 25

Transfection of plasmids into cells

DIPG cells were seeded in 48 well-plates until 60-70% confluent. Prior to start of experiment, cells were incubated in OptiMEM low serum media for 1-2 hours. For 48-well plates, the FuGENE HD transfection protocol (Promega) the protocol advises to aim for a media volume of 0.5-2.0ml, FuGENE volume of 0.6-1.8µl and DNA amount of 0.2-0.6µg. Transfection efficiency depends on the ration of FuGENE to DNA, in this case a ratio of 3:1 is used.

Eppendorf tubes were prepared and labelled for use in the experiment. OptiMEM was added to each tube up to 20µl, depending on the volume of FuGENE to be used. FuGENE is then directly added into the medium without contact with the walls of the

plastic tube, and the mixture is incubated for 5 minutes at RT. Plasmid DNA is added to the tubes and the transfection reagent:DNA complex is incubated for 15 minutes at RT. The complex is then added to DIPG cells in a drop-wise manner and the plate swirled to ensure even distribution. Cells were incubated at 37°C for 6 hours, following which the transfection media is changed TSM media supplemented with FBS without antibiotic. Cells are replaced in the incubator and observed for cell viability in the next 24-48 hours.

Results

DIPG cells express integrin receptors for RGD-4C ligand binding

To establish the suitability of this vector model in this cell line for targeted gene delivery, cells were first investigated for expression of the integrins αvβ3 and αvβ5, by means of fluorescence microscopy. As shown in Figure 47, DIPG cells tested were positive for expression for αv, β3 and β5 units, with no fluorescence observed in cells incubated without antibodies or secondary antibody alone.

RGD-4C ligand as displayed on pIII coat protein produced higher transduction efficiency and gene expression levels than pVIII coat protein

Previous data from the inventor's lab has shown that targeted RGD-4C pIII vector mediated gene delivery and expression was selective and efficient as compared to un-targeted vector. In order to optimise transduction, one of the hypotheses was to display the RGD-4C on the pVIII coat protein in order to increase the number of copies of ligand per phage (Figure 48).

Using reporter gene vectors (GFP or lucia), transduction efficiency was compared using human kidney embryonic cells, HEK293T, a cellular model that was continuously used to characterise phage vectors. Cells were incubated with un-targeted vector (control lacking RGD-4C), targeted RGD pIII PAAV-GFP or RGD pVIII PAAV-GFP vectors and viewed under fluorescent microscopy (Figure 49). Across all TUs, the highest GFP expression was seen in cells transduced with RGD pIII PAAV-GFP.

Similarly, in HEK293T cells transduced with un-targeted, RGD pIII PAAV-lucia or RGD pVIII PAAV-lucia vectors, the highest RLU is observed in RGD pIII PAAV-lucia (Figure 50). This suggested that RGD displayed on pIII was superior to pVIII in transduction and gene expression.

H5W peptide as displayed on recombinant pVIII coat protein improves transduction efficiency and gene expression

An alternative strategy to optimise the vector was to display H5W peptide on recombinant pVIII coat protein. Previous data from the lab showed that in a transduction experiment using vectors containing reporter gene lucia (Sajee Waramit, unpublished data), display of H5W peptide increases luciferase expression in HEK293T cells as compared to RGD pIII alone (Figure 51).

To investigate if this finding could be applied to DIPG cells, DIPG cells were incubated with un-targeted, RGD pIII PAAV-lucia or H5W RGD pIII PAAV-lucia vectors. Consistent with that of HEK293T data, the highest RLU in DIPG cells is observed in H5W RGD pIII PAAV-lucia (Figure 52). This suggested that H5W peptide was able to improve vector efficiency in DIPG cells and should be used in the final vector construct.

Transfection of PAAV-hTRAIL plasmid into DIPG cells induced cell death

Following optimisation of vector and before further experiments were conducted, it was essential to determine if the selected gene of interest, human TRAIL (hTRAIL), was able to induce cell death in DIPG cells via transfection.

DIPG cells were transfected with either PAAV-hTRAIL or control PAAV-GFP plasmid. There was significantly more cell death in cells transfected with PAAV-hTRAIL according to microscopic images taken 18 hours post-transfection at all DNA concentrations (Figure 53).

Discussion

Without wishing to be being bound to any given theory, the data thus far show that vectors expressing H5W and RGD-4C on pIII coat proteins were most optimal in targeting and inducing expression of desired genes in DIPG cells. Data from transfection experiments also suggests that the therapeutic gene TRAIL is able to induce DIPG cell death, which warrants further investigation.

*Example 12 – hybrid IL2-TNF*a

To increase targeted loco-regional production of TNFa within the tumour site, the inventors constructed a phagemid encoding a secreted TNFa by inserting a signal peptide from IL-2 to precede the TNFa sequence lacking the transmembrane domain. To the inventor's knowledge this is the first time that a hybrid IL2-TNFa was designed

and their data suggest that preceding TNFa gene with the IL2 signal peptide sequence significantly enhanced expression and secretion of TNFa in cancer cells. Such modifications represent a significant advance in the technique available for targeted production and release of TNFa in the tumour microenvironment and should be considered for increasing the therapeutic levels of TNFa.

Protocol

PAAV.Grp78.IL-2SP.hTNFa construction

The coding therapeutic sequence inserted in the phagemid is a hybrid sequence that contained a tumour specific promoter of the Glucose Regulated protein (Grp78), signal peptide (SP) sequence from IL-2 (Figure 57) and human sequence of TNFa. The *Grp78* promoter is stress-inducible and is strongly activated by conditions of glucose deprivation, chronic anoxia, and acidic pH that persist within aggressive and poorly perfused tumours. Moreover, the *Grp78* promoter is induced in a wide variety of tumours and thus makes it an attractive candidate for use in gene therapy. Previous studies have demonstrated several advantages of this promoter over viral promoters. The safety and tumour specificity of this promoter have also been elegantly reported in transgenic mice carrying a *LacZ* transgene. High *LacZ* expression was shown in tumours established in these transgenic mice, while no promoter activity was detected in major normal tissues. Furthermore, unlike viral promoters used in gene therapy vectors, mammalian promoters such as *Grp78* are not silenced in eukaryotic cells. In the inventor's previously published work, they reported that the double-targeted RGD4C/phage-*Grp78* provides persistent transgene expression over RGD4C/Phage-*CMV* carrying the cytomegalovirus *CMV* promoter. Inclusion of both RGD4C ligand and Grp78 promoter generates a vector with dual tumour targeting at both cell entry and transcriptional levels.

The TNFα is transmembrane, to generate a secreted TNFα with better availability for its receptor on the cell surface of tumour cells, we generated a secreted form of TNFα, by removing the transmembrane domain of TNFα and replacing it with the signal peptide of interleukin-2 (IL-2). The IL-2 was previously used as an effective signal peptide tested in various studies to enhance the secretion of other cytokines or growth factors into the extracellular milieu and subsequently increased their efficacy and availability for their corresponding receptors. IL-2 signal peptide sequence flanked by *BamHI* and *EcoRI* restriction sites (Thermoscientific, UK) was ligated to PAAV.Grp78

backbone. hTNFα_ sequence flanked by *EcoRI* and *SalI* restriction sites was provided from pUNO1-hTNFα_plasmid (Invivogen, France) by polymerase chain reaction (PCR), then ligated to PAAV.Grp78.IL-2 backbone. Molecular cloning steps were conducted using restriction enzyme digestion (NEB, UK) and quick T4 DNA ligase for ligation (NEB, UK). The modified plasmid was transformed into TG1 competent *E. coli* (Zymo research, USA). The bacteria carrying the plasmid were then selected by ampicillin selection on 2xYT agar plate. The construct was validated by restriction digestion and gel electrophoresis, and DNA sequencing (MRC CSC Genomics Core Laboratory, UK) see Figure 58.

Example 13 - Application of PAAV–delivered TNFα cytokine gene therapy in DIPG

Introduction

Diffuse intrinsic pontine glioma (DIPG) is the most aggressive brain tumour that arise exclusively in children with poor survival of only 6-10% beyond two years. Due to its diffuse nature and its sensitive location in the brainstem, surgical removal is not feasible and there is no effective therapeutic strategy for this type of cancer. The current standard treatment for DIPG is radiotherapy, which is not showing any success, as all children relapse afterwards, even in combination with radio-sensitizers. Many clinical trials have tested the effect of chemotherapy in combination with the conventional radiotherapy, yet no improvement on increasing the overall survival even when combining high doses of chemotherapeutic drugs. This resistance to chemotherapy is due to the intact blood brain barrier as well as the anatomical location of DIPG in the pons makes it more difficult for any drug to reach the targeted location. To overcome this issue, a clinical trial has applied the use of convection-enhanced delivery (CED) to deliver the chemodrug, topotecan, for the treatment of DIPG in 2 children. In this pilot study, both patients died after treatment although initially there was a reduction in tumour size. CED is a technique that directs the delivery of chemotherapeutic drugs to the brain tumours through sustained flow. This technique is being used lately by clinical trials to overcome the drug delivery challenges by bypassing the blood brain barrier.

Targeted therapy for DIPG seems to be the best solution due to its diffuse nature and sensitive location. In an attempt to target DIPG tumour, an ongoing clinical trial applying the concept of targeted radiation through labelling a radioactive substance,124I, with 8H9 antibody, which is known to bind selectively to cancer cells sparing the healthy brain cells (clinicaltrials.gov ID NCT01502917) [8]. Another

candidate to target brain tumours, as suggested by our group (Hajitou's lab) and showed high selectivity in glioblastoma, are the integrins $\alpha v\beta 3$ and $\alpha v\beta 5$ through the binding of RGD4C peptide as discussed in chapter 3. In fact, RGD4C targeting DIPG is currently being used in an ongoing phase I clinical trial (clinicaltrials.gov ID NCT03178032). In this trial an oncolytic adenovirus, DNX2401, is used to target tumour cells and induce cell death. Among thirty ongoing clinical trials for DIPG, the majority are focused on testing different drugs and radiotherapy, other treatments including gene therapy has not yet been applied. TNFα is an inflammatory cytokine that is known for its anticancer properties by mediating apoptosis, necrosis, and immune cell activation. Depending on the cell context, it can also induce cell proliferation and angiogenesis. Its anti-tumour activity has been studied in many solid tumours including colon cancer, oesophageal adenocarcinoma, melanoma, pancreatic cancer and many others. Yet, the efficiency as a therapeutic agent was very limited due to its significant toxicities when introduced systemically. This limited the clinical use of TNFα in the clinic as a therapeutic agent for cancer to be used for soft tissue sarcoma, melanoma and other irresectable tumours confined to the limb in the form of isolated limb perfusion (ILP) to avoid limb amputation. Indeed, TNFα works synergistically with chemotherapeutic drugs in vivo through targeting tumour vasculature disrupting the VE-cadherins and thus increase the penetration of the chemotherapeutic drugs to tumour environment. Thus, efforts to overcome the limitation of systemic toxicity are required to enhance therapeutic efficacy of TNFα. One strategy was made to decrease TNFα toxicity and yet retain its anti-tumour activity by making a mutant form of TNFα. This mutated TNFα was further targeted to the tumour environment by the signalling peptide RGD4C and enhanced the efficiency of the chemotherapeutic drugs in hepatoma and sarcoma allografts.

In vitro studies for DIPG are very limited with little human tissue available for study, restricting the understanding of this devastating type of brain cancer. Therapeutic applications, other than chemotherapeutic drugs and radiotherapy, are also hindered due to the lack of in vitro applications and understanding. Thus, in the inventors investigated the therapeutic effect of two forms of TNFα, transmembrane (tmTNFα) and secreted(sTNFα), against DIPG. TNFα was used in the form of gene therapy to be expressed and delivered to the cells using PAAV targeted by the signalling peptide RGD4C. Without wishing to be bound to any particular theory, the use of RGD4C-targeted PAAV as a therapeutic gene delivery vehicle to the tumour site will ensure safer, selective and efficient gene transfer for the treatment of DIPG.

In order to understand the mechanism of cell death induced by TNFα, the inventors studied the viability and apoptotic activity in response to transduction of PAAV carrying two different forms of TNFα, tmTNFα and sTNFα. The apoptotic activity was determined by measuring the activity of caspase3/7, caspase 8, and caspase 9.

Finally, the effect of chemotherapeutic drug, cisplatin, in enhancing gene therapy was studied. Thus, two different promoters, GRP78 and CMV, upstream of TNFα were used to understand the synergestic effect of gene therapy with cisplatin. Lucia reporter gene under the control of GRP78 and CMV promoters was used for quantitative analysis for gene expression after cisplatin treatment and further understand the role of cisplatin in enhancing gene expression.

Results

Transduction of DIPG with PAAV carrying the transmembrane tmTNFα and secreted sTNFα transgene:

Transmembrane TNFα gene and secreted TNFα were cloned into PAAV vector, PAAV-tmTNFα and PAAV-sTNFα (comprising the vector as shown in example 12), followed by the production of targeted (RGD4C) and non-targeted (M13/control) viruses. DIPG cells were transduced and cell viability was measured at day 7 post-transduction. The transduction efficiency was further enhanced with 40ng/μg of phage protein DEAE dextran. DIPG cells showed better response to PAAV-sTNFα in inducing cell death compared to PAAV-tmTNFα, where the former shows about 50% cell death on day 7 compared to only 20% cell death that was induced by the transmembrane form as shown in figure 59. Together, these data suggest that sTNFα is a good candidate for the treatment of DIPG.

Gene delivery and TNFα expression after transduction with PAAV:

The efficiency of PAAV gene delivery was assessed by measuring mRNA levels after transduction. Both forms of TNFα were expressed in the transduced cells with high specificity as the non-targeted (M13) form shows negligible mRNA expression of TNFα compared to the targeted PAAV (RGD4C) as shown in figure 60A. At the protein level, Transduction of PAAV- tmTNFα did not lead to the secretion of TNFα in the medium as was detected by ELISA (figure 60B). Although transduction with both forms shows TNFα expression at the mRNA level, only the secreted form was related to the level of TNFα protein. This further confirms that the transduction of the transgene was

efficient and may suggest that the inefficiency of inducing cell death by the transmembrane form is due to the lack of soluble secreted protein. As the release of sTNFα from the initial membrane bound form requires the enzymatic activity or expression of TNFα converting enzyme (TACE). Thus, TACE enzyme expression was measured by western blot and compared with other paediatric brain tumour cells (medulloblastoma). DIPG expression of TACE enzyme was 4X lower than the expression level of UW228 and 2X lower than the expression level of Daoy cells.

CLAIMS

1. A recombinant phagemid particle for expressing a transgene in a target tumour cell transduced with the particle, for use in a method for treating, preventing, or ameliorating cancer, wherein the phagemid particle comprises at least one transgene expression cassette comprising a nucleic acid sequence encoding one or more cytokine, and comprises a genome which lacks at least 50% of its bacteriophage genome, and wherein the method comprises delivering the nucleic acid sequence to at least adjacent to the tumour cell, such that one or more cytokine is expressed.

2. The recombinant phagemid particle for use according to claim 1, wherein the transgene expression cassette encodes a cytokine which has the effect of apoptosis induction in the tumour cell, alternation of the tumour cell to promote endogenous anti-tumour responses, alternation of the tumour cell to facilitate other therapies, or alternation of the tumour microenvironment to facilitate therapy.

3. The recombinant phagemid particle for use according to either claim 1 or 2, wherein the cytokine is IL-4, IL-12, IL-15, TNFα, TRAIL, IFN-γ, or any combination thereof, optionally wherein the cytokine is IL-15.

4. The recombinant phagemid particle for use according to any preceding claim, wherein the cytokine is a hybrid cytokine comprising a non-endogenous signal peptide configured to increase expression and/or secretion of the cytokine.

5. The recombinant phagemid particle for use according to claim 4, wherein the non-endogenous signal peptide is an IL-2 signal peptide.

6. The recombinant phagemid particle for use according to claim 5, wherein the hybrid cytokine is a hybrid TNFα comprising a IL-2 signal peptide configured to increase expression and/or secretion of TNFα.

7. The recombinant phagemid particle for use according to claim 6, wherein the hybrid TNFα comprises an amino acid sequence substantially as set out in SEQ ID No: 22 or a fragment or variant thereof.

8. The recombinant phagemid particle for use according to either claim 6 or 7, wherein the hybrid TNFα is encoded by a nucleic acid sequence comprising SEQ ID No: 23 or a fragment or variant thereof.

9. The recombinant phagemid particle for use according to any preceding claim, for use in treating, preventing or ameliorating paediatric brain tumours, optionally diffuse intrinsic pontine glioma (DIPG) or medulloblastoma.

10. A system for producing a recombinant phagemid particle from a prokaryotic host, the system comprising:-

(i) a first vector configured to persist inside a prokaryotic host, and comprising at least one transgene expression cassette comprising a nucleic acid sequence encoding one or more cytokine, and a packaging signal for enabling replication of the vector into single-stranded DNA; and

(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.

11. A method for producing a recombinant phagemid particle from a prokaryotic host, the method comprising:-

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 comprising a nucleic acid sequence encoding one or more cytokine, and a packaging signal for enabling replication of the vector into single-stranded DNA;

ii) introducing, into the host, a helper phage comprising nucleic acid encoding bacteriophage structural proteins; and

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.

12. A method for producing a recombinant phagemid particle from a prokaryotic host, the method comprising:-

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 comprising a nucleic acid sequence encoding one or more cytokine, 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

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.

13. A pharmaceutical composition comprising the recombinant phagemid viral particle produced by the system according to claim 10, or produced by the method of clam 11 or 12, and a pharmaceutically acceptable vehicle.

14. A process for making the pharmaceutical composition according to claim 13, the process comprising contacting a therapeutically effective amount of the recombinant phagemid particle produced by the system according to claim 10, or produced by the method of claim 11 or 12, and a pharmaceutically acceptable vehicle.

15. A recombinant phagemid particle for expressing a transgene in a target tumour cell transduced with the particle, wherein the phagemid particle comprises at least one transgene expression cassette comprising a nucleic acid sequence encoding one or more cytokine, and comprises a genome which lacks at least 50% of its bacteriophage genome, and wherein, in use, the particle is configured to deliver the nucleic acid sequence to at least adjacent to the tumour cell, such that one or more cytokine is expressed, and wherein the cytokine is any one of IL-4, IL-12, IL-15, TRAIL, IFN-γ, hybrid TNFα or any combination thereof, optionally wherein the cytokine is IL-15.

16. The recombinant phagemid particle of claim 15, for use in therapy or diagnosis.

17. A recombinant phagemid particle for expressing at least one antigen in a target tumour cell transduced with the particle, the phagemid particle comprising at least one transgene expression cassette comprising a nucleic acid sequence

encoding one or more cytokine, and comprising a genome which lacks at least 50% of its bacteriophage genome, and wherein, in use, the particle is configured to deliver the nucleic acid sequence to at least adjacent to the target tumour cell, such that the one or more cytokine is expressed.

18. The recombinant phagemid particle according to claim 17, for use in therapy or diagnosis.

19. The recombinant phagemid particle according to claim 17, for use in treating, preventing or ameliorating cancer.

20. Use of a helper phage comprising nucleic acid encoding viral vector structural proteins to produce the recombinant phagemid particle according to claim 17 from a prokaryotic host.

21. A recombinant phagemid viral particle according to claim 17, produced by the system of claim 10, produced by the methods of claim 11 or 12, or produced according to the use of claim 20, wherein the recombinant phagemid particle is for production of a recombinant viral vector comprising or derived from the viral genome within the genome of the phagemid particle, wherein the recombinant viral vector is used for delivering the nucleic acid sequence encoding one or more antigen, to at least adjacent to the tumour cell, such that one or more cytokine is expressed.

22. A recombinant vector comprising rAAV, rep-cap, adenohelper genes, and a nucleic acid sequence encoding one or more antigen or cytokine, for use in the treatment, prevention, or amelioration of cancer.

23. A recombinant phagemid particle comprising the vector of claim 22, for use in a method for the treatment, prevention, or amelioration of cancer.

24. The recombinant phagemid particle according to claim 15 or 17, or produced by the system of claim 10, or produced by the methods of claim 11 or 12, or produced according to the use of claim 20 or 21, or for use according to any of claims 1-9 and 22-23, or according to the pharmaceutical composition of claim 13, or according to the process for making the pharmaceutical composition of

claim 14, wherein the phagemid particle is configured to deliver the transgene expression cassette to the target tumour cell.

25. A particle according to claims 15, 17 or 24, or produced by the system of claim 10, produced by the methods of claim 11 or 12, or produced according to the use of claim 20 or 21, or for use according to any of claims 1-9 and 22-23, or according to the pharmaceutical composition of claim 13, or according to the process for making the pharmaceutical composition of claim 14,wherein 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, optionally wherein the packaging signal comprises an origin of replication, optionally an F1 ori.

26. A particle according to claims 15, 17, 24 or 25, or produced by the system of claim 10, or produced by the methods of claim 11 or 12, or produced according to the use of claim 20 or 21, or for use according to any of claims 1-9 and 22-23, or according to the pharmaceutical composition of claim 13, or according to the process for making the pharmaceutical composition of claim 14,wherein the genome of the recombinant phagemid particle comprises an origin of replication for enabling replication of double-stranded vector inside a prokaryotic host, optionally a pUC ori.

27. A particle according to any one of claims 15, 17, 24-26, or produced by the system of claim 10, or produced by the methods of claim 11 or 12, or produced according to the use of claim 20 or 21, or for use according to any of claims 1-9 and 22-23, or according to the pharmaceutical composition of claim 13, or according to the process for making the pharmaceutical composition of claim 14, wherein the genome of the recombinant phagemid particle comprises one or more DNA sequence, which favours targeted integration into a host genome.

28. A particle according to any one of claims 15, 17, 24-27, or produced by the system of claim 10, or produced by the methods of claim 11 or 12, or produced according to the use of claim 20 or 21, or for use according to any of claims 1-9 and 22-23, or according to the pharmaceutical composition of claim 13, or according to the process for making the pharmaceutical composition of claim

14, wherein the at least one transgene expression cassette comprises a viral transgene expression cassette.

29. A particle according to any one of claims 15, 17, 24-28, or produced by the system of claim 10, or produced by the methods of claim 11 or 12, or produced according to the use of claim 20, or for use according to any of claims 1-9 and 22-23, or according to the pharmaceutical composition of claim 13, or according to the process for making the pharmaceutical composition of claim 14, comprising multiple transgene expression cassettes.

30. A particle according to any one of claims 15, 17, 24-29,or produced by the system of claim 10, produced by the methods of claim 11 or 12, or produced according to the use of claim 20 or 21, or for use according to any of claims 1-9 and 22-23, or according to the pharmaceutical composition of claim 13, or according to the process for making the pharmaceutical composition of claim 14, wherein the at least one transgene expression cassette comprises a mammalian viral transgene expression cassette.

31. A particle according to any one of claims 15, 17, 24-30, or produced by the system of claim 10, or produced by the methods of claim 11 or 12, or produced according to the use of claim 20 or 21, or for use according to any of claims 1-9 and 22-23, or according to the pharmaceutical composition of claim 13, or according to the process for making the pharmaceutical composition of claim 14, wherein the transgene expression cassette comprises one or more functional elements required for expression of the nucleic acid in the target cell selected from the groups consisting of: a promoter, nucleic acid for encoding a polyA tail attachable to the expressed agent, and either left and/or right Inverted Terminal Repeat sequences (ITRs) or left and/or right Long Terminal repeat sequences (LTRs).

32. A particle according to any one of claims 15, 17, 24-31, or produced by the system of claim 10, or produced by the methods of claim 11 or 12, or produced according to the use of claim 20 or 21, or for use according to any of claims 1-9 and 22-23, or according to the pharmaceutical composition of claim 13, or according to the process for making the pharmaceutical composition of claim 14, wherein the at least one transgene expression cassette comprises a

lentivirus transgene expression cassette.

33. A particle according to any one of claims 15, 17, 24-32, or produced by the system of claim 10, or produced by the methods of claim 11 or 12, or produced according to the use of claim 20 or 21, or for use according to any of claims 1-9 and 22-23, or according to the pharmaceutical composition of claim 13, or according to the process for making the pharmaceutical composition of claim 14, wherein the at least one transgene expression cassette comprises an adeno-associated virus (AAV) transgene expression cassette.

34. A particle according to any one of claims 15, 17, 24-33, or produced by the system of claim 10, or produced by the methods of claim 11 or 12, or produced according to the use of claim 20 or 21, for use according to any of claims 1-9 and 22-23, or according to the pharmaceutical composition of claim 13, or according to the process for making the pharmaceutical composition of claim 14, wherein the transgene expression cassette further comprises a nucleic acid encoding an agent, which has therapeutic or industrial utility in the target cell or tissue, optionally wherein the agent encoded by the nucleic acid is polypeptide or protein.

35. A particle according to any one of claims 15, 17, 24-34, or produced by the system of claim 10, or produced by the methods of claim 11 or 12, or produced according to the use of claim 20 or 21, or for use according to any of claims 1-9 and 22-23, or according to the pharmaceutical composition of claim 13, or according to the process for making the pharmaceutical composition of claim 14, wherein the recombinant phagemid particle comprises one or more capsid minor coat protein, optionally wherein the recombinant phagemid particle comprises a pIII capsid minor coat protein that is configured to display a cell-targeting ligand for enabling delivery of the particle to the target tumour cell.

36. A particle according to any one of claims 15, 17, 24-35, or produced by the system of claim 10, or produced by the methods of claim 11 or 12, or produced according to the use of claim 20 or 21, or for use according to any of claims 1-9 and 22-23, or according to the pharmaceutical composition of claim 13, or according to the process for making the pharmaceutical composition of claim 14, wherein the recombinant phagemid particle comprises one or more capsid

major coat protein, optionally wherein the recombinant phagemid particle comprises at least one pVIII capsid major coat protein that is configured to display a foreign peptide thereon.

37. A particle according to any one of claims 15, 17, 24-36, or produced by the system of claim 10, or produced by the methods of claim 11 or 12, or produced according to the use of claim 20 or 21, or for use according to any of claims 1-9 and 22-23, or according to the pharmaceutical composition of claim 13, or according to the process for making the pharmaceutical composition of claim 14, wherein the recombinant phagemid particle is combined with a cationic polymer to form a complex having a net positive charge, optionally wherein the cationic polymer is selected from a group consisting of: chitosan; poly-D-lysine (PDL); diethylaminoethyl (DEAE); diethylaminoethyl-dextran (DEAE.DEX); polyethyleneimine (PEI); polybrene; protamine sulphate; 5 and a cationic lipid.

38. A particle according to any one of claims 15, 17, 24-37, or produced by the system of claim 10, or produced by the methods of claim 11 or 12, or produced according to the use of claim 20 or 21, or for use according to any of claims 1-9 and 22-23, or according to the pharmaceutical composition of claim 13, or according to the process for making the pharmaceutical composition of claim 14, wherein:

a. the genome of the recombinant phagemid particle lacks at least 60%, 70%, or at least 80% of the bacteriophage genome from which it is derived;
b. a particle according to any preceding claim, wherein the genome of the recombinant phagemid particle lacks at least 90%, 95%, or at least 99% of the bacteriophage genome from which it is derived; or
c. a particle according to any preceding claim, wherein the phagemid particle lacks bacteriophage structural genes in its genome required for the formation, packaging or extrusion of the particle from a prokaryotic host, preferably structural genes which encode the capsid proteins.

Figure 1

Comparing AAVP and PAAV

	Next-generation PAAV vectors
Relative genome size	c. 6000 bases (42% of AAVP)
Relative virus size	~50% shorter than AAVP
Production yield	up to 100,000X current AAVP yields
Payloads per particle	Multiple (AAVP can only carry 1 payload)
Biodistribution	*Potentially better*

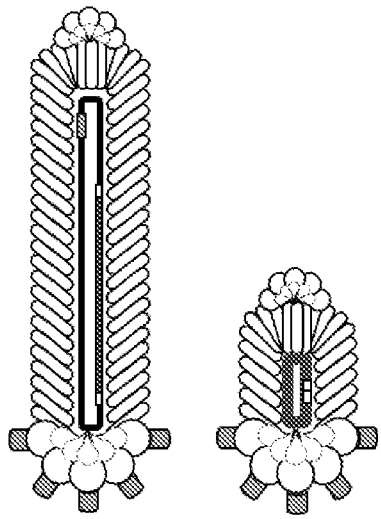
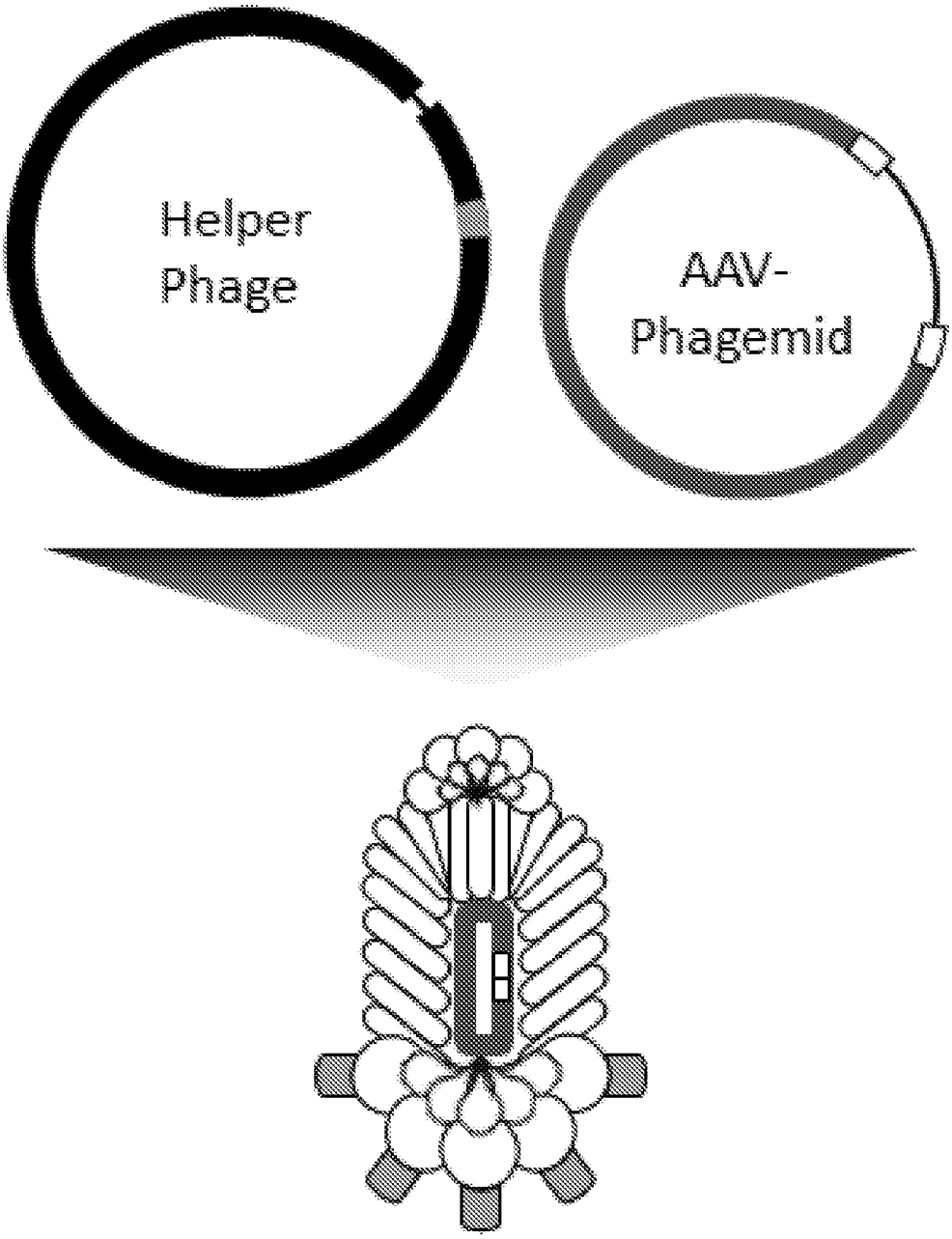


Figure 2



Phagemid/Adeno-associated Virion (PAAV)

Figure 3

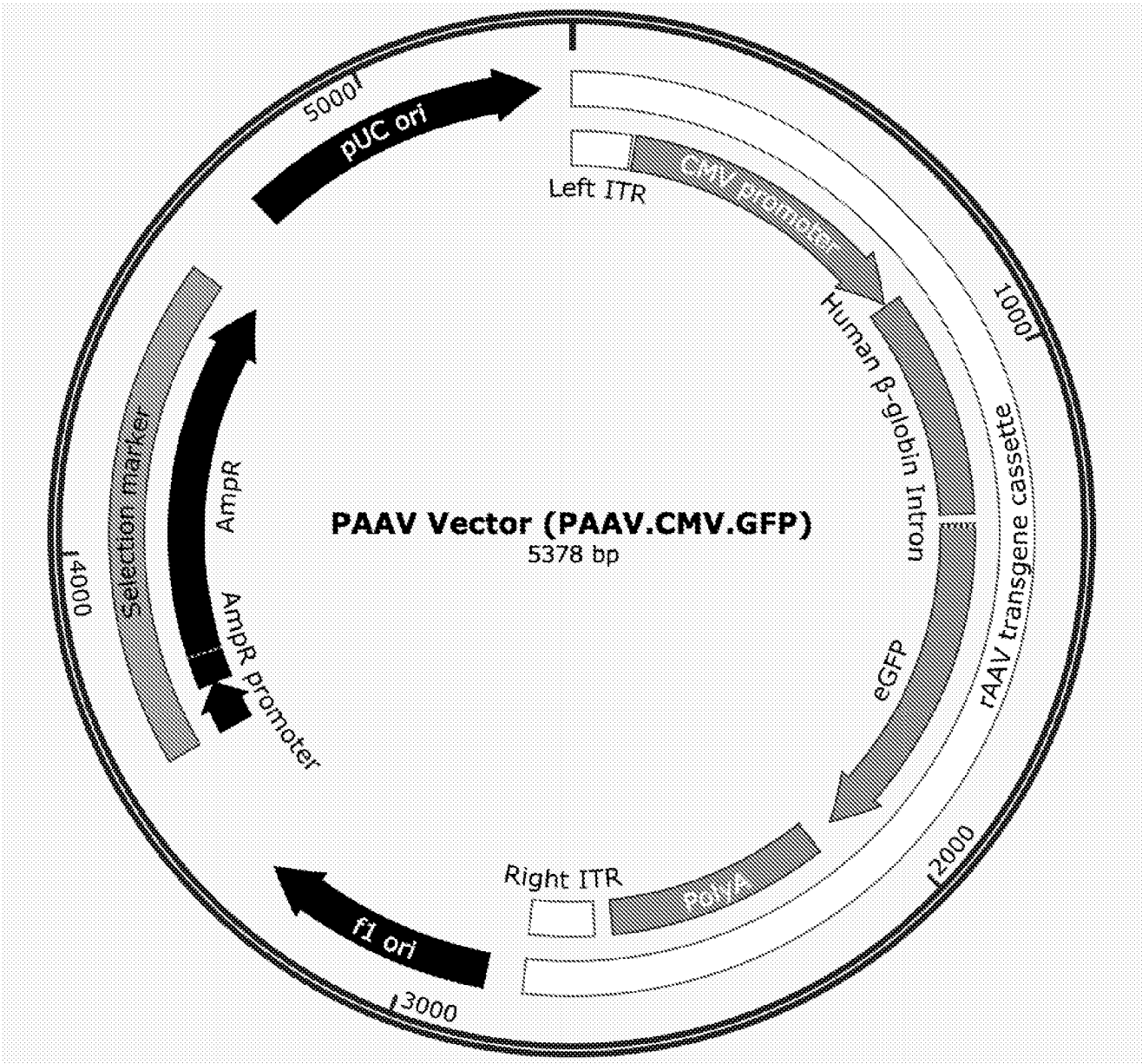

5000
pUC ori
Left ITR
CMV promoter
1000
Human β-globin Intron
rAAV transgene cassette
eGFP
2000
Right ITR
PolyA
3000
f1 ori
4000
Selection marker
AmpR
AmpR promoter
PAAV Vector (PAAV.CMV.GFP)
5378 bp

Figure 4

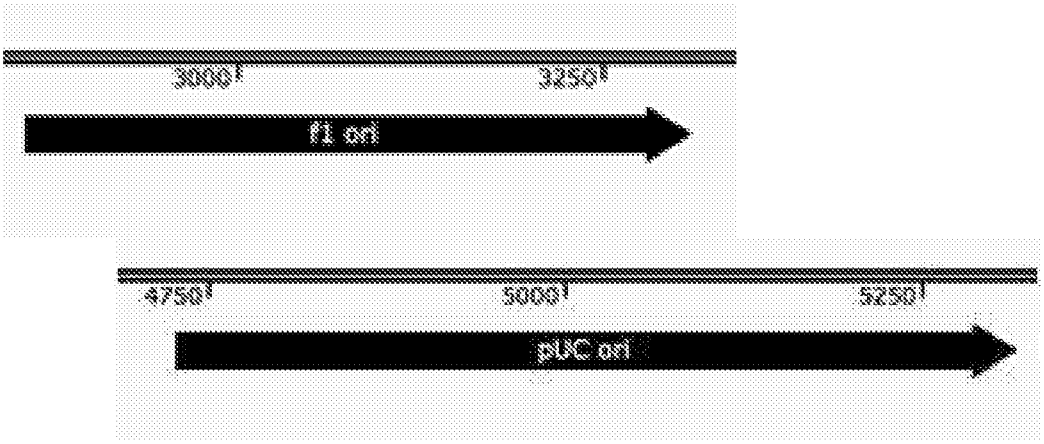


Figure 5

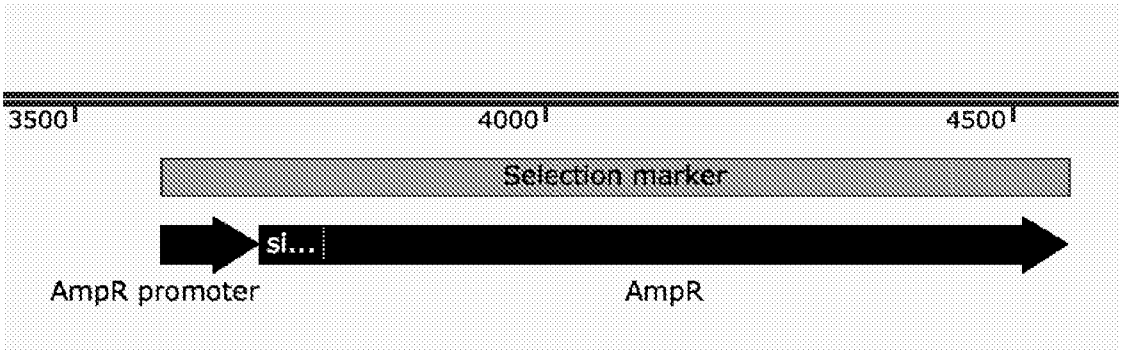


Figure 6

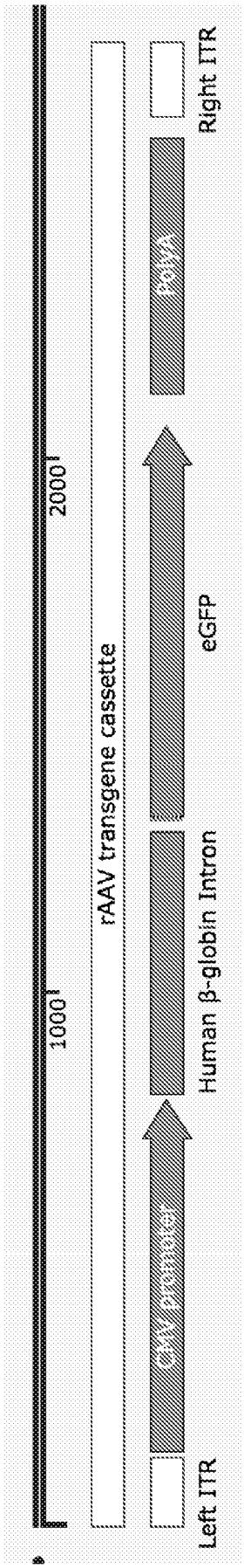

1000
2000
rAAV transgene cassette
Left ITR
CMV promoter
Human β-globin Intron
eGFP
polyA
Right ITR

Figure 7

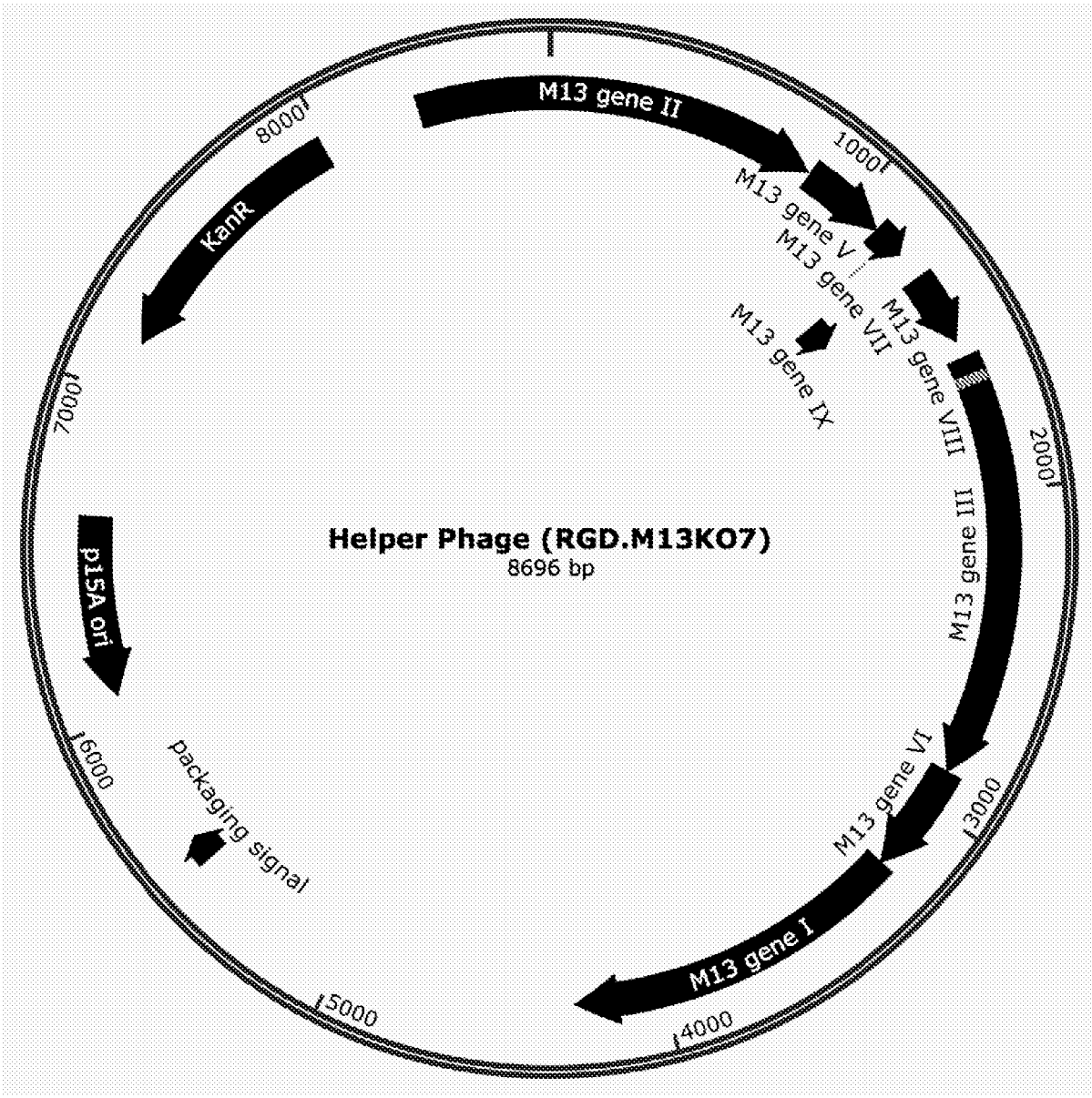

M13 gene II
1000
M13 gene V
M13 gene VII
M13 gene IX
M13 gene VIII
2000
M13 gene III
M13 gene VI
3000
M13 gene I
4000
5000
6000
p15A ori
packaging signal
7000
KanR
8000
Helper Phage (RGD.M13KO7)
8696 bp

Figure 8

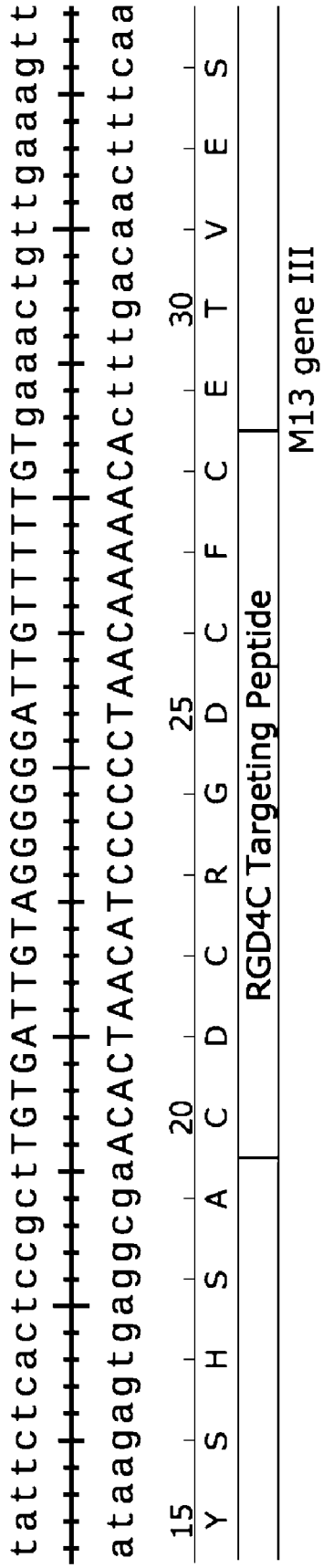

tattctcactccgcttGTGATTGTAGGGGGGATTGTTTTTTGTgaaactgttgaaagtt
ataagagtgaggcgaACACTAACATCCCCCCTAACAAAAACACtttgacaactttcaa
15
20
25
30
Y S H S A C D C R G D C F C E T V E S
RGD4C Targeting Peptide
M13 gene III

Figure 9

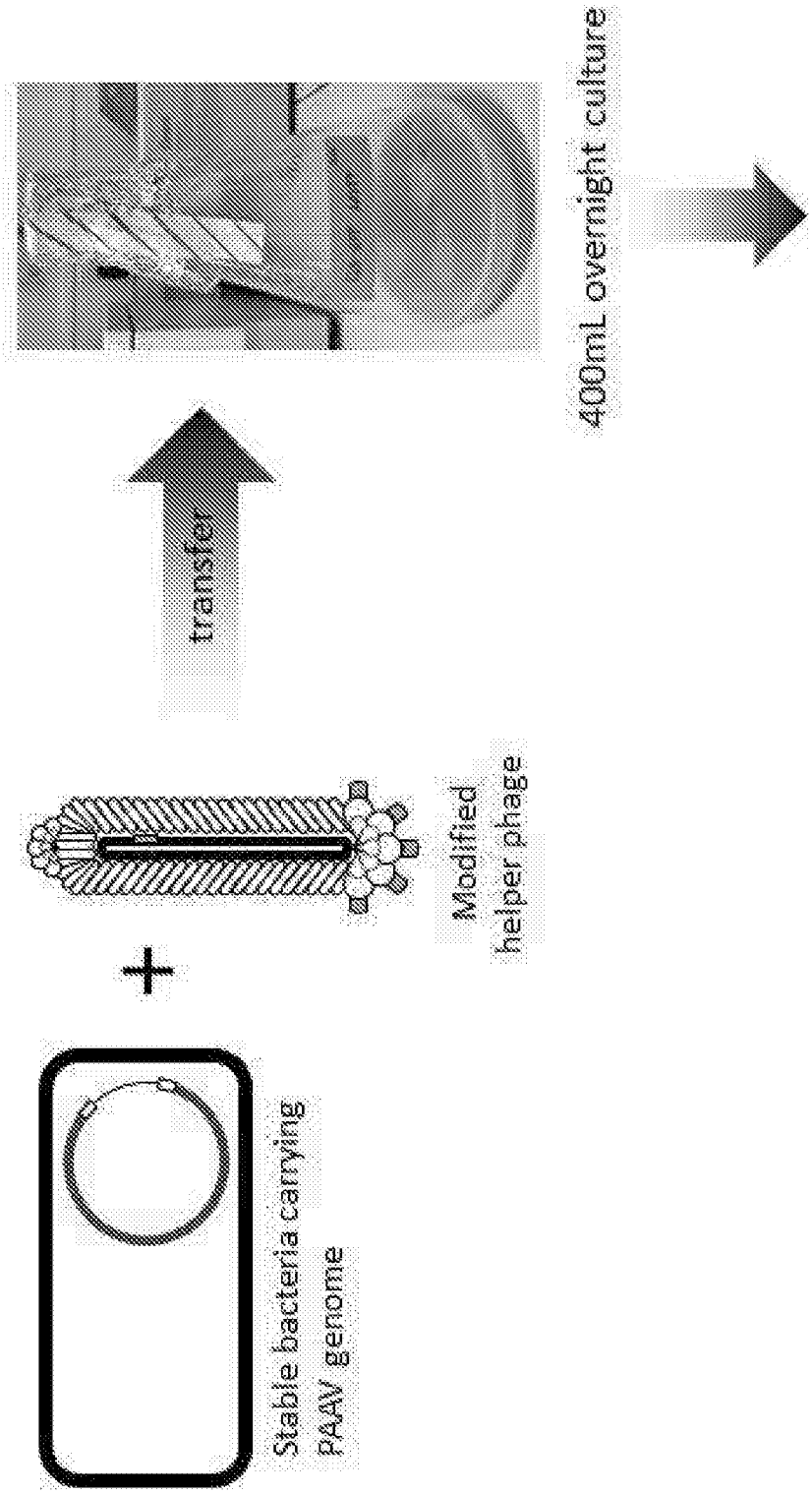

400mL overnight culture
transfer
Modified
helper phage
Stable bacteria carrying
PAAV genome

Figure 9 continued

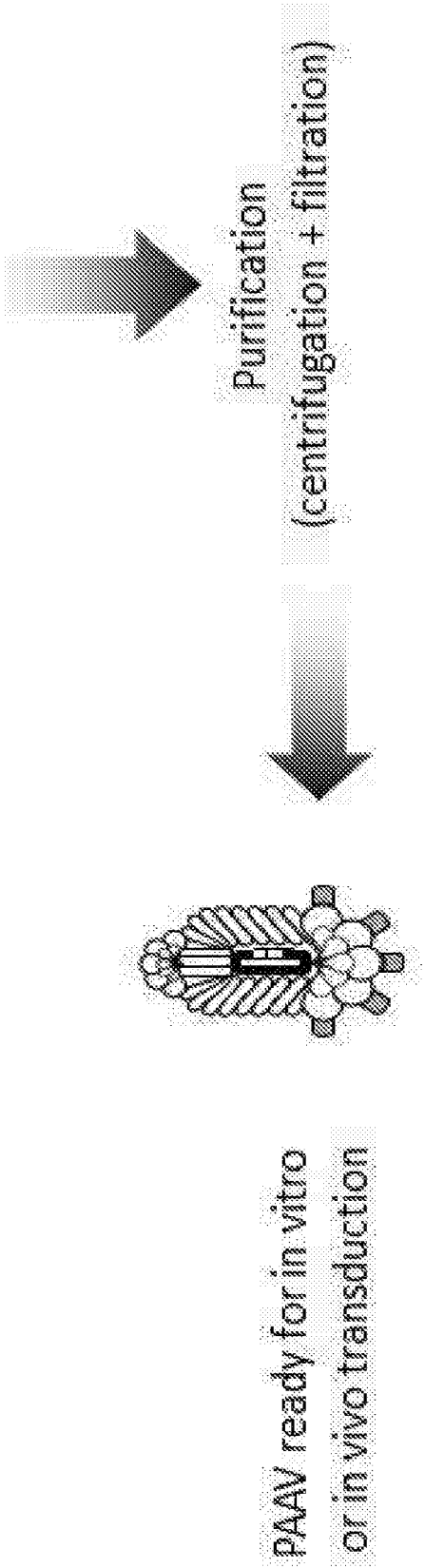


Figure 10

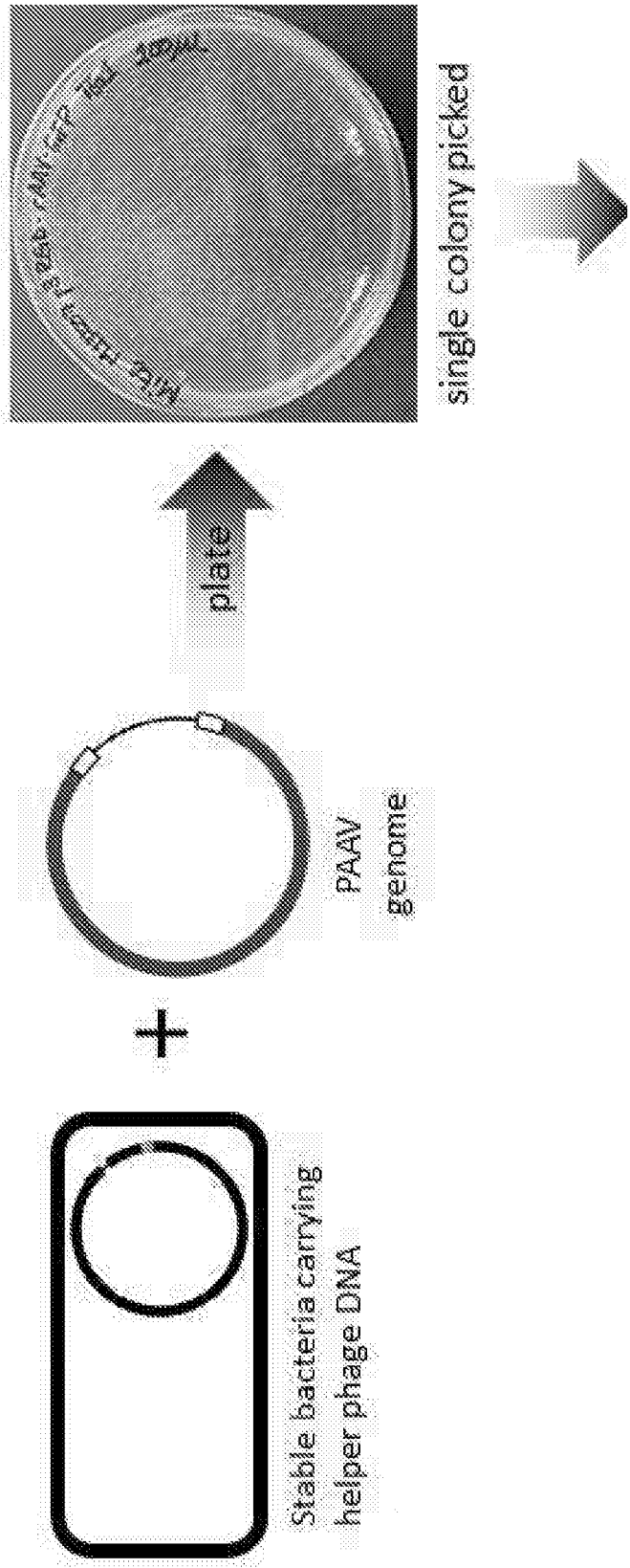

single colony picked
plate
PAAV genome
Stable bacteria carrying helper phage DNA

Figure 10 continued

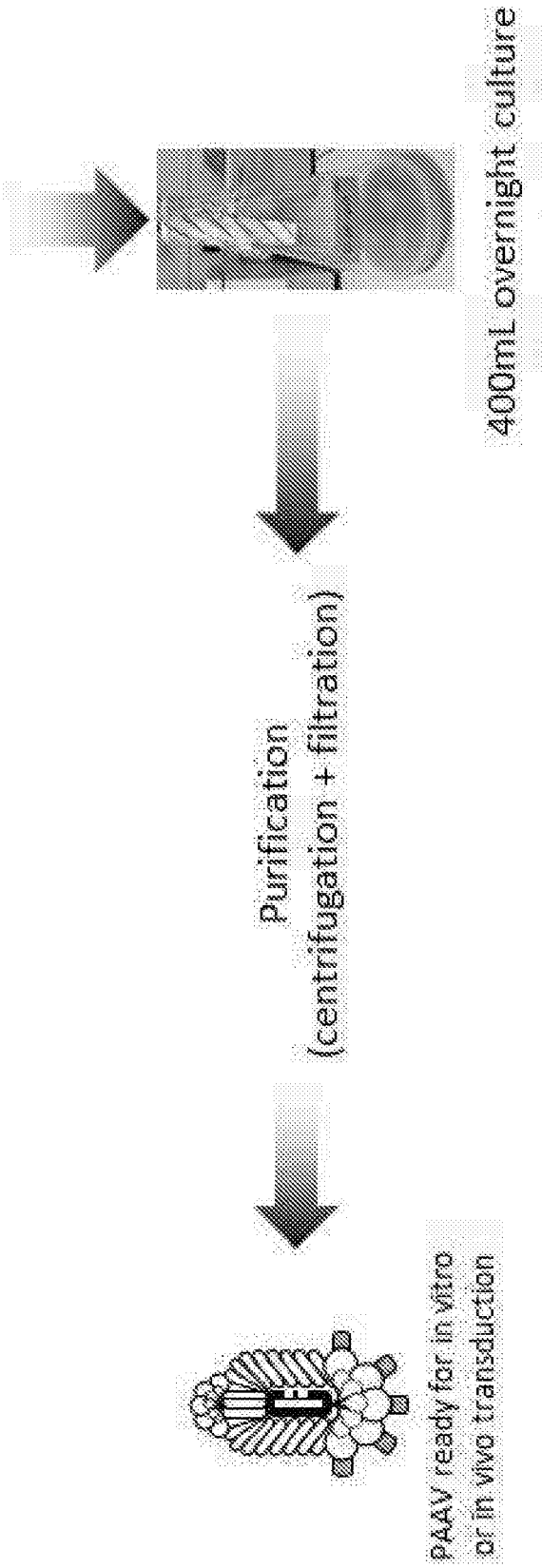


Figure 11

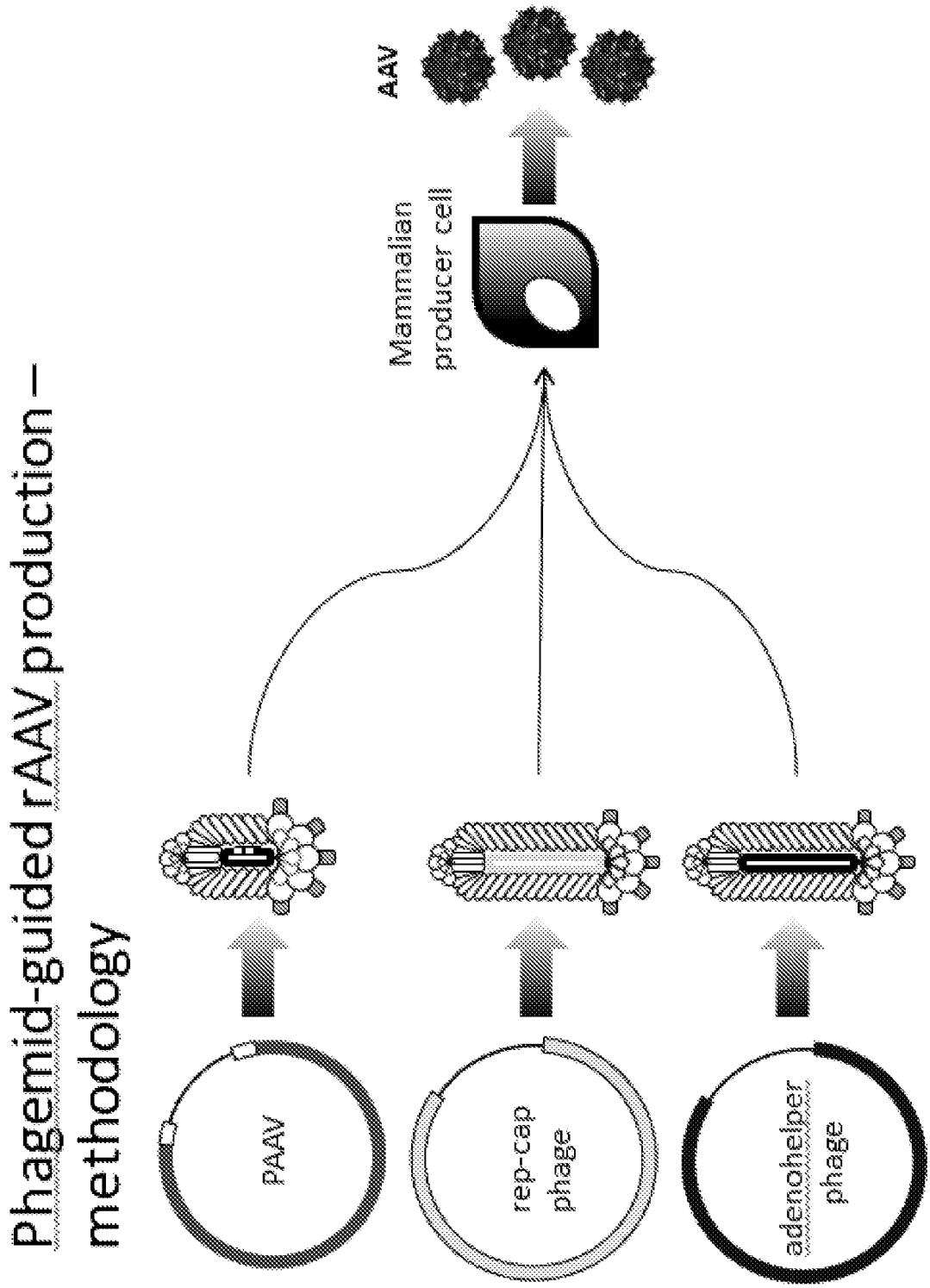

Phagemid-guided rAAV production – methodology
AAV
Mammalian producer cell
PAAV
rep-cap phage
adenohelper phage

Figure 12

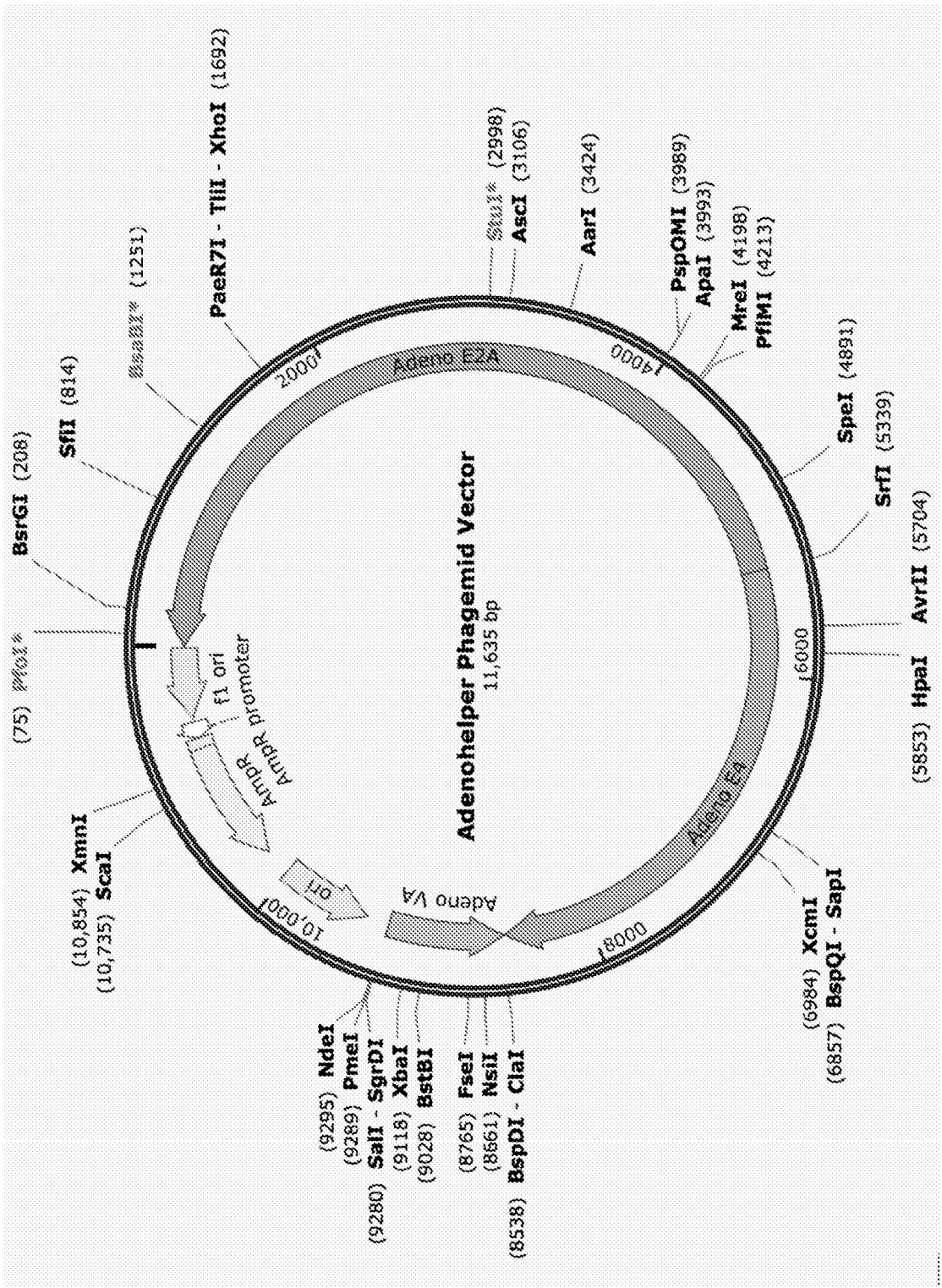

Adenohelper Phagemid Vector
11,635 bp
Adeno E2A
Adeno E4
Adeno VA
f1 ori
AmpR promoter
AmpR
ori
2000
4000
6000
8000
10,000
(75) PmlI
BsrGI (208)
SfiI (814)
(1251) SmaI
PaeR7I - TliI - XhoI (1692)
(2998) SmiI
AscI (3106)
AarI (3424)
PspOMI (3989)
ApaI (3993)
MreI (4198)
PflMI (4213)
SpeI (4891)
SrfI (5339)
AvrII (5704)
(5853) HpaI
(6857) BspQI - SapI
(6984) XcmI
(8538) BspDI - ClaI
(8661) NsiI
(8765) FseI
(9028) BstBI
(9118) XbaI
(9280) SalI - SgrDI
(9289) PmeI
(9295) NdeI
(10,735) ScaI
(10,854) XmnI

Figure 13

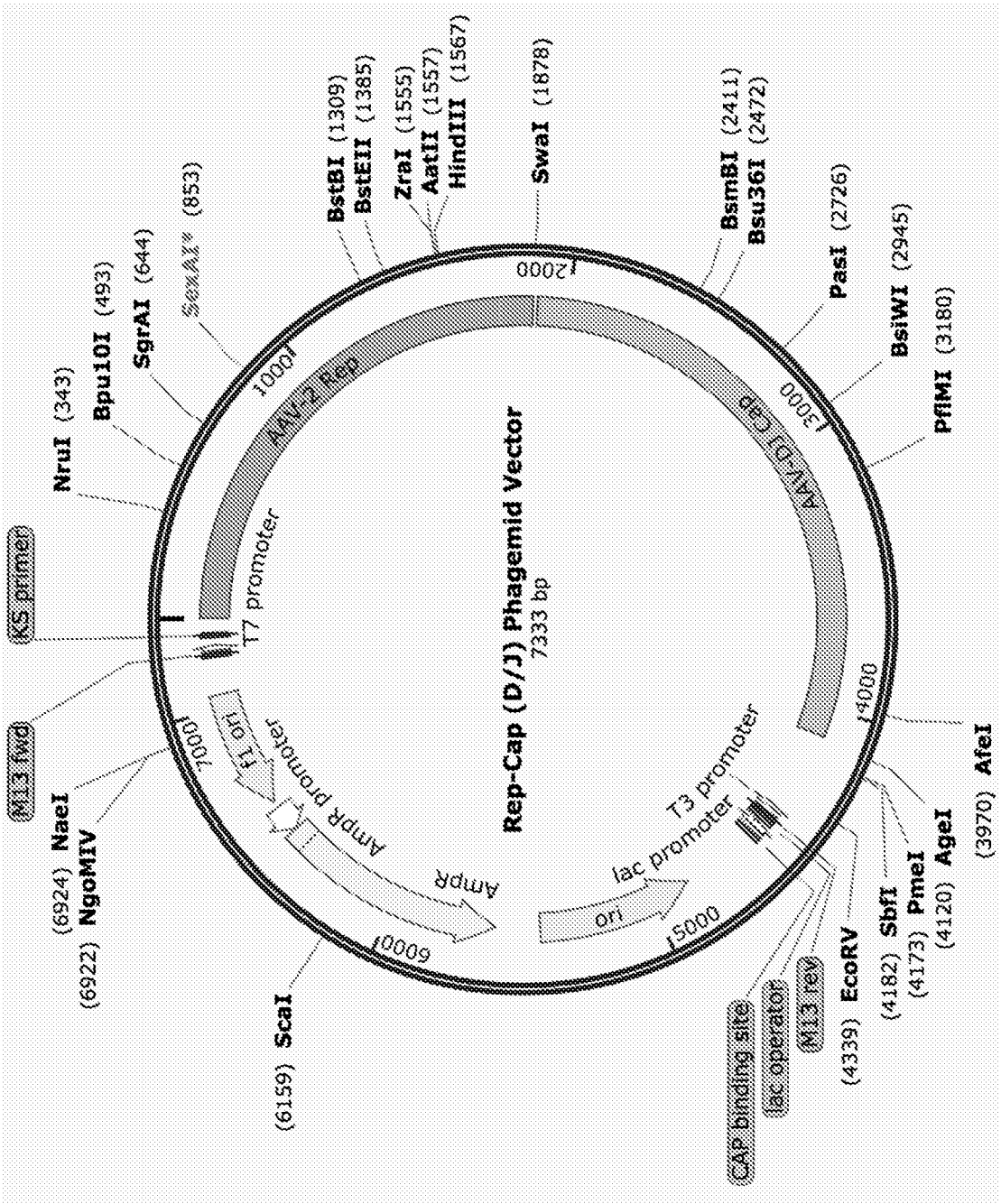

Rep-Cap (D/J) Phagemid Vector
7333 bp
AAV-DJ Cap
AAV-2 Rep
T7 promoter
T3 promoter
lac promoter
AmpR promoter
AmpR
ori
f1 ori
KS primer
M13 fwd
M13 rev
CAP binding site
lac operator
NruI (343)
Bpu10I (493)
SgrAI (644)
SexAI* (853)
BstBI (1309)
BstEII (1385)
ZraI (1555)
AatII (1557)
HindIII (1567)
SwaI (1878)
BsmBI (2411)
Bsu36I (2472)
PasI (2726)
BsiWI (2945)
PflMI (3180)
AfeI (3970)
AgeI (4120)
PmeI (4173)
SbfI (4182)
EcoRV (4339)
ScaI (6159)
NgoMIV (6922)
NaeI (6924)
1000
2000
3000
4000
5000
6000
7000

Figure 14

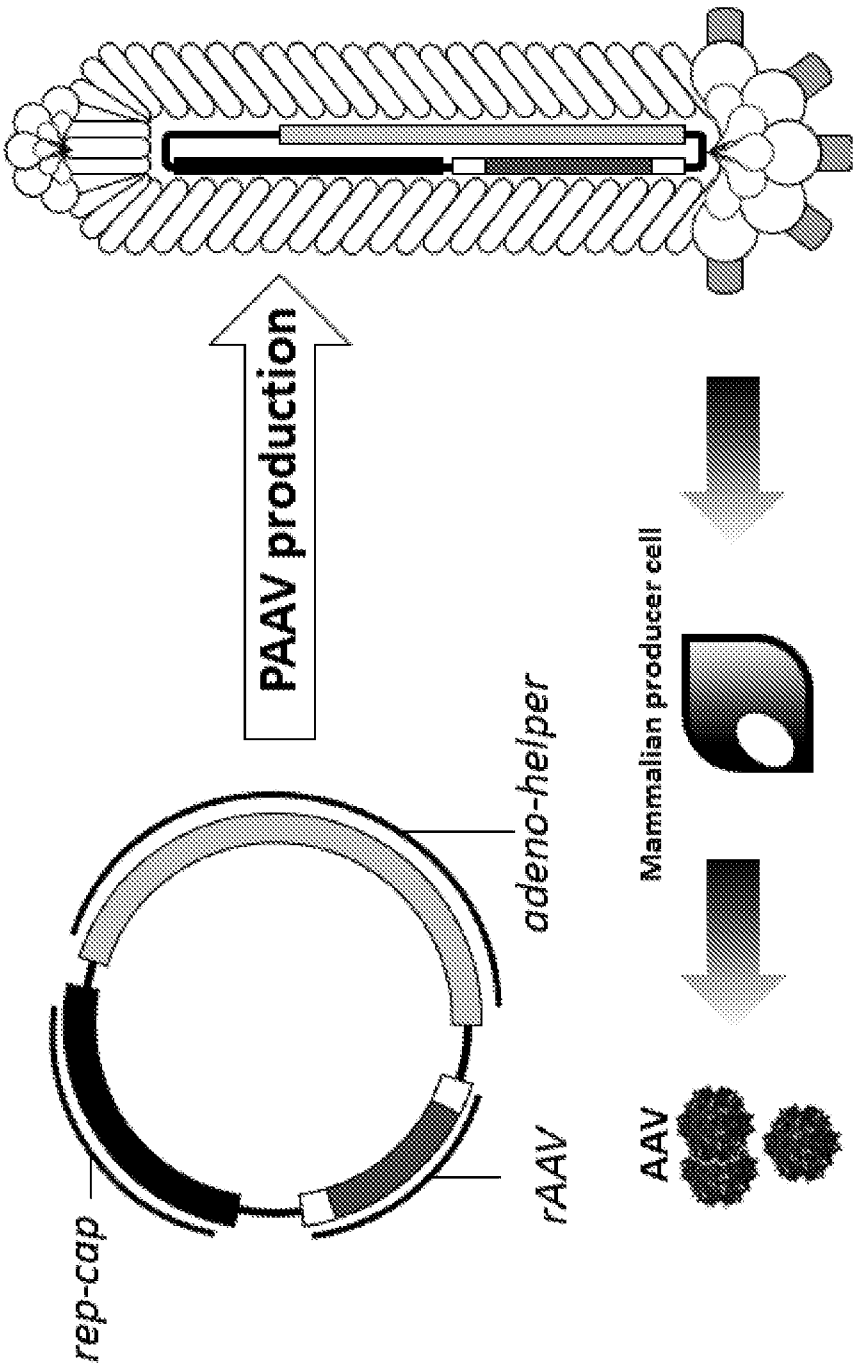

PAAV production
adeno-helper
rAAV
rep-cap
Mammalian producer cell
AAV

Figure 15

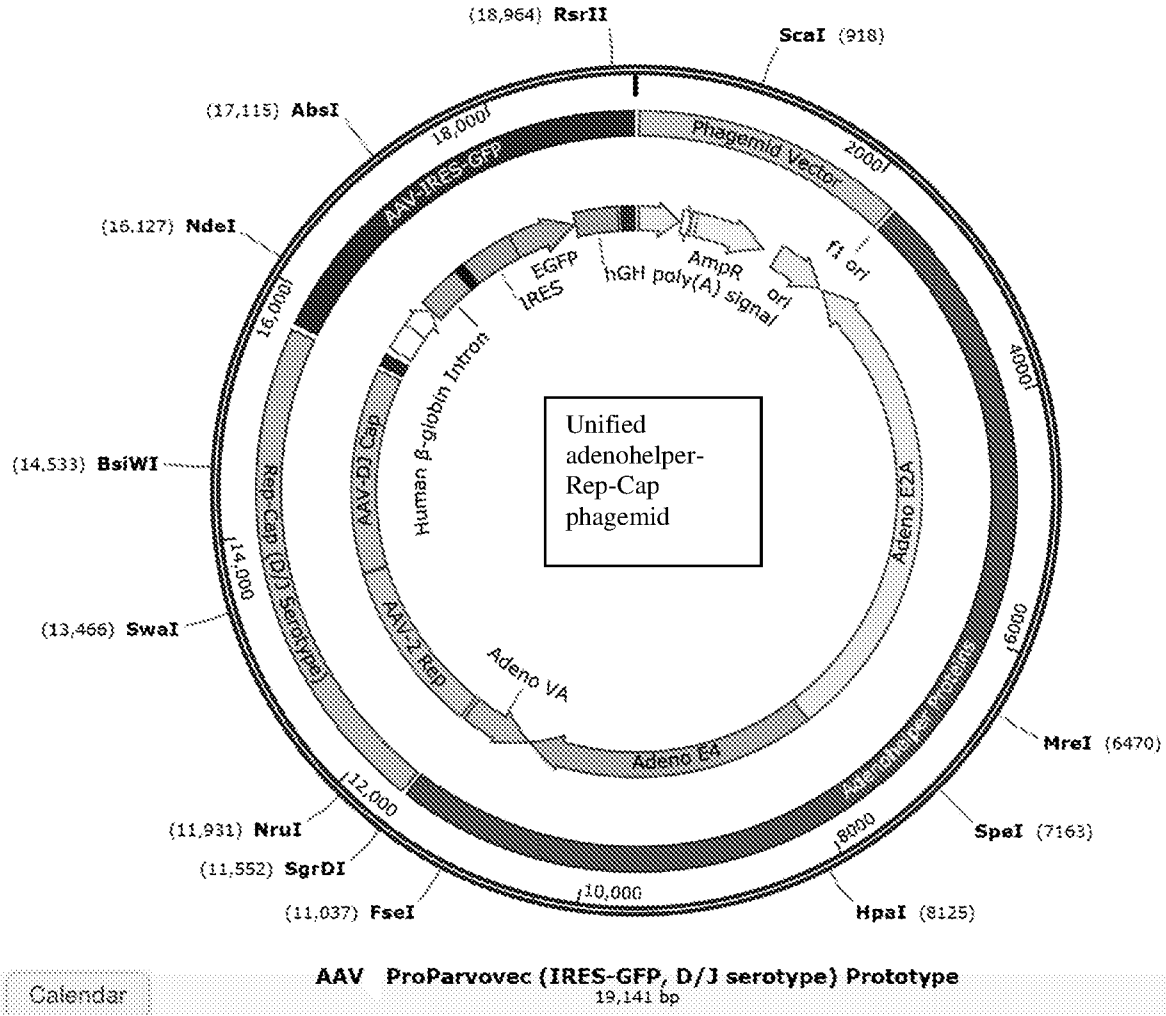

(18,964) RsrII
ScaI (918)
(17,115) AbsI
(16,127) NdeI
(14,533) BsiWI
(13,466) SwaI
(11,931) NruI
(11,552) SgrDI
(11,037) FseI
MreI (6470)
SpeI (7163)
HpaI (8125)
18,000
2000
4000
6000
8000
10,000
12,000
14,000
16,000
AAV ITR-5' ITR
Phagemid Vector
EGFP
IRES
hGH poly(A) signal
AmpR
ori
f1 ori
Human β-globin Intron
AAV-2 Cap
Rep-Cap (D/J Serotype)
AAV-2 Rep
Adeno VA
Adeno E4
Adeno E2A
Adeno E1A
Unified adenohelper-Rep-Cap phagemid
Calendar
AAV ProParvovec (IRES-GFP, D/J serotype) Prototype
19,141 bp

Figure 16

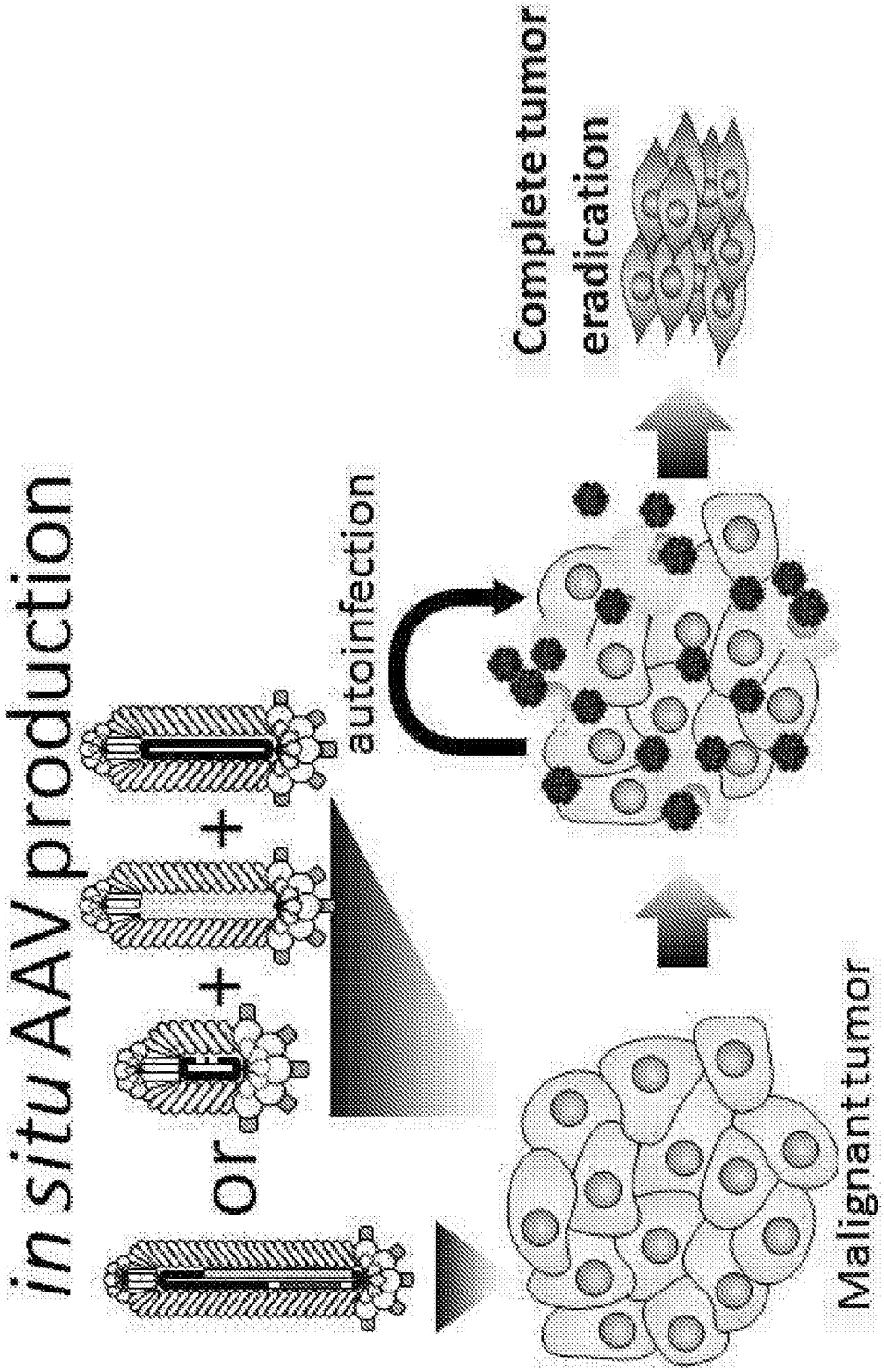

Complete tumor eradication
autoinfection
in situ AAV production
+
+
or
Malignant tumor

Figure 17

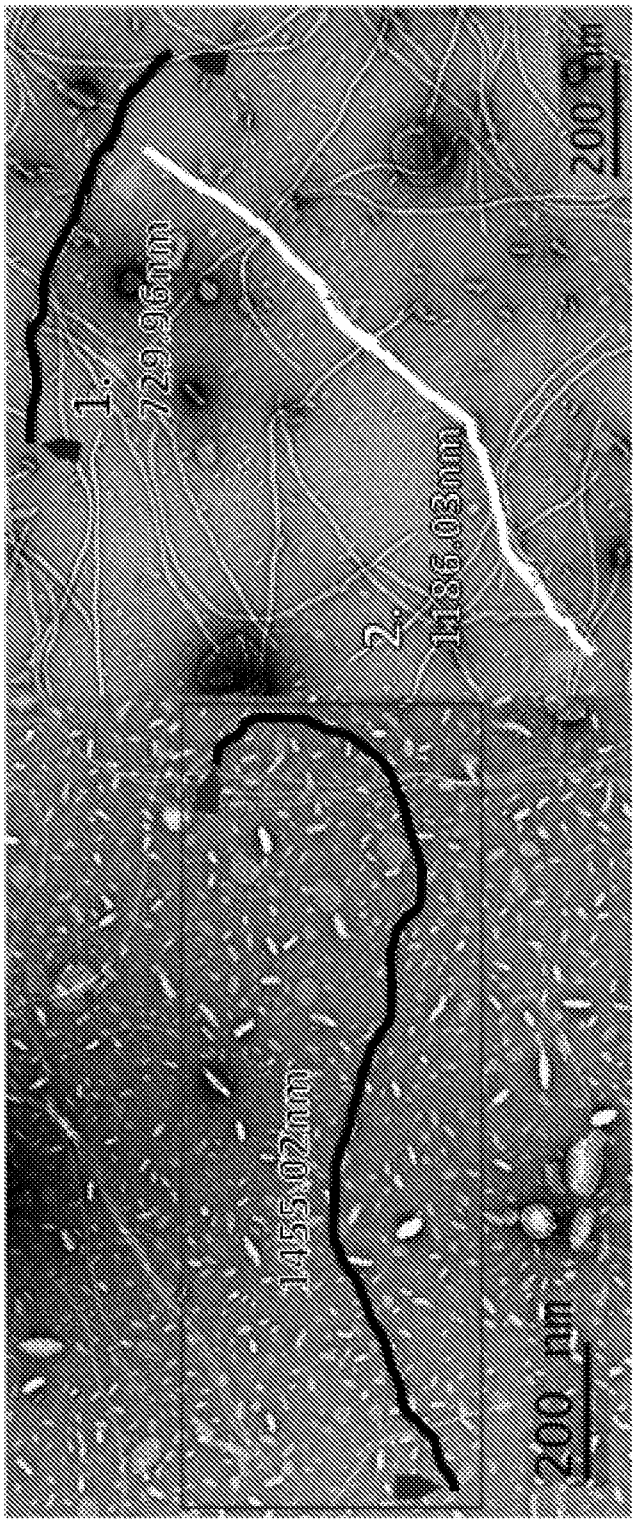


Figure 18

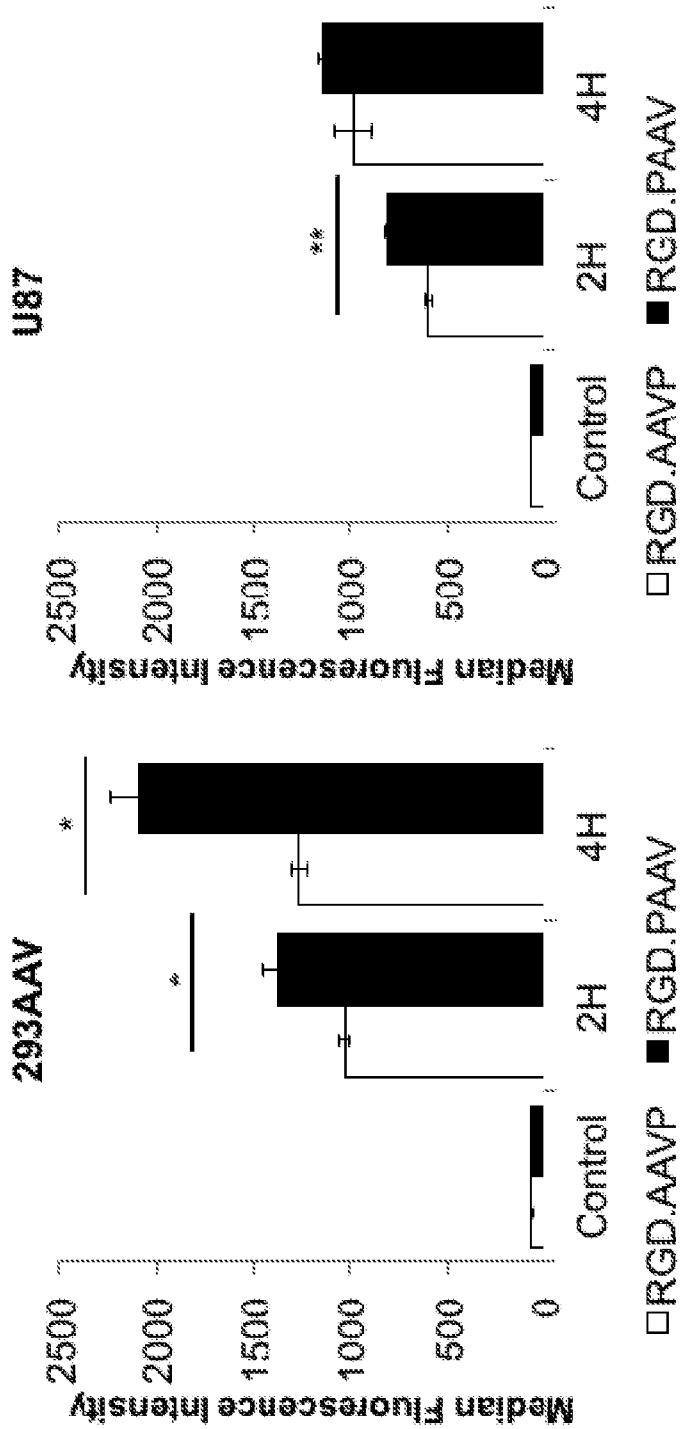

U87
293AAV
Median Fluorescence Intensity
2500
2000
1500
1000
500
0
Control
2H
4H
*
**
RGD.AAVP
RGD.PAAV

Figure 19

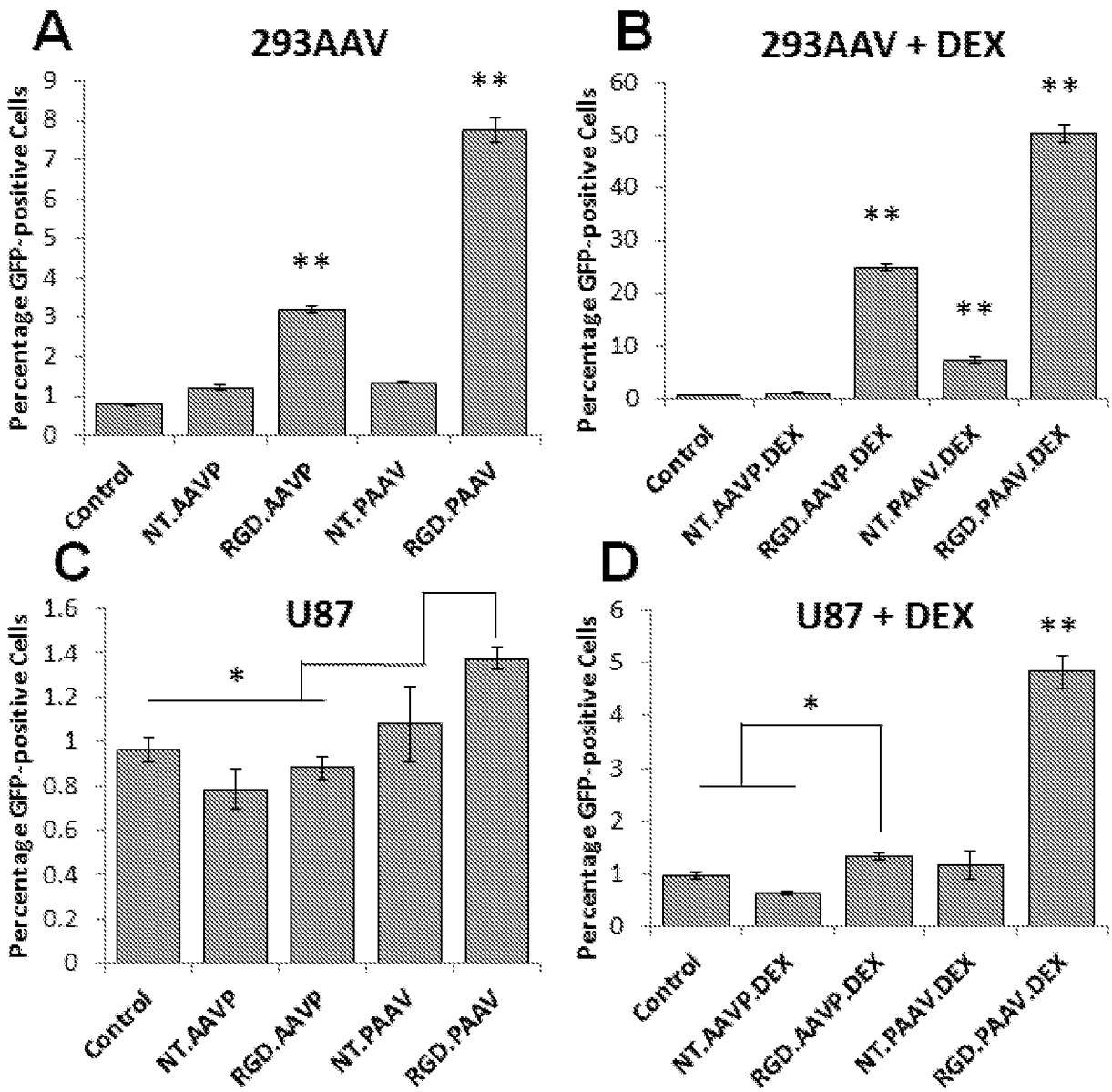

A
293AAV
Percentage GFP-positive Cells
0
1
2
3
4
5
6
7
8
9
**
**
Control
NT.AAVP
RGD.AAVP
NT.PAAV
RGD.PAAV
B
293AAV + DEX
Percentage GFP-positive Cells
0
10
20
30
40
50
60
**
**
**
Control
NT.AAVP.DEX
RGD.AAVP.DEX
NT.PAAV.DEX
RGD.PAAV.DEX
C
U87
Percentage GFP-positive Cells
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
*
Control
NT.AAVP
RGD.AAVP
NT.PAAV
RGD.PAAV
D
U87 + DEX
Percentage GFP-positive Cells
0
1
2
3
4
5
6
*
**
Control
NT.AAVP.DEX
RGD.AAVP.DEX
NT.PAAV.DEX
RGD.PAAV.DEX

Figure 20

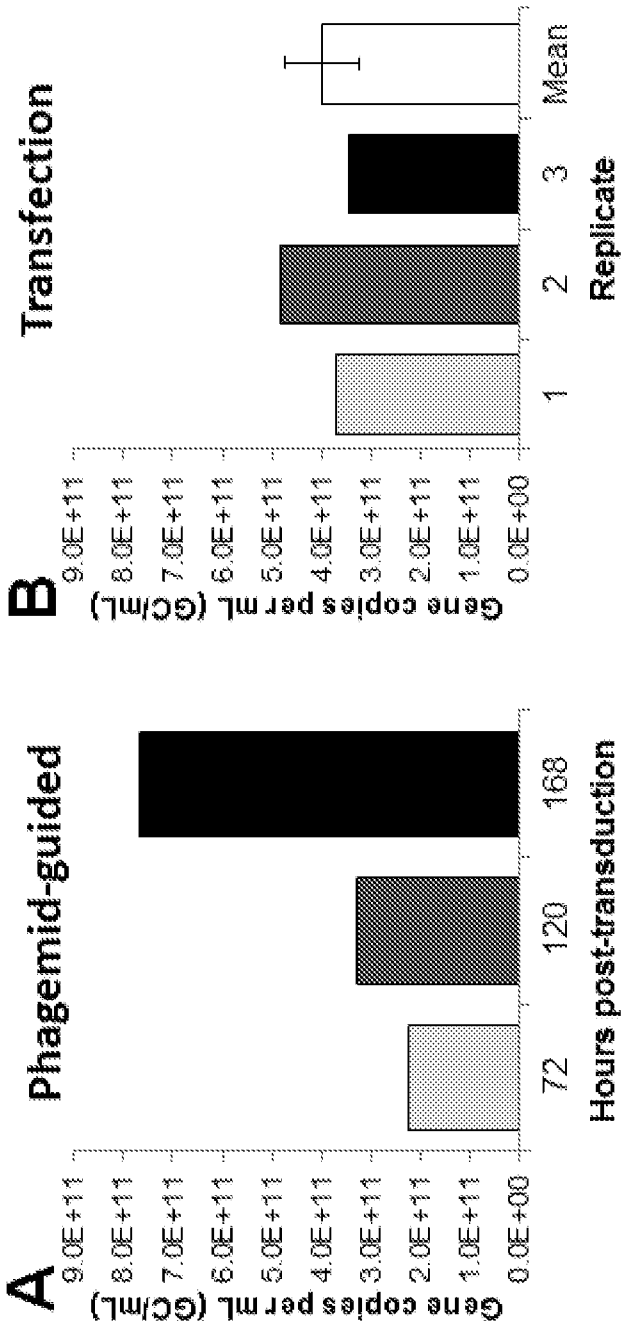

A
Phagemid-guided
Gene copies per mL (GC/mL)
9.0E+11
8.0E+11
7.0E+11
6.0E+11
5.0E+11
4.0E+11
3.0E+11
2.0E+11
1.0E+11
0.0E+00
72
120
168
Hours post-transduction
B
Transfection
Gene copies per mL (GC/mL)
9.0E+11
8.0E+11
7.0E+11
6.0E+11
5.0E+11
4.0E+11
3.0E+11
2.0E+11
1.0E+11
0.0E+00
1
2
3
Mean
Replicate

Figure 21

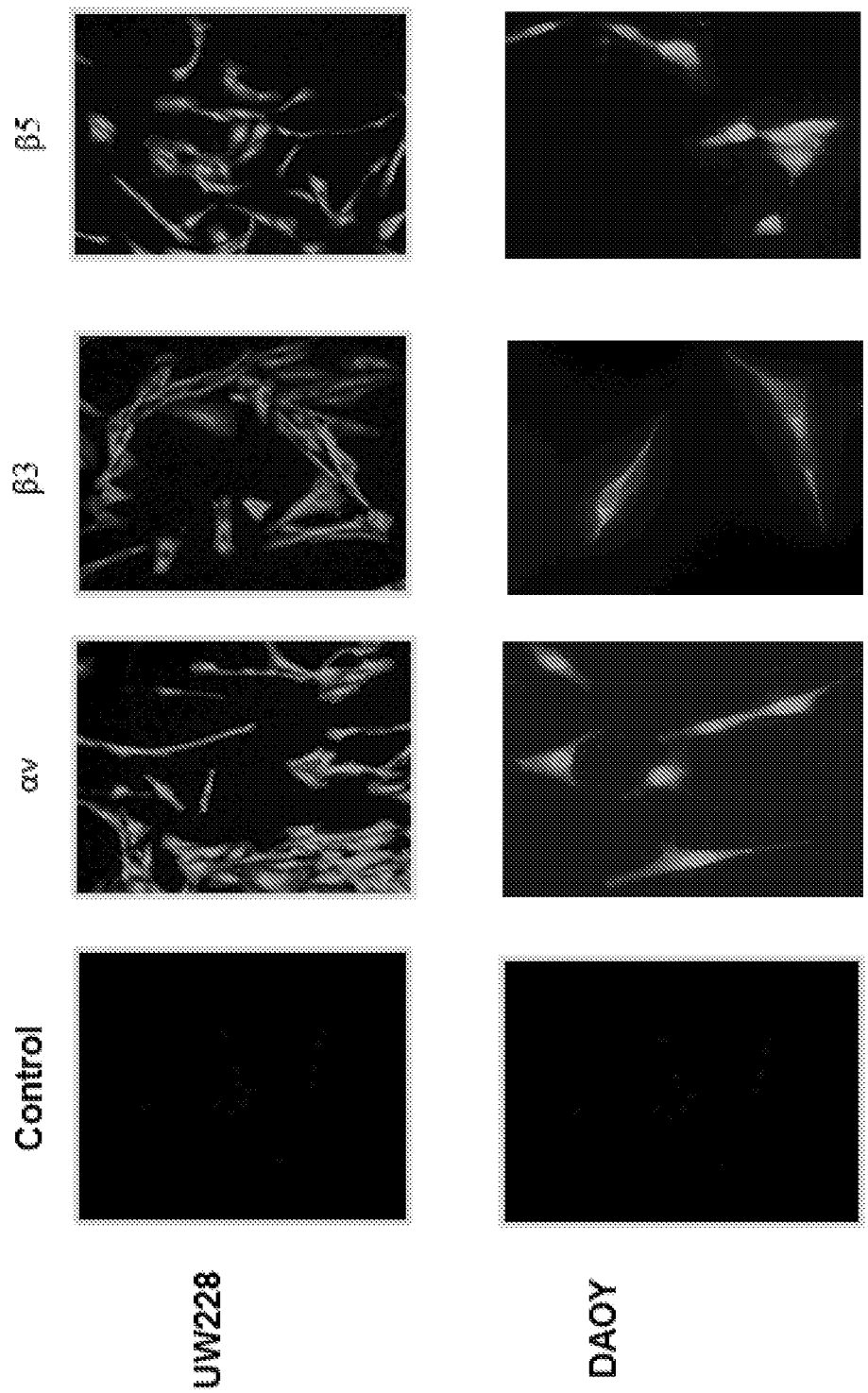


Figure 22

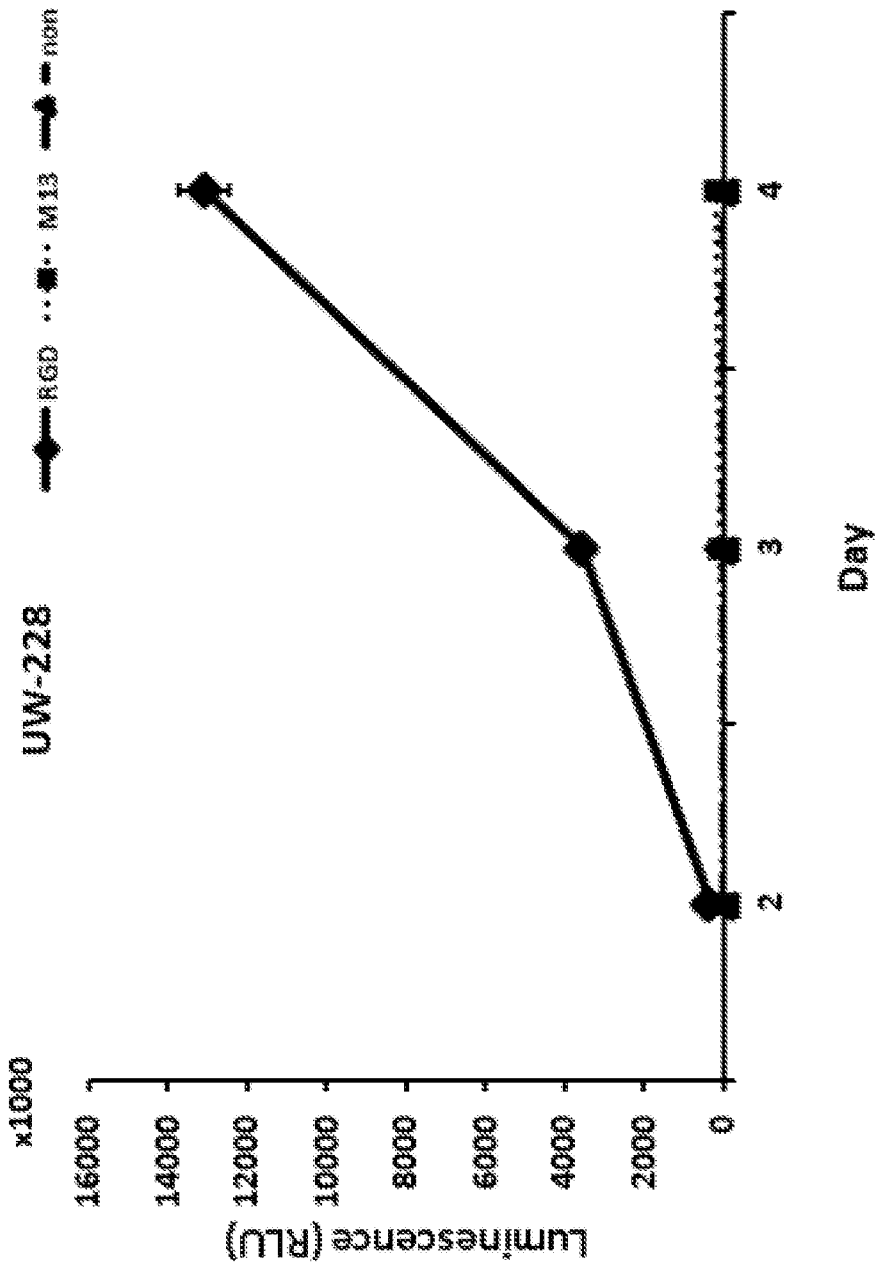

UW-228
RGD
M13
non
×1000
16000
14000
12000
10000
8000
6000
4000
2000
0
Luminescence (RLU)
2
3
4
Day

Figure 23

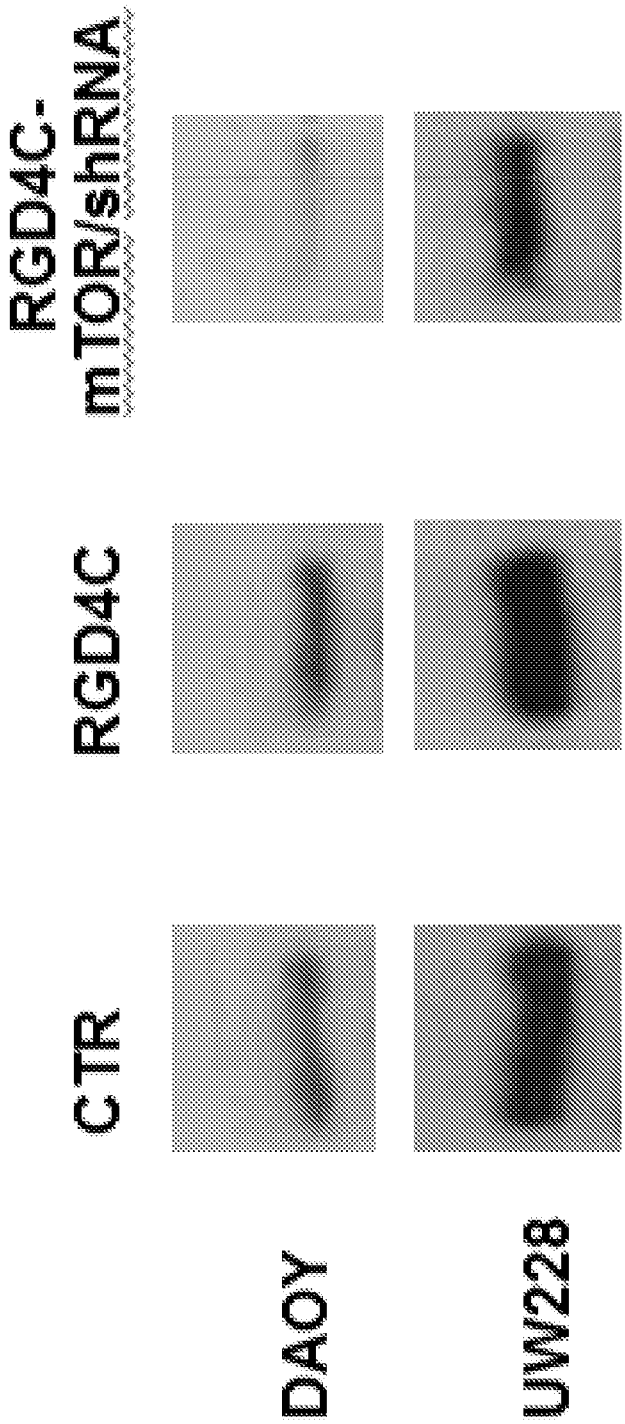


Figure 24

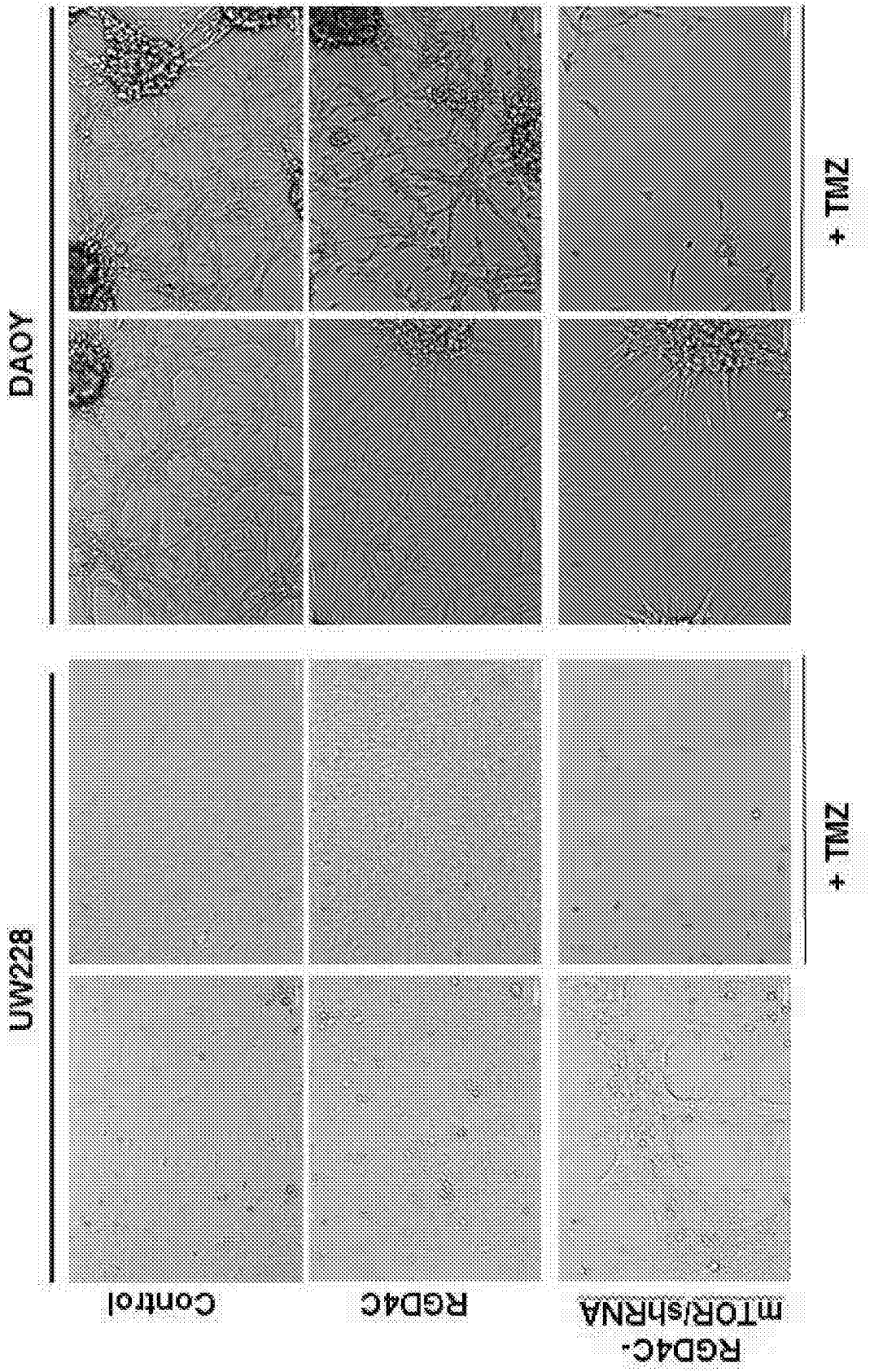
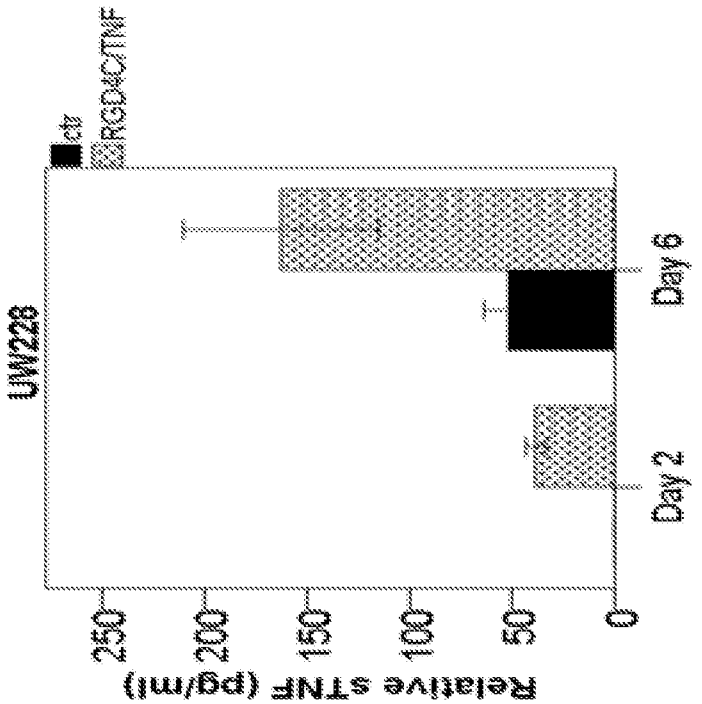


Figure 25


ctr
RGD4C/TNF
UW228
Day 6
Day 2
Relative sTNF (pg/ml)
250
200
150
100
50
0

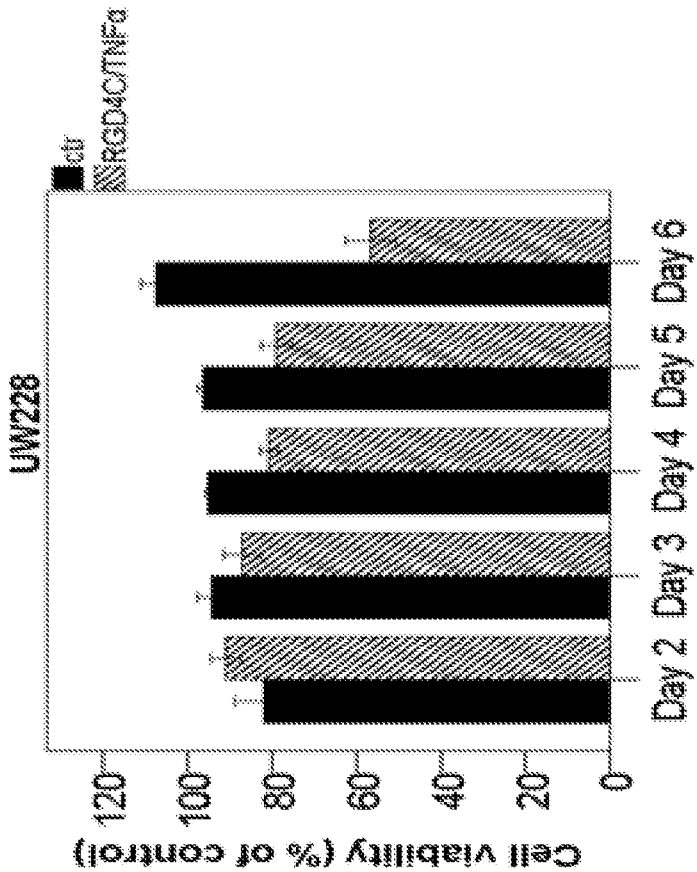

ctr
RGD4C/TNFα
UW228
Day 2
Day 3
Day 4
Day 5
Day 6
Cell viability (% of control)
120
100
80
60
40
20
0

Figure 26

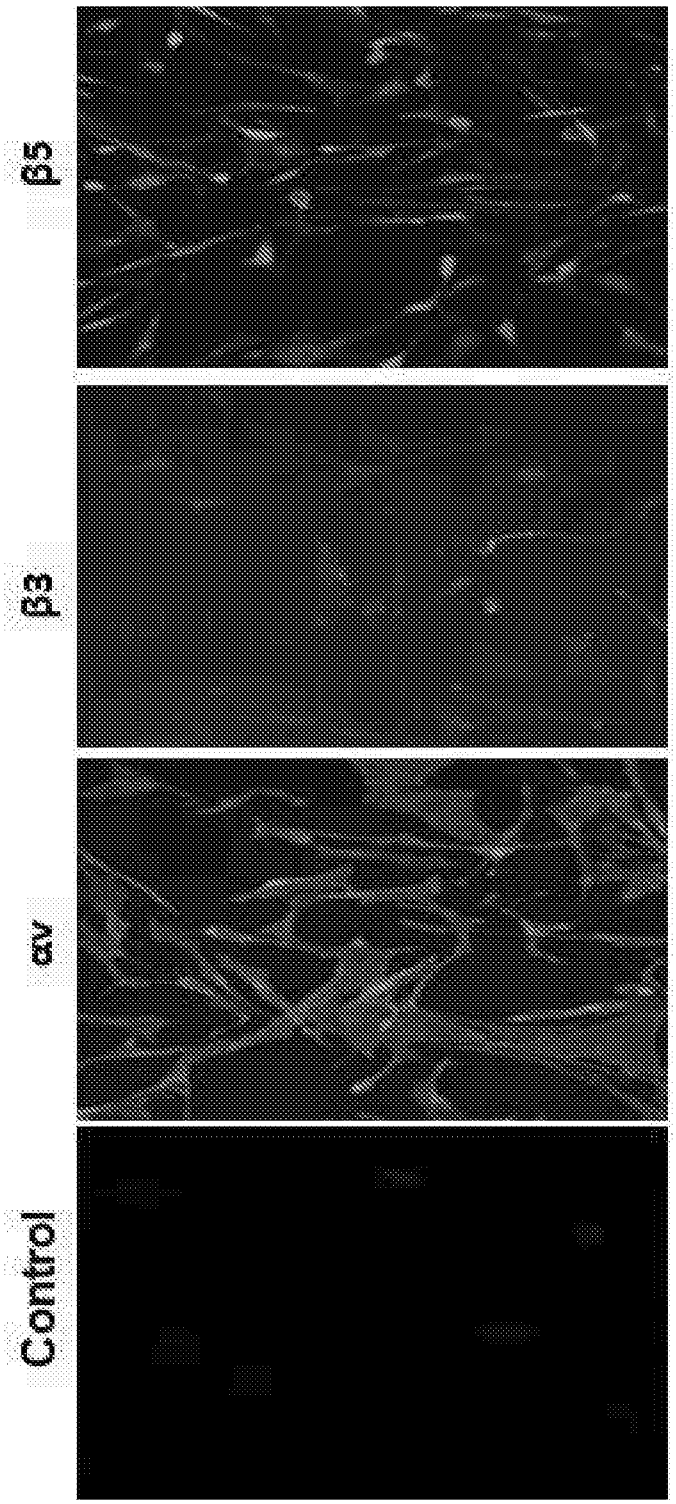


Figure 27A

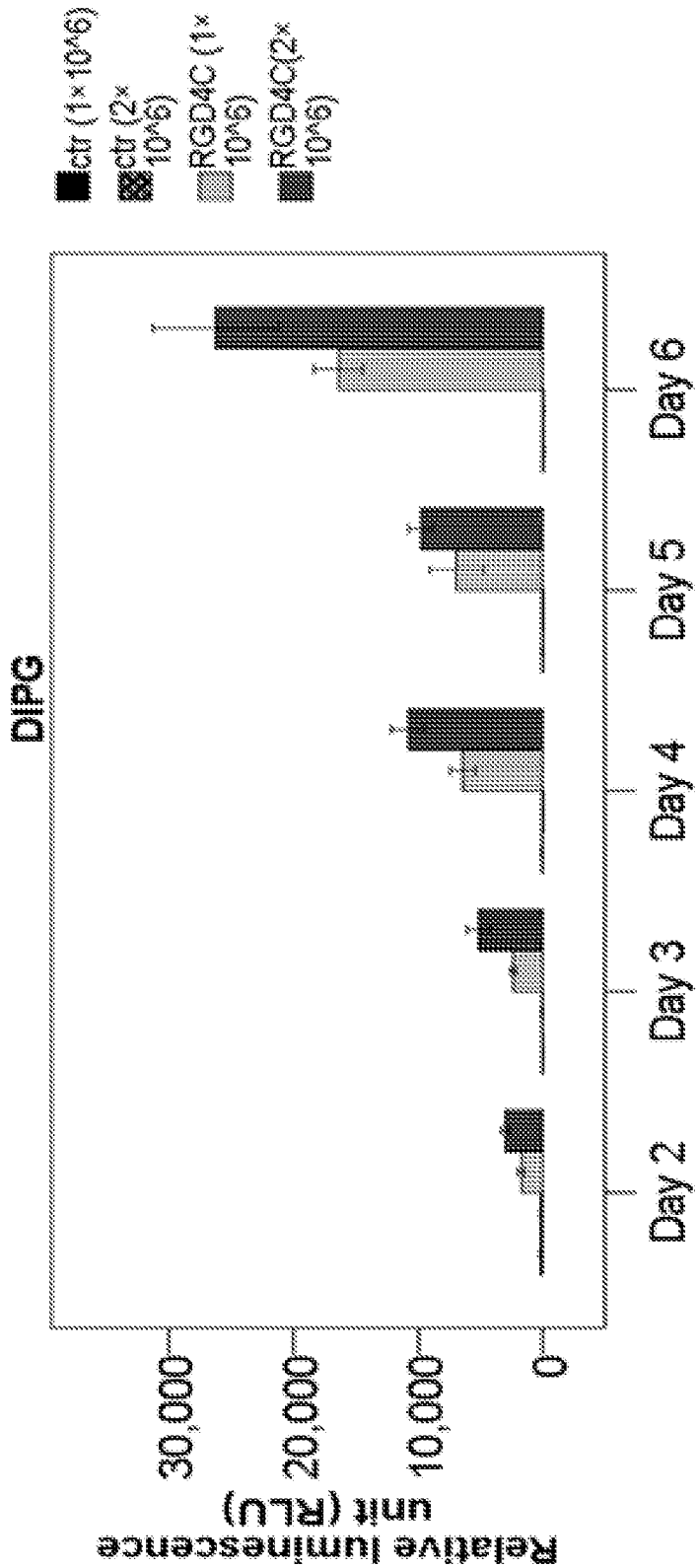

ctr (1×10^6)
ctr (2×10^6)
RGD4C (1×10^6)
RGD4C(2×10^6)
DIPG
Day 2
Day 3
Day 4
Day 5
Day 6
0
10,000
20,000
30,000
Relative luminescence unit (RLU)

Figure 27B

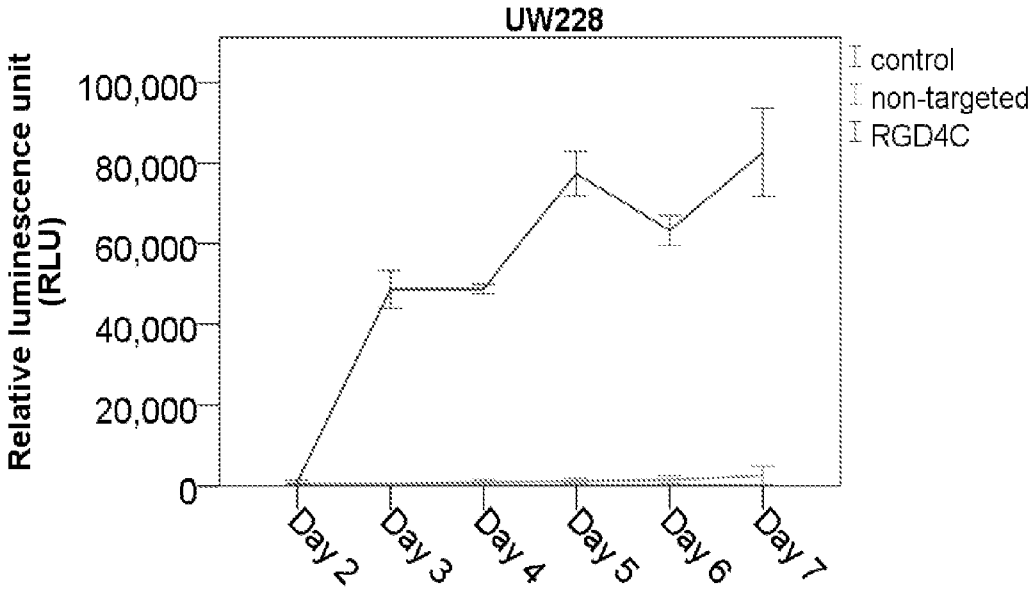


Figure 27C

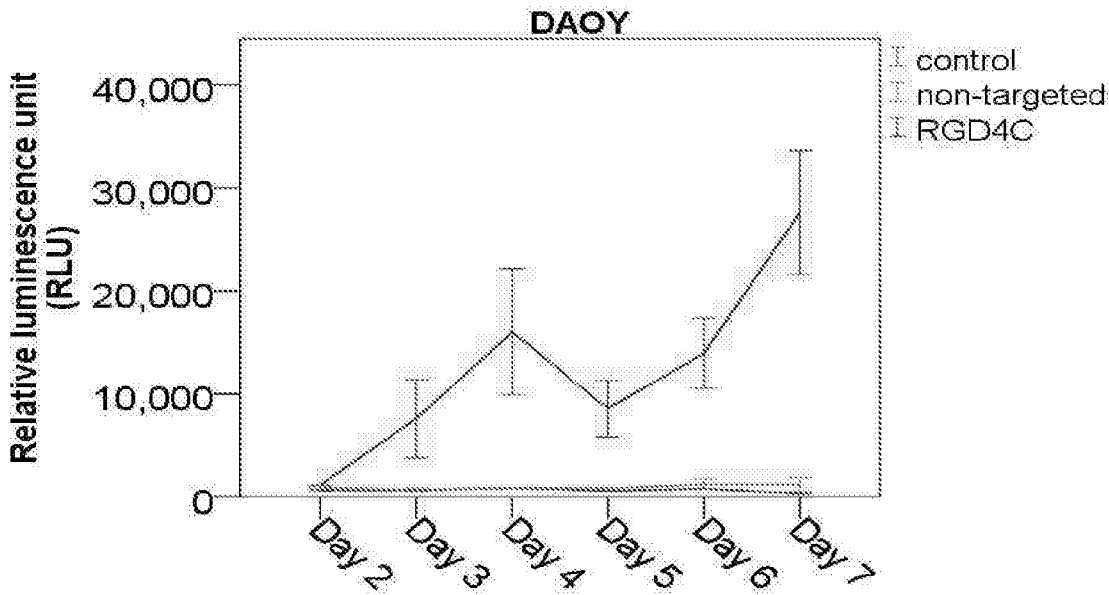


Figure 28

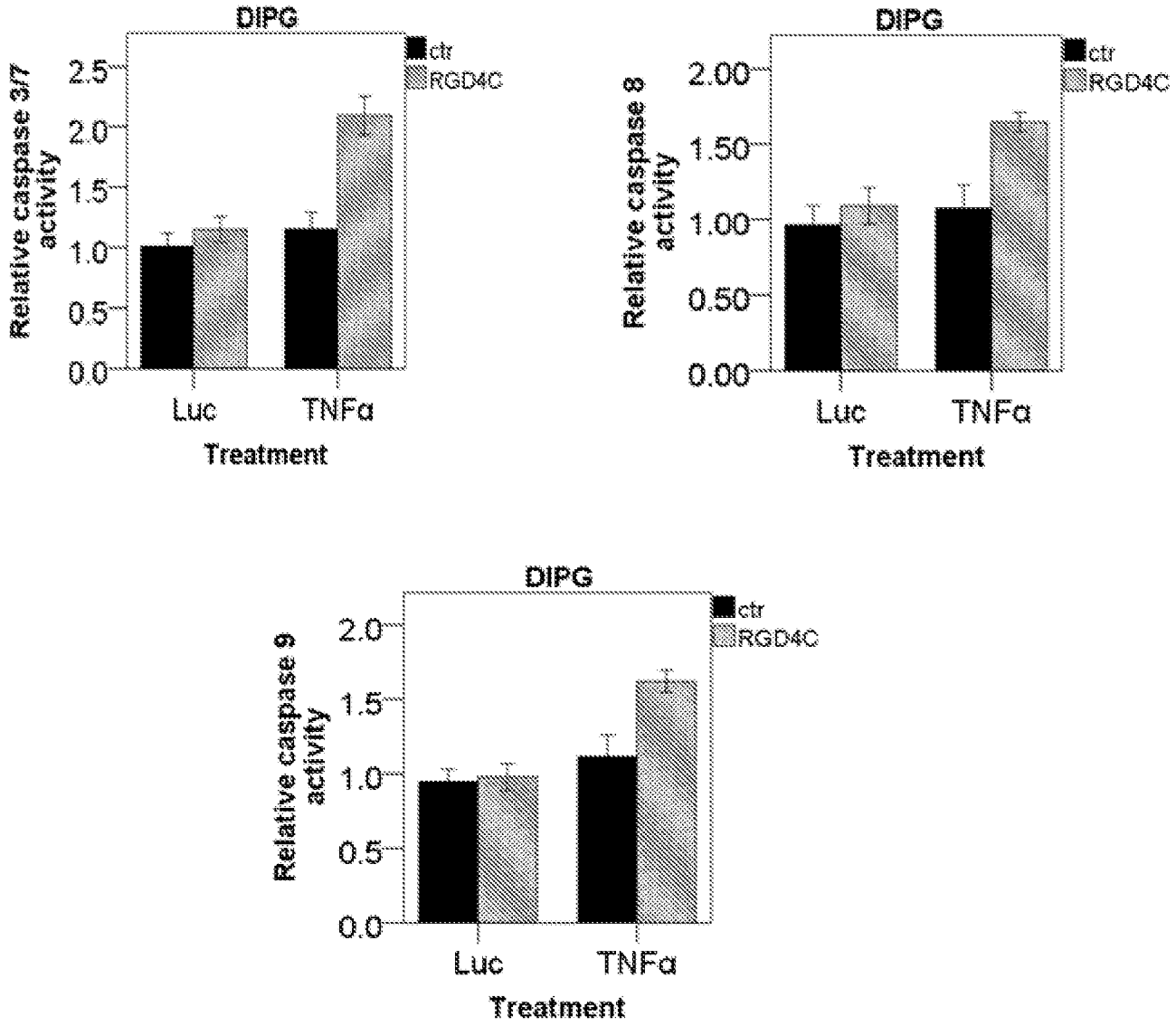

DIPG
ctr
RGD4C
Relative caspase 3/7 activity
2.5
2.0
1.5
1.0
0.5
0.0
Luc
TNFα
Treatment
DIPG
ctr
RGD4C
Relative caspase 8 activity
2.00
1.50
1.00
0.50
0.00
Luc
TNFα
Treatment
DIPG
ctr
RGD4C
Relative caspase 9 activity
2.0
1.5
1.0
0.5
0.0
Luc
TNFα
Treatment

Figure 28 (cont)

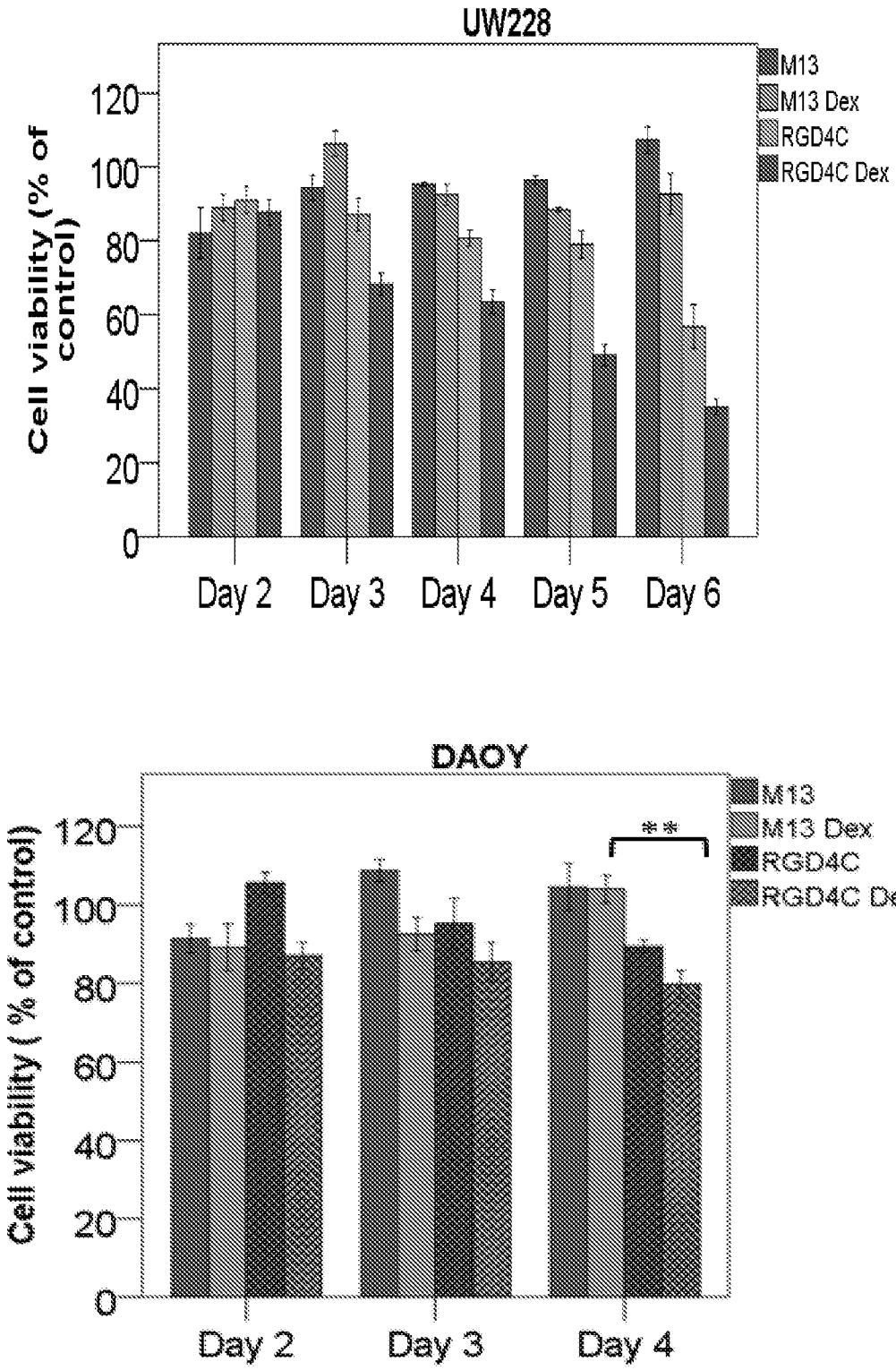


Figure 29

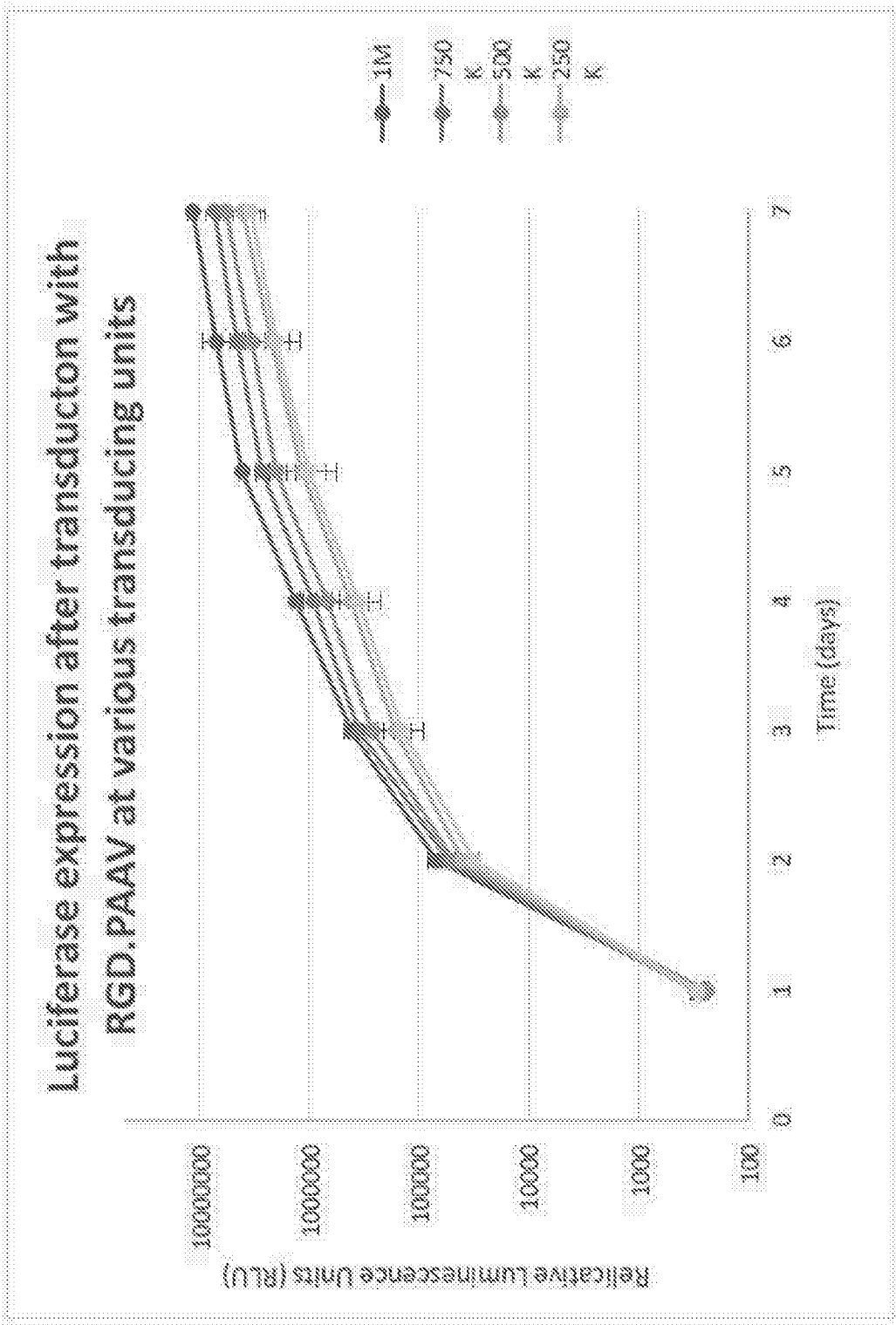

Luciferase expression after transducton with RGD.PAAV at various transducing units
1M
750 k
500 k
250 k
Relicative Luminescence Units (RLU)
10000000
1000000
100000
10000
1000
100
0
1
2
3
4
5
6
7
Time (days)

Figure 30

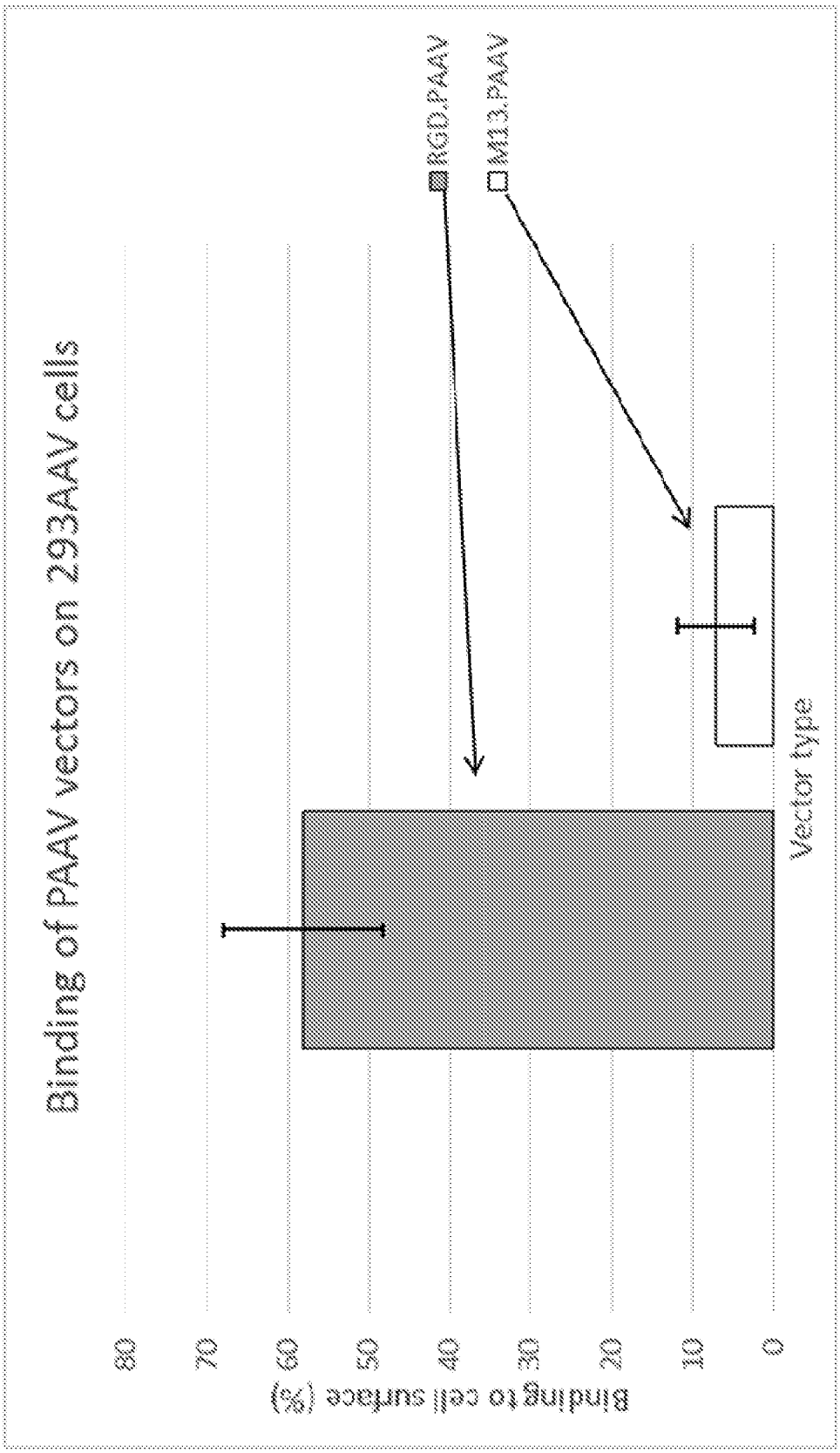

Binding of PAAV vectors on 293AAV cells
RGD.PAAV
M13.PAAV
Vector type
Binding to cell surface (%)
0
10
20
30
40
50
60
70
80

Figure 31a

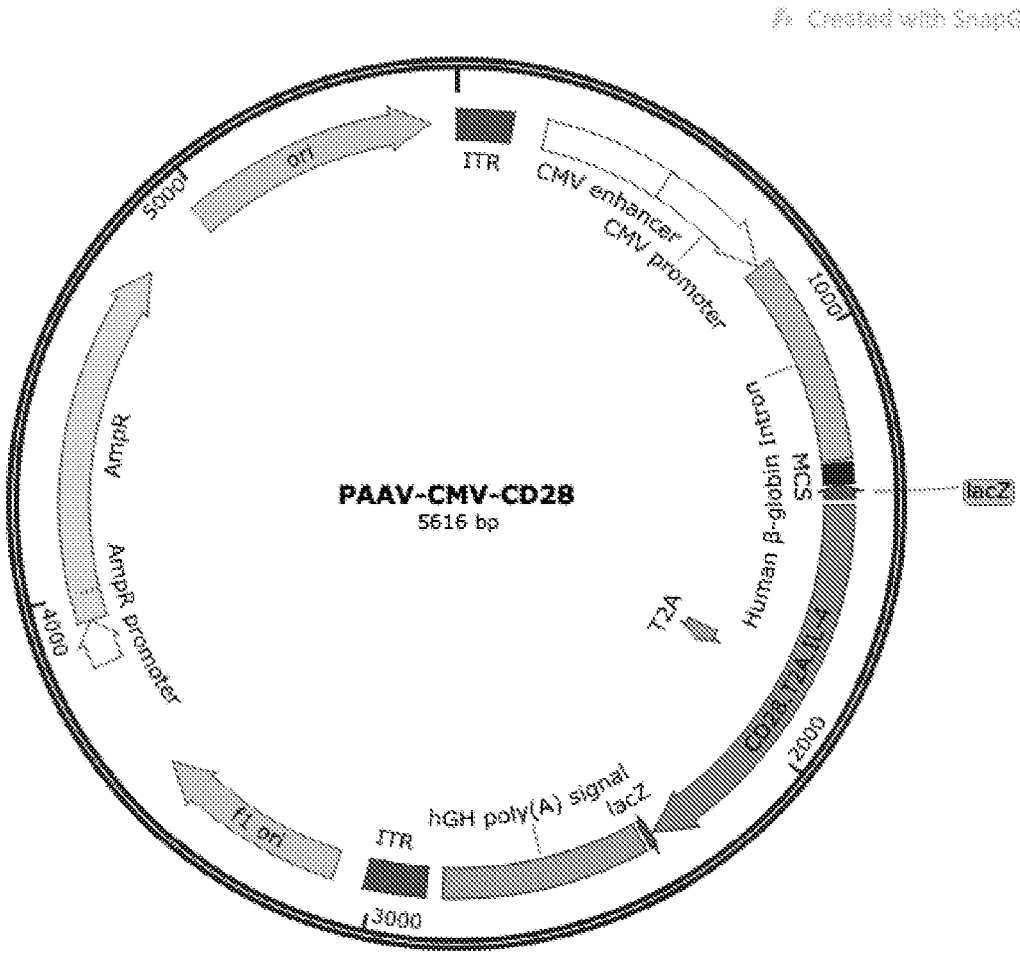

Created with SnapGene®
ori
ITR
CMV enhancer
CMV promoter
Human β-globin intron
MCS
lacZ
T2A
hGH poly(A) signal
lacZ
ITR
f1 ori
AmpR promoter
AmpR
PAAV-CMV-CD28
5616 bp
1000
2000
3000
4000
5000

Figure 31b

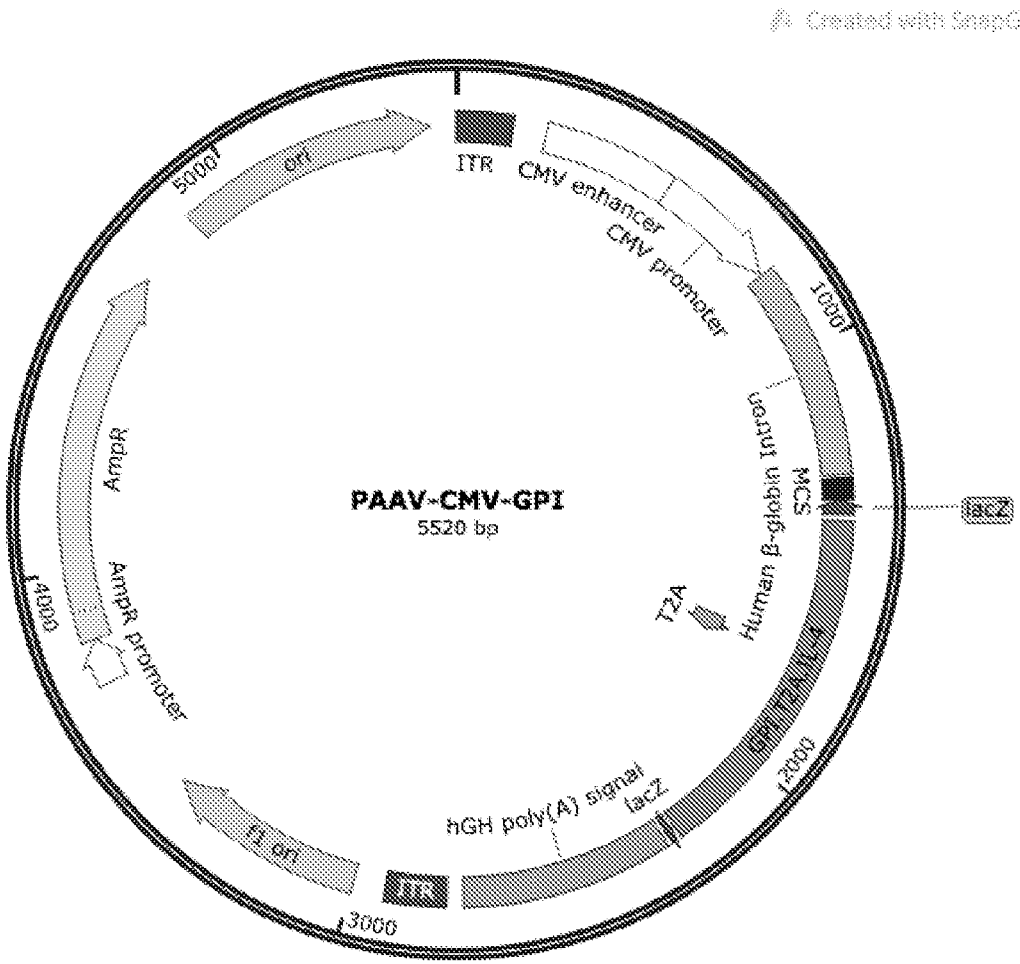

Created with SnapGene®
PAAV-CMV-GPI
5520 bp
ori
ITR
CMV enhancer
CMV promoter
Human β-globin intron
MCS
lacZ
T2A
hGH poly(A) signal
lacZ
f1 ori
ITR
AmpR
AmpR promoter
5000
10000
12000
13000
4000

Figure 31c

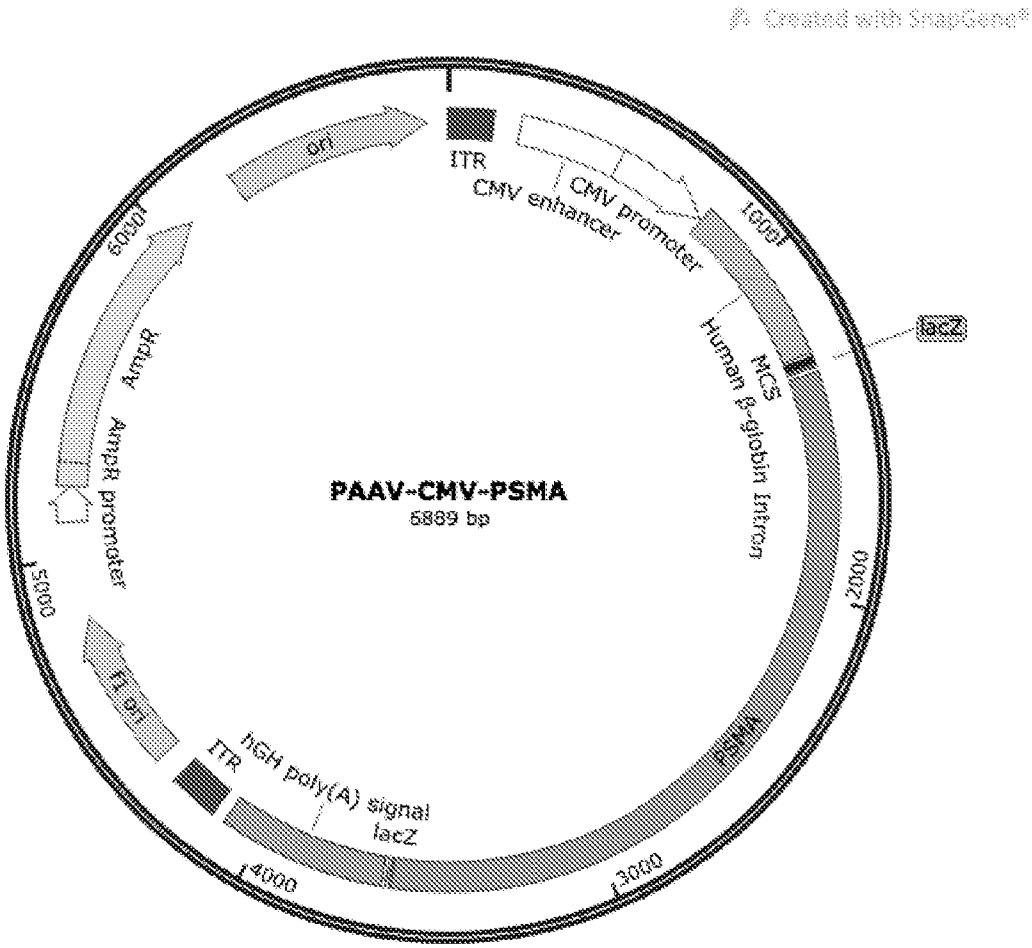

Created with SnapGene®
ori
ITR
CMV enhancer
CMV promoter
1000
lacZ
MCS
Human β-globin intron
2000
PSMA
3000
hGH poly(A) signal
lacZ
4000
ITR
f1 ori
5000
AmpR promoter
AmpR
6000
PAAV-CMV-PSMA
6889 bp

Figure 31d

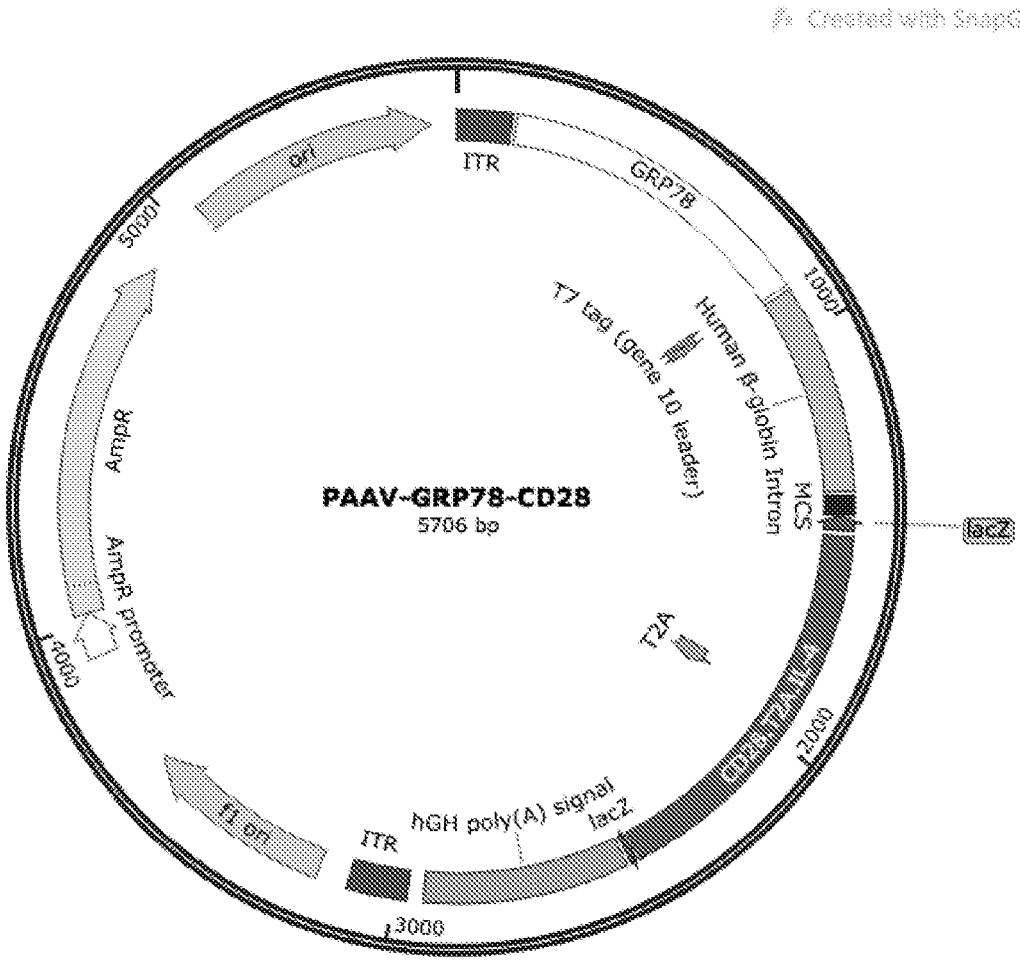

Created with SnapGene®
ori
ITR
GRP78
1000
T7 tag (gene 10 leader)
Human ß-globin Intron
MCS
lacZ
PAAV-GRP78-CD28
5706 bp
AmpR
AmpR promoter
4000
5000
T2A
2000
f1 ori
ITR
hGH poly(A) signal
lacZ
3000

Figure 31e

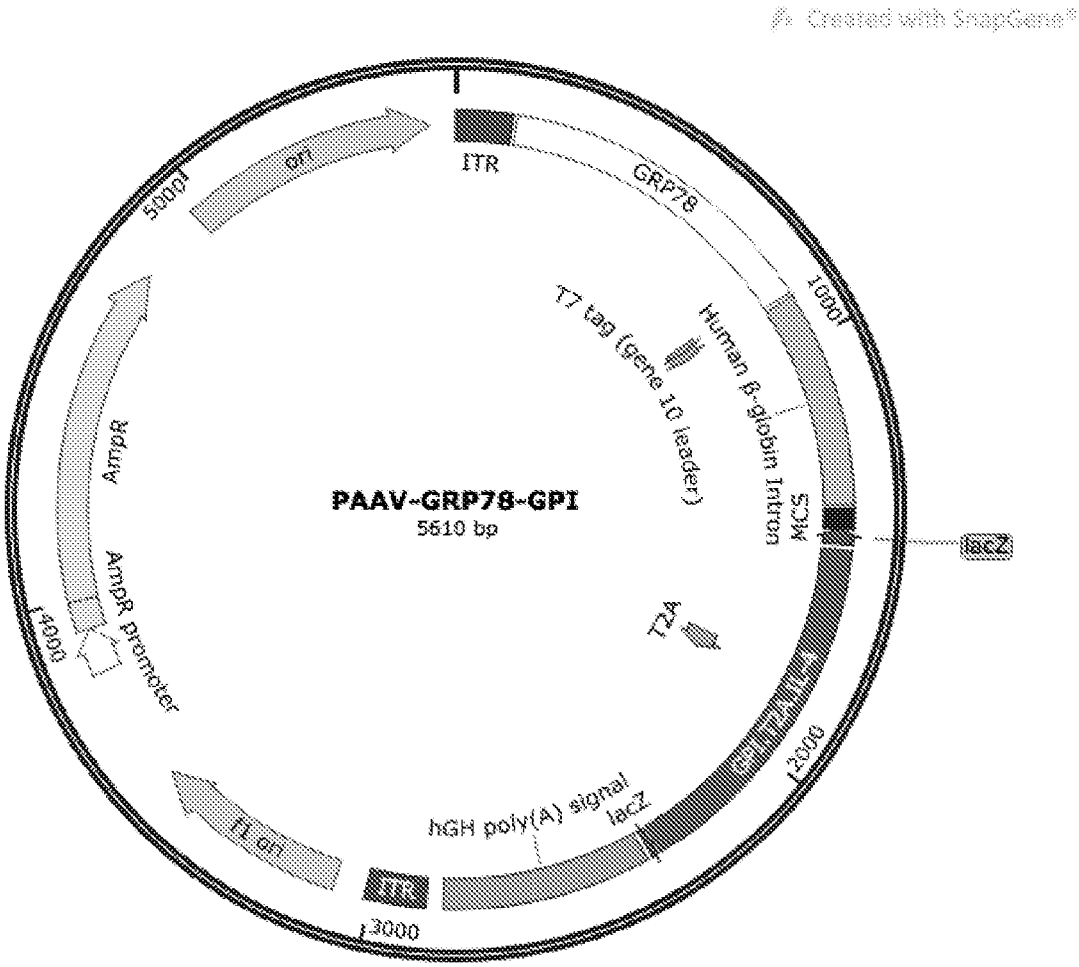

Created with SnapGene®
ori
ITR
GRP78
1000
T7 tag (gene 10 leader)
Human β-globin Intron
SD
SA
lacZ
PAAV-GRP78-GPI
5610 bp
Amp
AmpR promoter
4000
2000
f1 ori
hGH poly(A) signal
lacZ
ITR
3000

Figure 31f

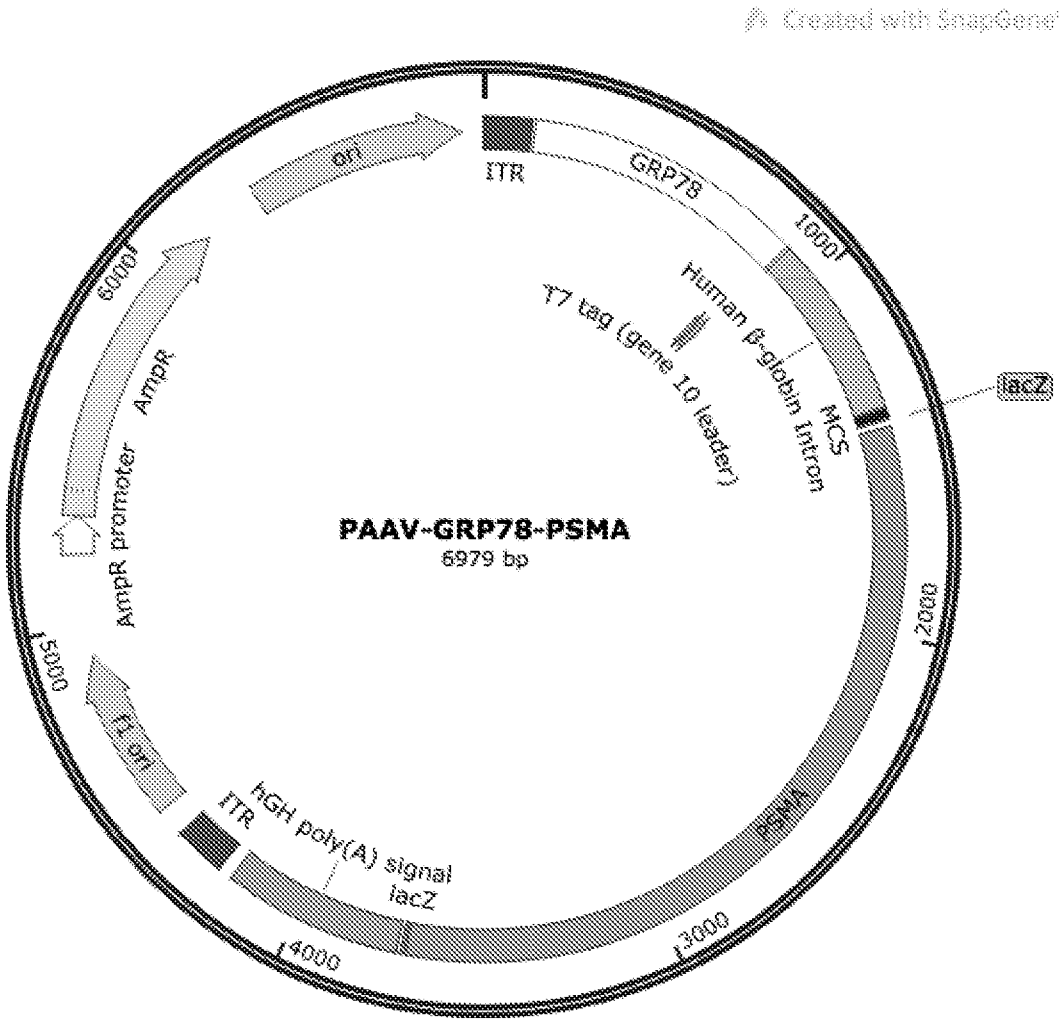

Created with SnapGene®
ori
ITR
GRP78
1000
human β-globin Intron
T7 tag (gene 10 leader)
MCS
lacZ
2000
3000
pA
4000
hGH poly(A) signal
ITR
f1 ori
5000
AmpR promoter
AmpR
6000
PAAV-GRP78-PSMA
6979 bp

Figure 32

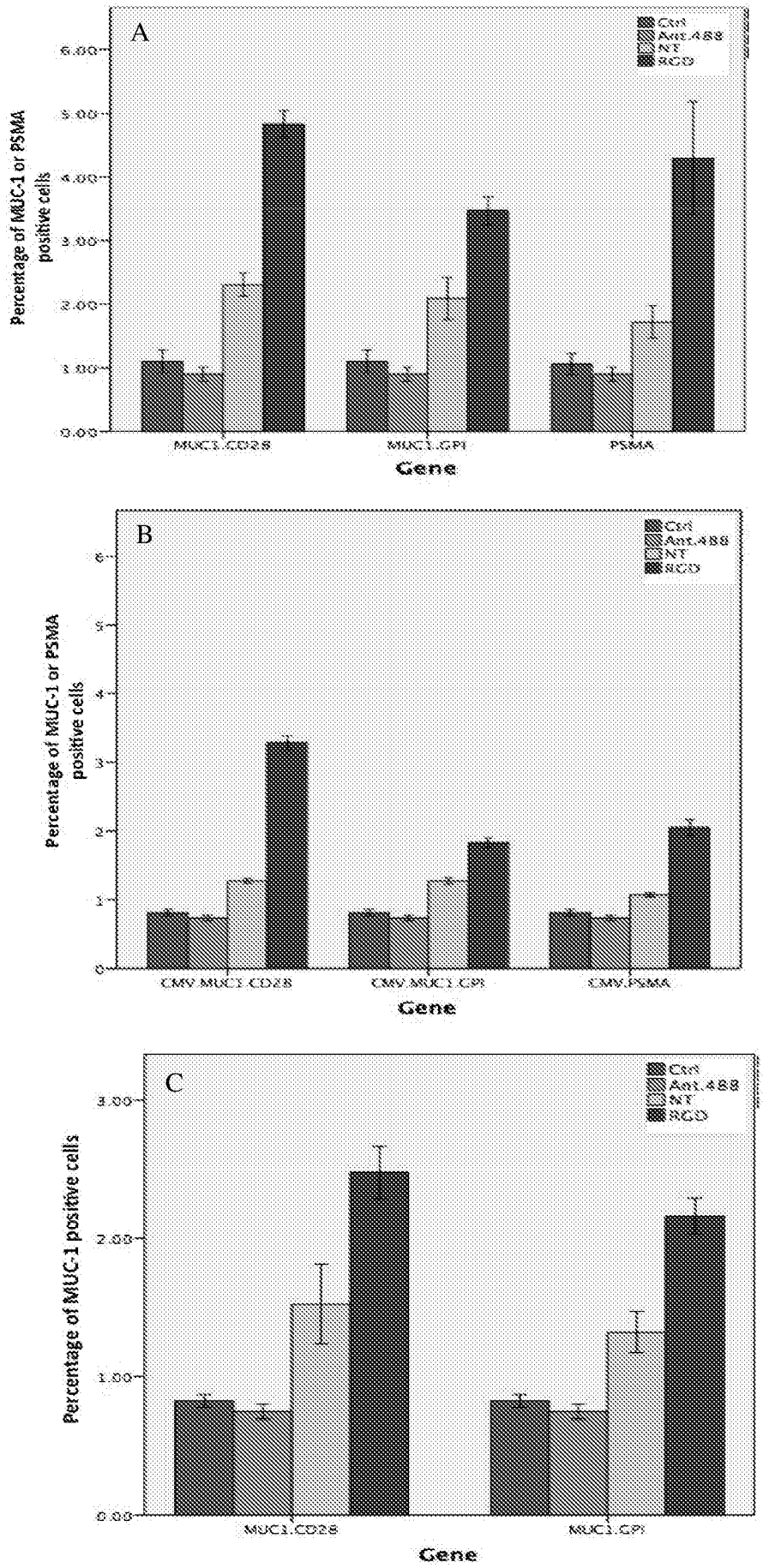

A
Ctrl
Ant.488
NT
RGD
Percentage of MUC-1 or PSMA positive cells
6.00
5.00
4.00
3.00
2.00
1.00
0.00
MUC1.CD28
MUC1.GPI
PSMA
Gene
B
Ctrl
Ant.488
NT
RGD
Percentage of MUC-1 or PSMA positive cells
6
5
4
3
2
1
0
CMV.MUC1.CD28
CMV.MUC1.GPI
CMV.PSMA
Gene
C
Ctrl
Ant.488
NT
RGD
Percentage of MUC-1 positive cells
3.00
2.00
1.00
0.00
MUC1.CD28
MUC1.GPI
Gene

Figure 33a

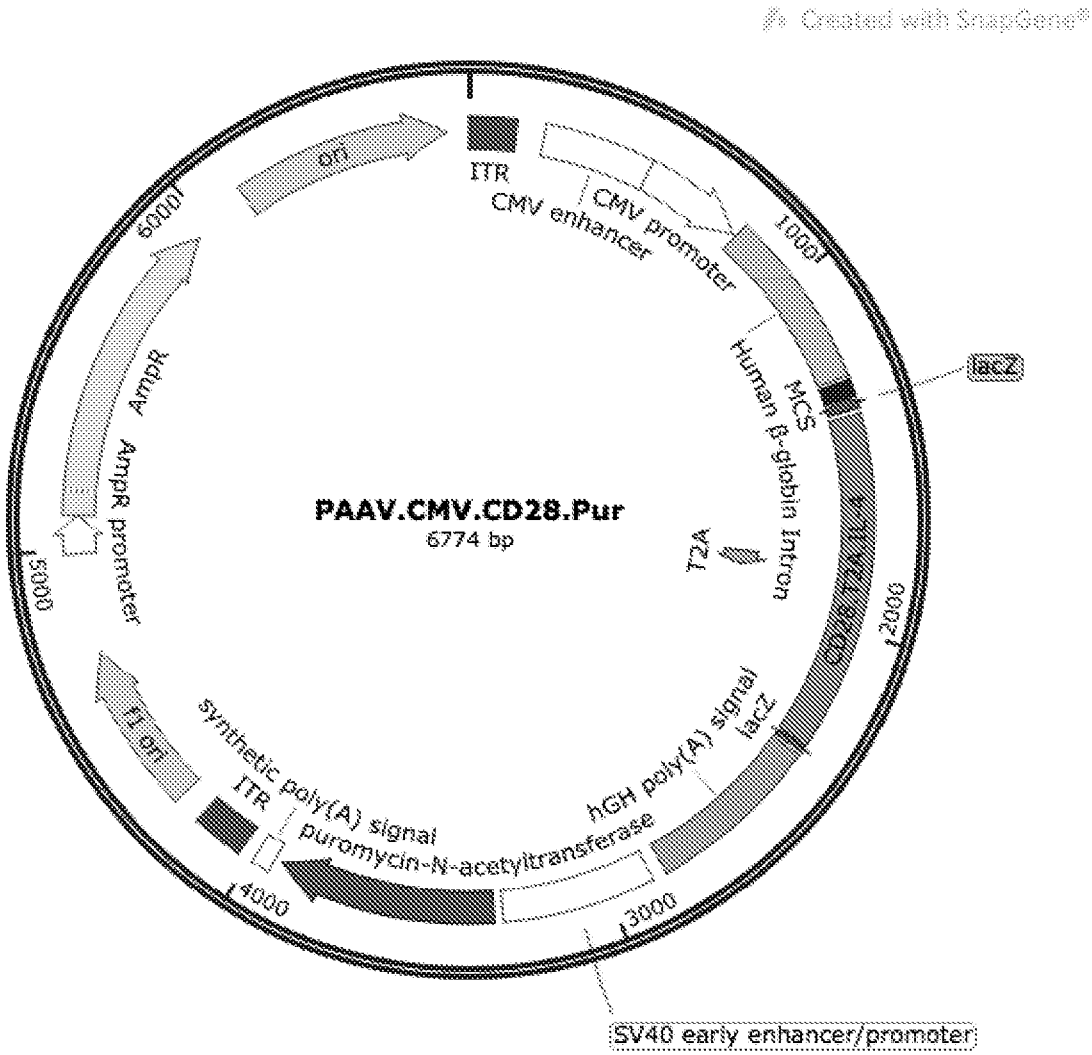

Created with SnapGene®
ori
ITR
CMV enhancer
CMV promoter
1000
MCS
Human β-globin intron
lacZ
T2A
2000
hGH poly(A) signal
lacZ
3000
SV40 early enhancer/promoter
puromycin-N-acetyltransferase
synthetic poly(A) signal
ITR
4000
f1 ori
5000
AmpR promoter
AmpR
6000
PAAV.CMV.CD28.Pur
6774 bp

Figure 33b

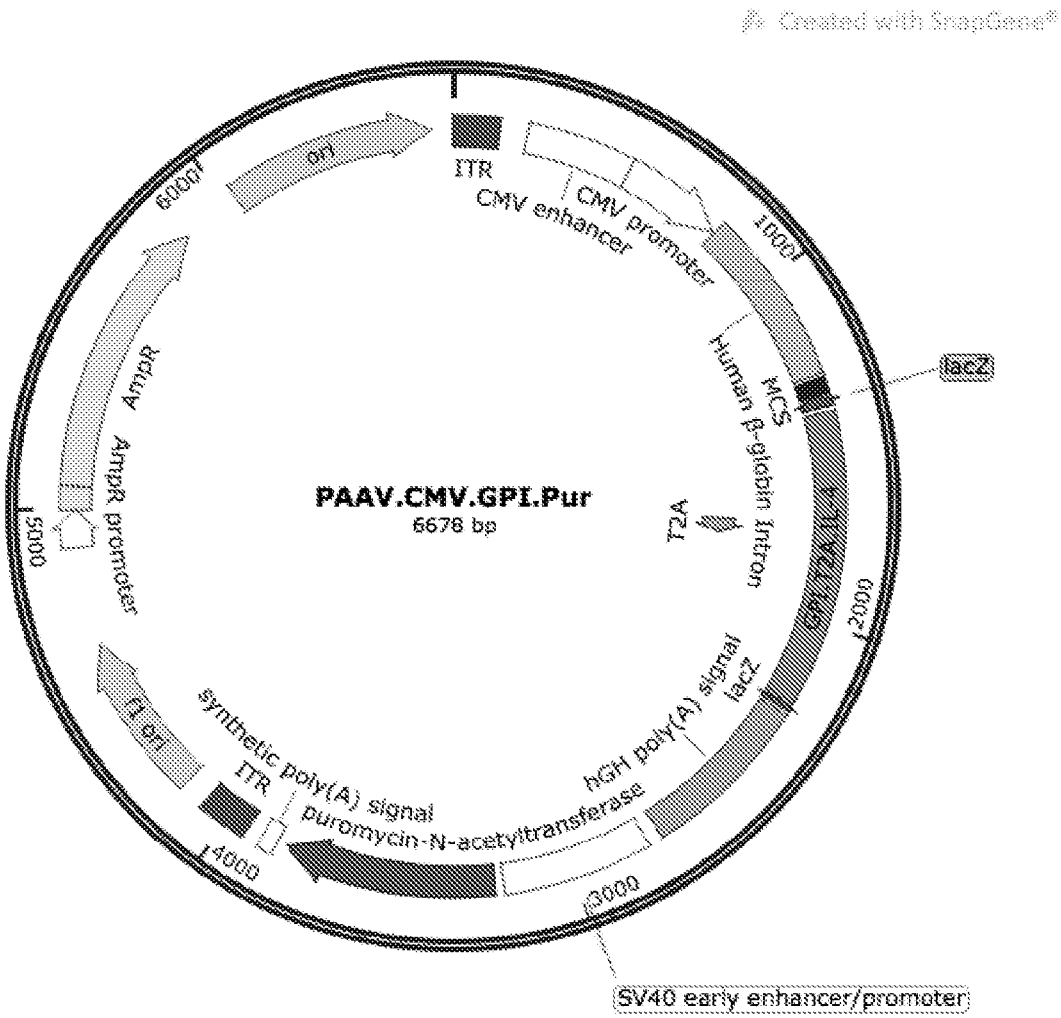

Created with SnapGene®
PAAV.CMV.GPI.Pur
6678 bp
ori
ITR
CMV enhancer
CMV promoter
Human β-globin Intron
MCS
lac2
hGH poly(A) signal
lacZ
synthetic poly(A) signal
puromycin-N-acetyltransferase
ITR
f1 ori
AmpR promoter
AmpR
1000
2000
3000
4000
5000
6000
SV40 early enhancer/promoter

Figure 33c

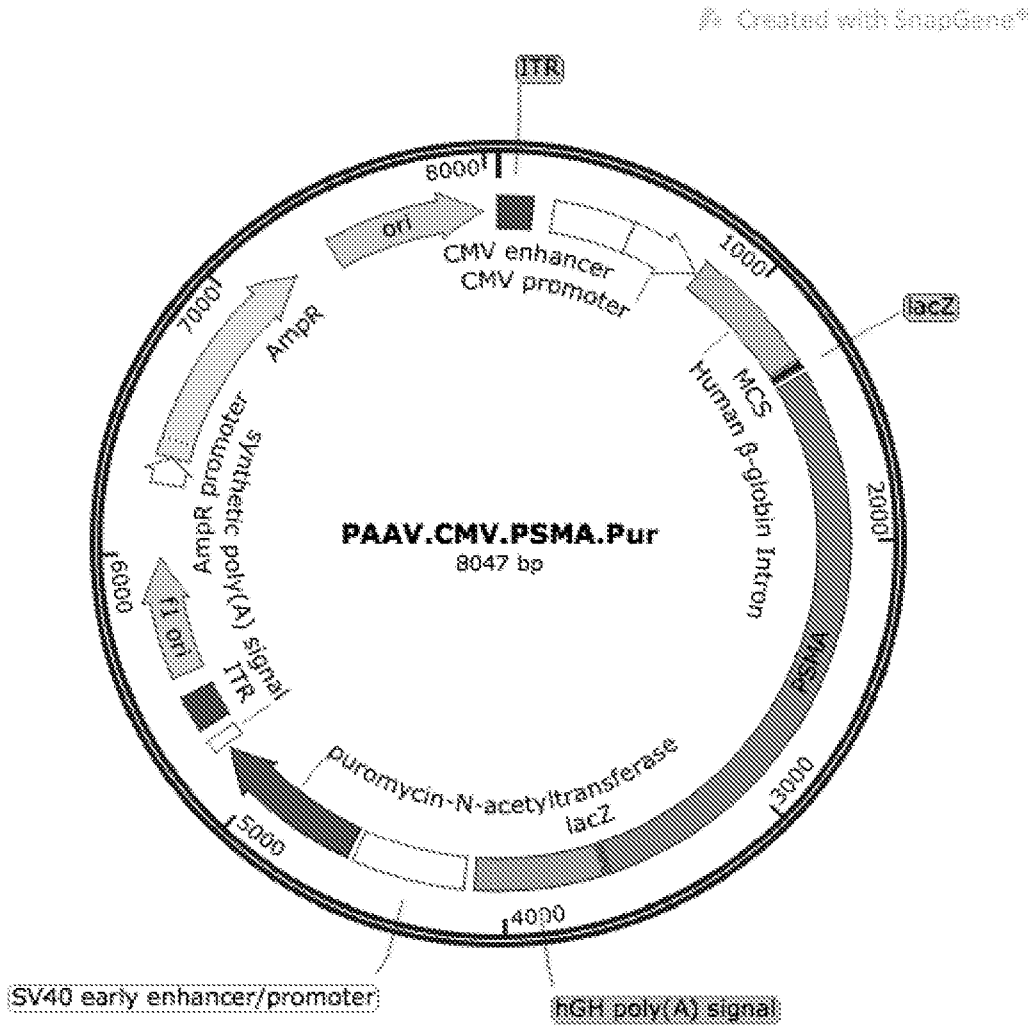

Created with SnapGene®
ITR
8000
ori
CMV enhancer
CMV promoter
1000
lacZ
MCS
Human β-globin Intron
PSMA
2000
3000
4000
5000
puromycin-N-acetyltransferase
lacZ
SV40 early enhancer/promoter
hGH poly(A) signal
6000
f1 ori
ITR
AmpR promoter
Synthetic poly(A) signal
7000
AmpR
PAAV.CMV.PSMA.Pur
8047 bp

Figure 33d

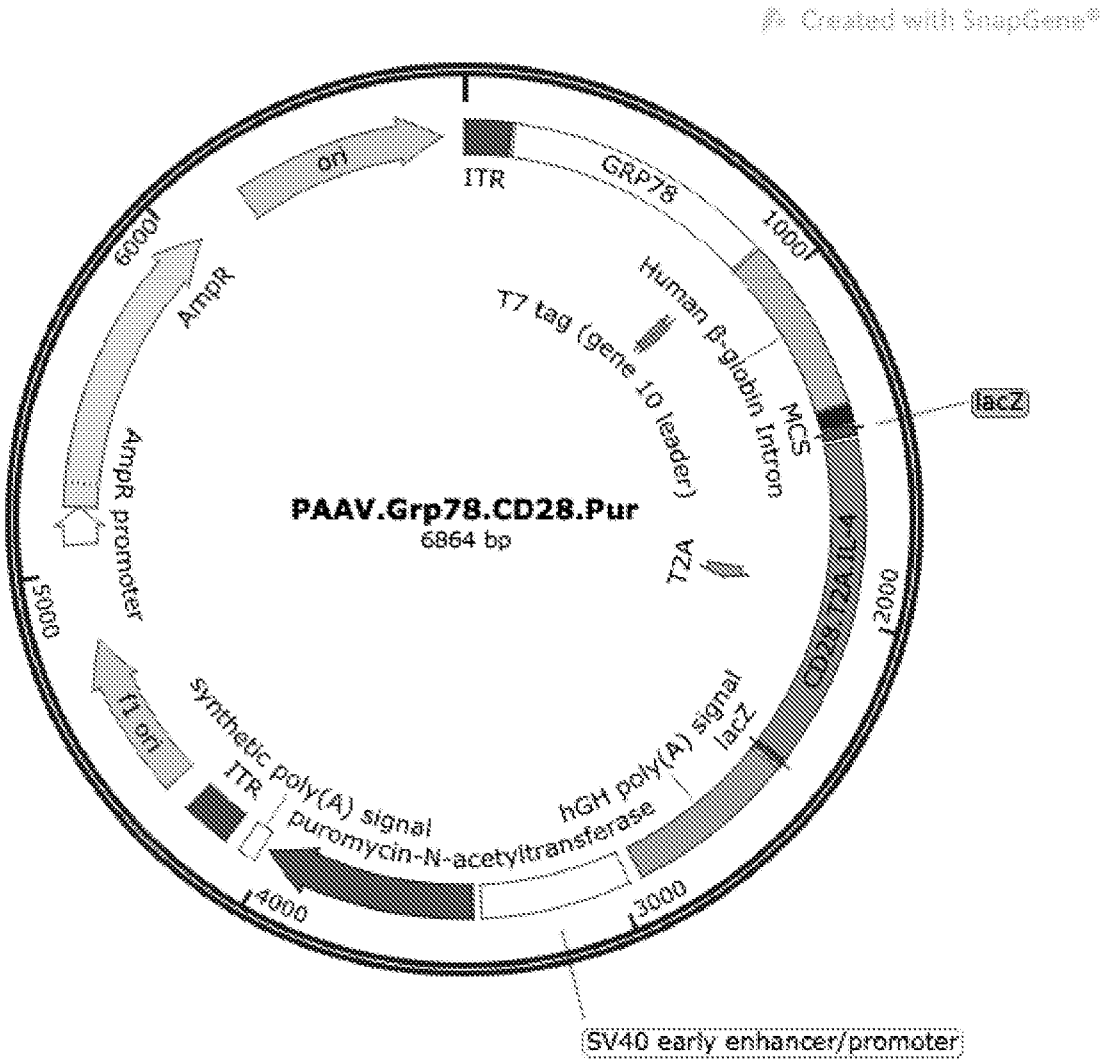

Created with SnapGene®
ori
ITR
GRP78
1000
Human β-globin Intron
T7 tag (gene 10 leader)
MCS
lacZ
PAAV.Grp78.CD28.Pur
6864 bp
T2A
2000
AmpR
6000
AmpR promoter
5000
f1 ori
synthetic poly(A) signal
ITR
puromycin-N-acetyltransferase
hGH poly(A) signal
lacZ
4000
3000
SV40 early enhancer/promoter

Figure 33e

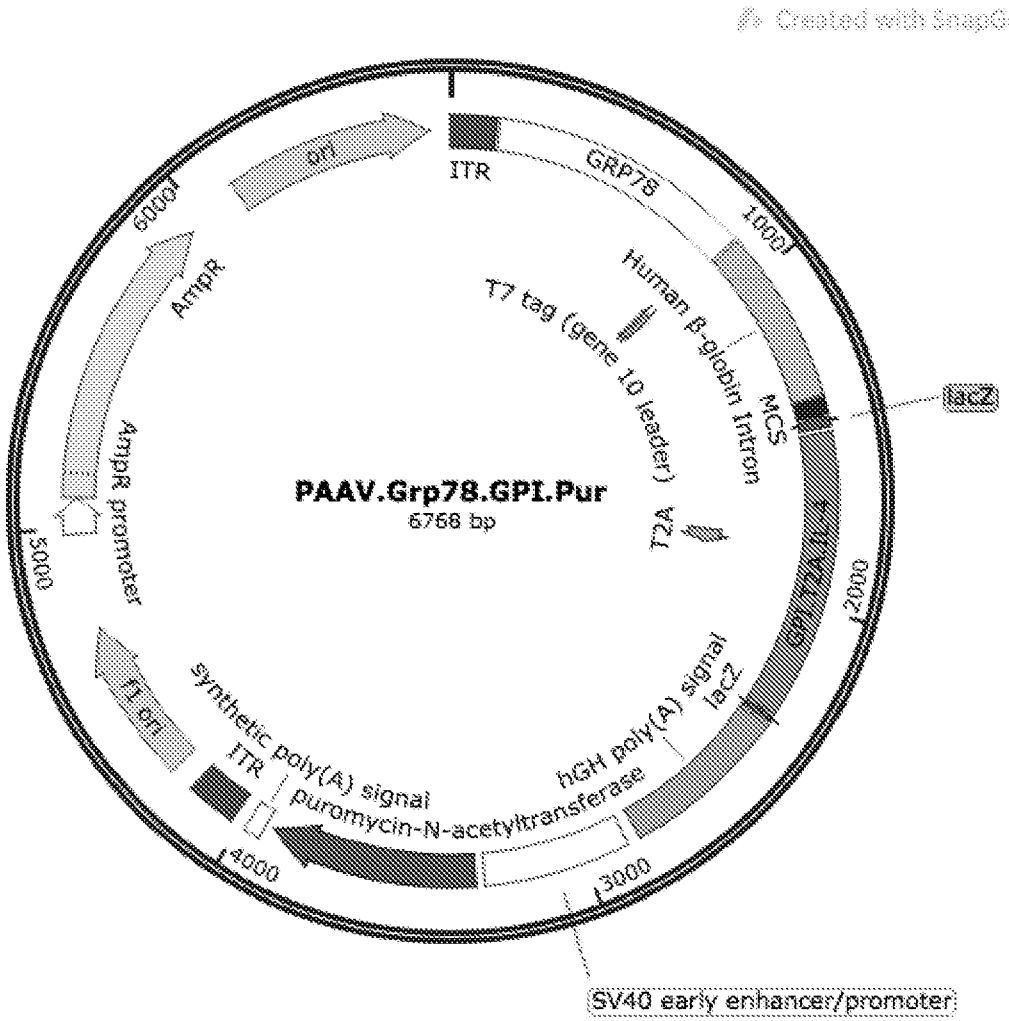

Created with SnapGene®
ori
ITR
GRP78
1000
Human β-globin Intron
MCS
lacZ
T7 tag (gene 10 leader)
T2A
PAAV.Grp78.GPI.Pur
6768 bp
GPI-T2A-LacZ
2000
hGH poly(A) signal
lacZ
3000
SV40 early enhancer/promoter
puromycin-N-acetyltransferase
synthetic poly(A) signal
ITR
4000
f1 ori
5000
AmpR promoter
AmpR
6000

Figure 33f

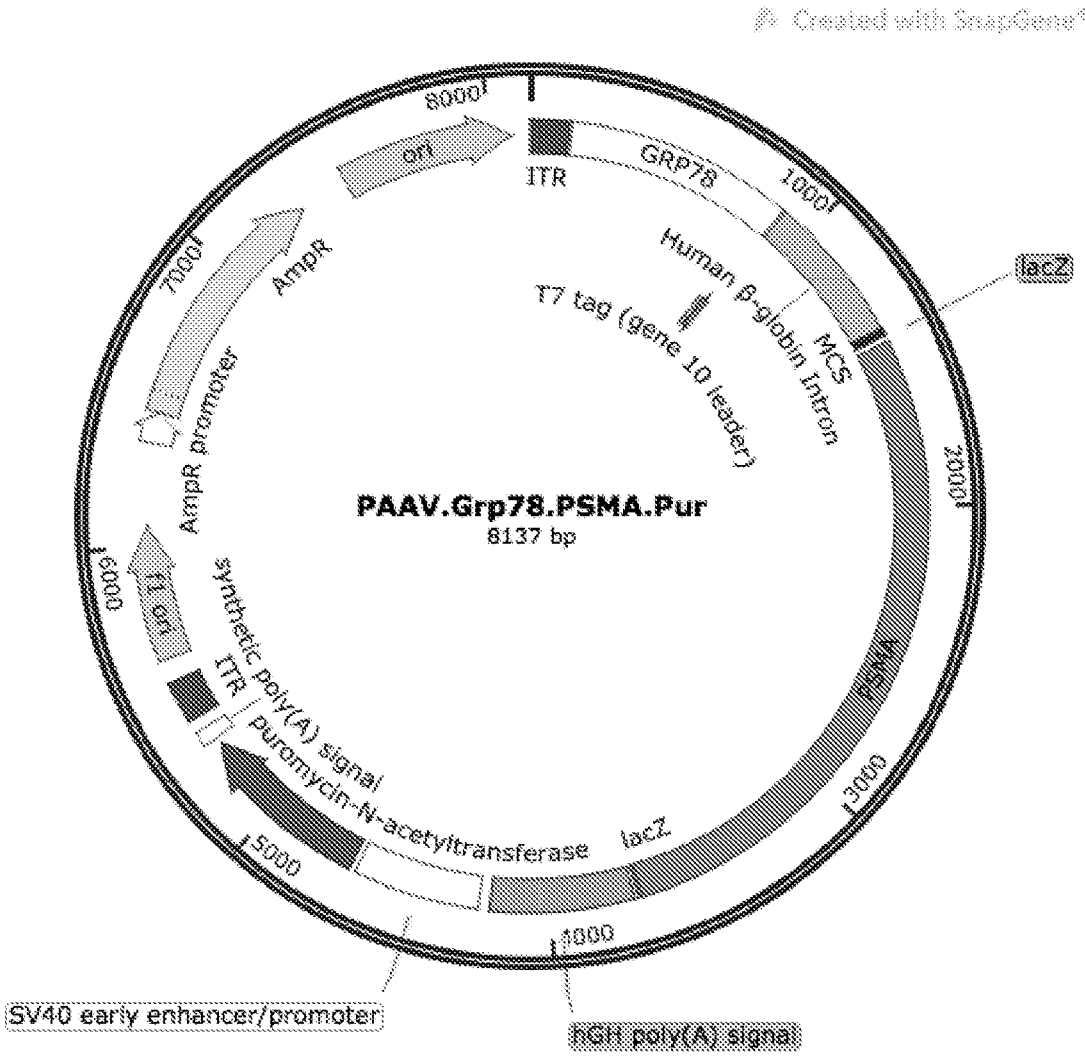

Created with SnapGene®
PAAV.Grp78.PSMA.Pur
8137 bp
8000
ori
ITR
GRP78
1000
Human β-globin intron
T7 tag (gene 10 leader)
MCS
lacZ
2000
PSMA
3000
lacZ
4000
hGH poly(A) signal
5000
puromycin-N-acetyltransferase
synthetic poly(A) signal
ITR
6000
f1 ori
AmpR promoter
7000
AmpR
SV40 early enhancer/promoter

Figure 34

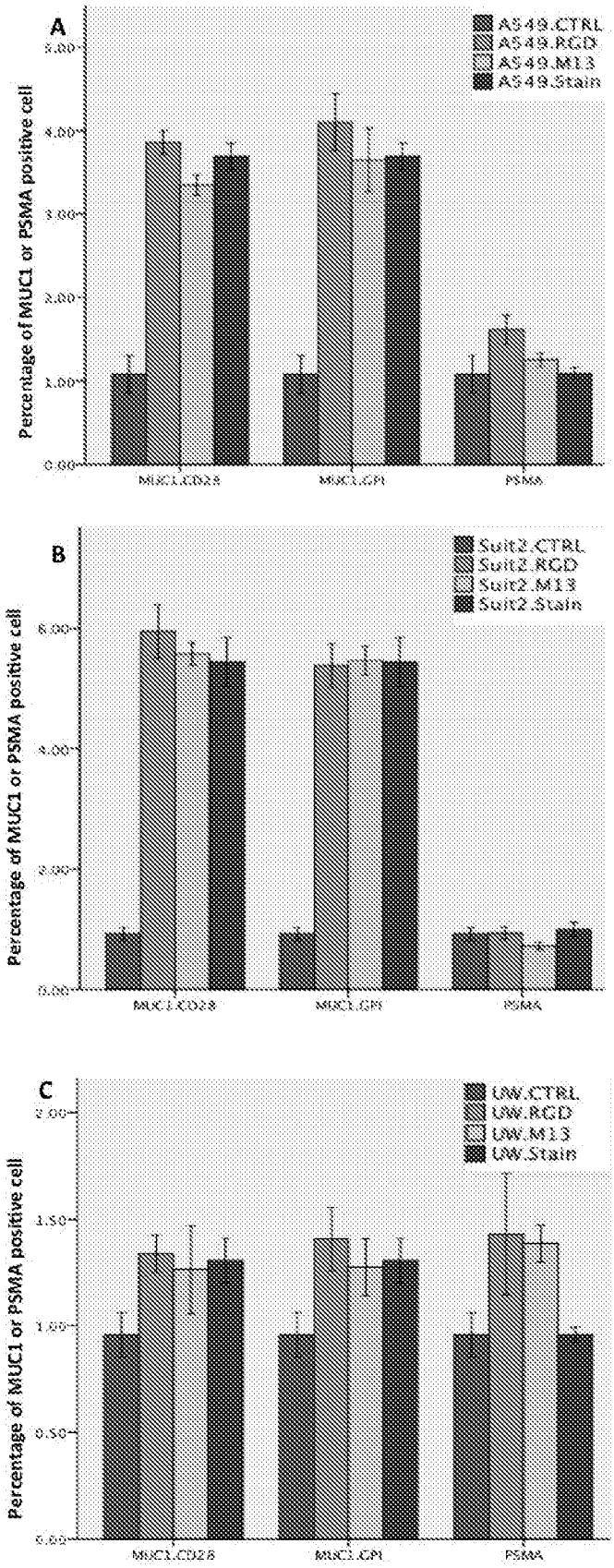

A
A549.CTRL
A549.RGD
A549.M13
A549.Stain
Percentage of MUC1 or PSMA positive cell
5.00
4.00
3.00
2.00
1.00
0.00
MUC1.CD28
MUC1.GPI
PSMA
B
Suit2.CTRL
Suit2.RGD
Suit2.M13
Suit2.Stain
Percentage of MUC1 or PSMA positive cell
6.00
4.00
2.00
0.00
MUC1.CD28
MUC1.GPI
PSMA
C
UW.CTRL
UW.RGD
UW.M13
UW.Stain
Percentage of MUC1 or PSMA positive cell
2.00
1.50
1.00
0.50
0.00
MUC1.CD28
MUC1.GPI
PSMA

Figure 35

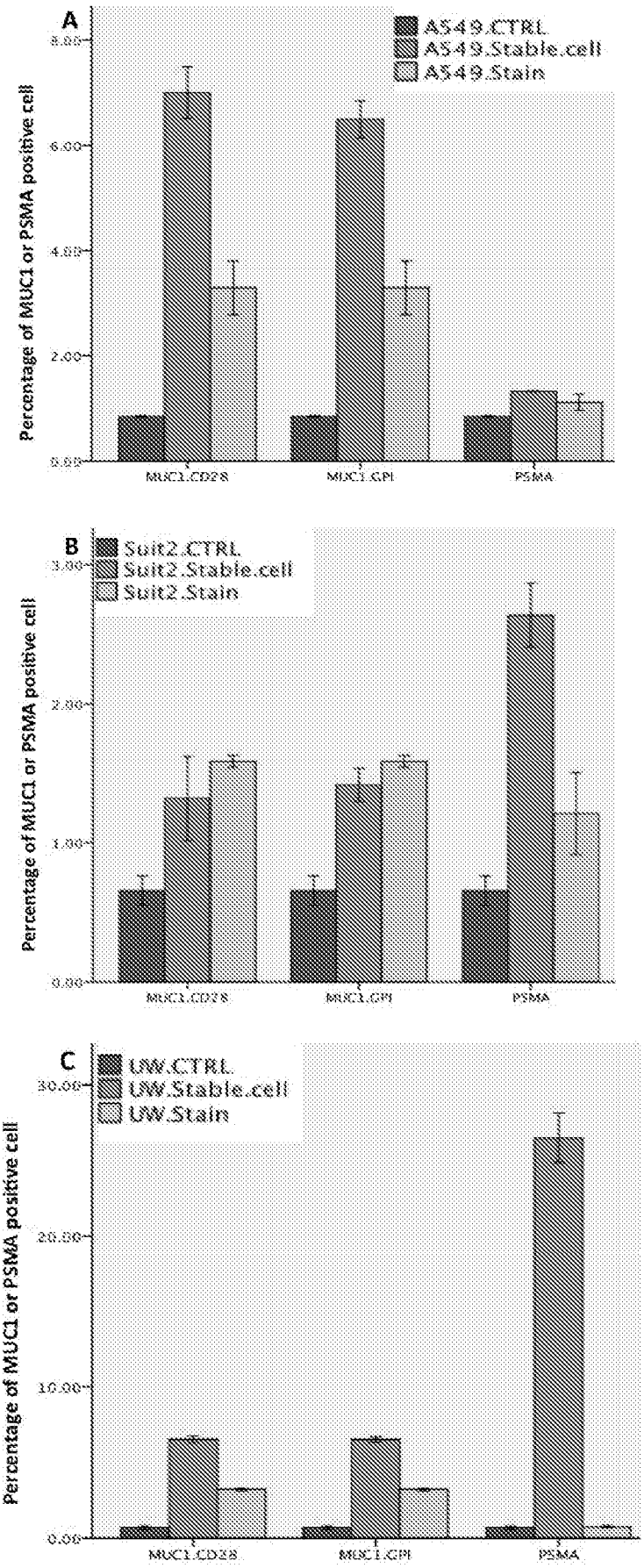
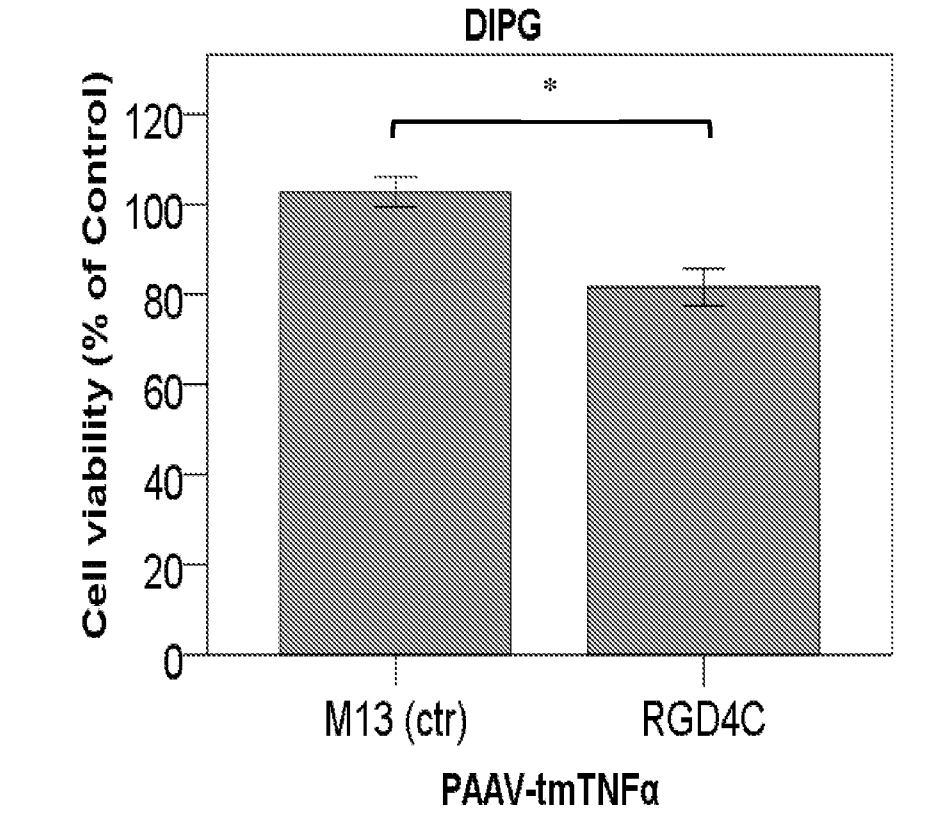

A
A549.CTRL
A549.Stable.cell
A549.Stain
Percentage of MUC1 or PSMA positive cell
8.00
6.00
4.00
2.00
0.00
MUC1.CD28
MUC1.GPI
PSMA
B
Suit2.CTRL
Suit2.Stable.cell
Suit2.Stain
Percentage of MUC1 or PSMA positive cell
3.00
2.00
1.00
0.00
MUC1.CD28
MUC1.GPI
PSMA
C
UW.CTRL
UW.Stable.cell
UW.Stain
Percentage of MUC1 or PSMA positive cell
30.00
20.00
10.00
0.00
MUC1.CD28
MUC1.GPI
PSMA

Figure 36


DIPG
*
Cell viability (% of Control)
120
100
80
60
40
20
0
M13 (ctr)
RGD4C
PAAV-tmTNFα

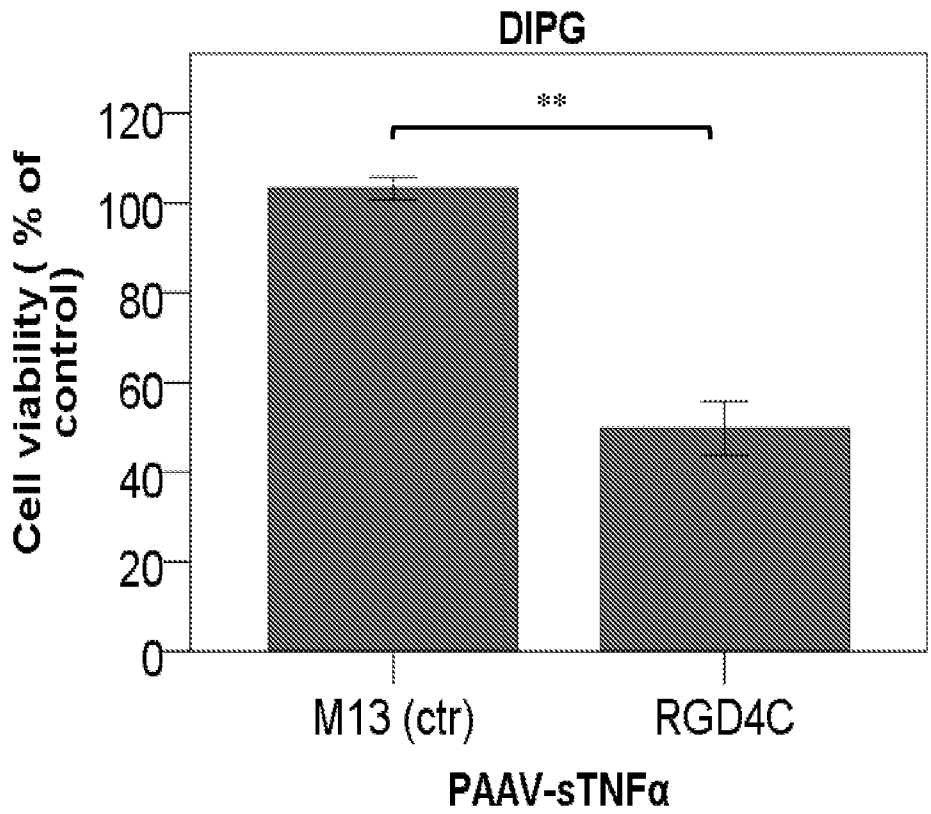

DIPG
**
Cell viability (% of control)
120
100
80
60
40
20
0
M13 (ctr)
RGD4C
PAAV-sTNFα

Figure 37

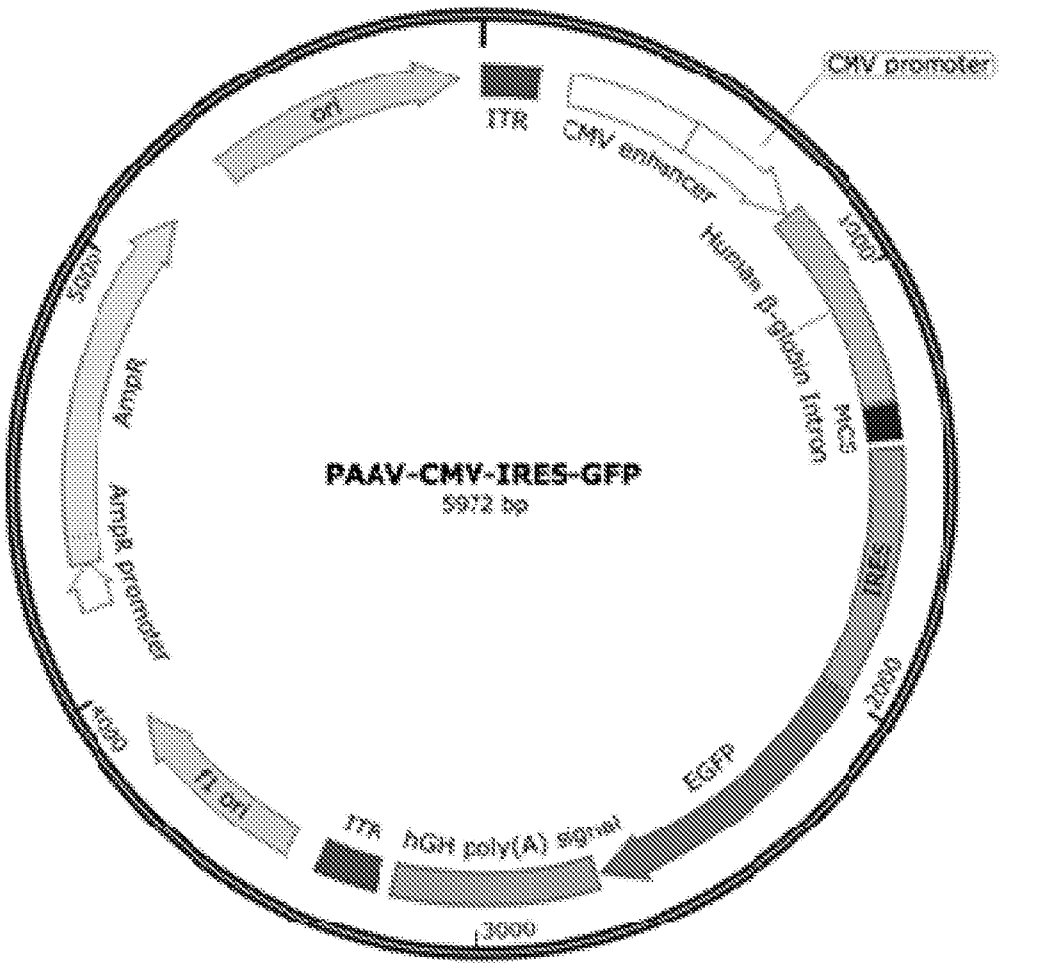

Created with SnapGene
CMV promoter
ori
ITR
CMV enhancer
Human β-globin intron
MCS
EGFP
hGH poly(A) signal
ITR
f1 ori
KanR
Amp^R promoter
AmpR
SV40
PAAV-CMV-IRES-GFP
5972 bp

Figure 37 continued

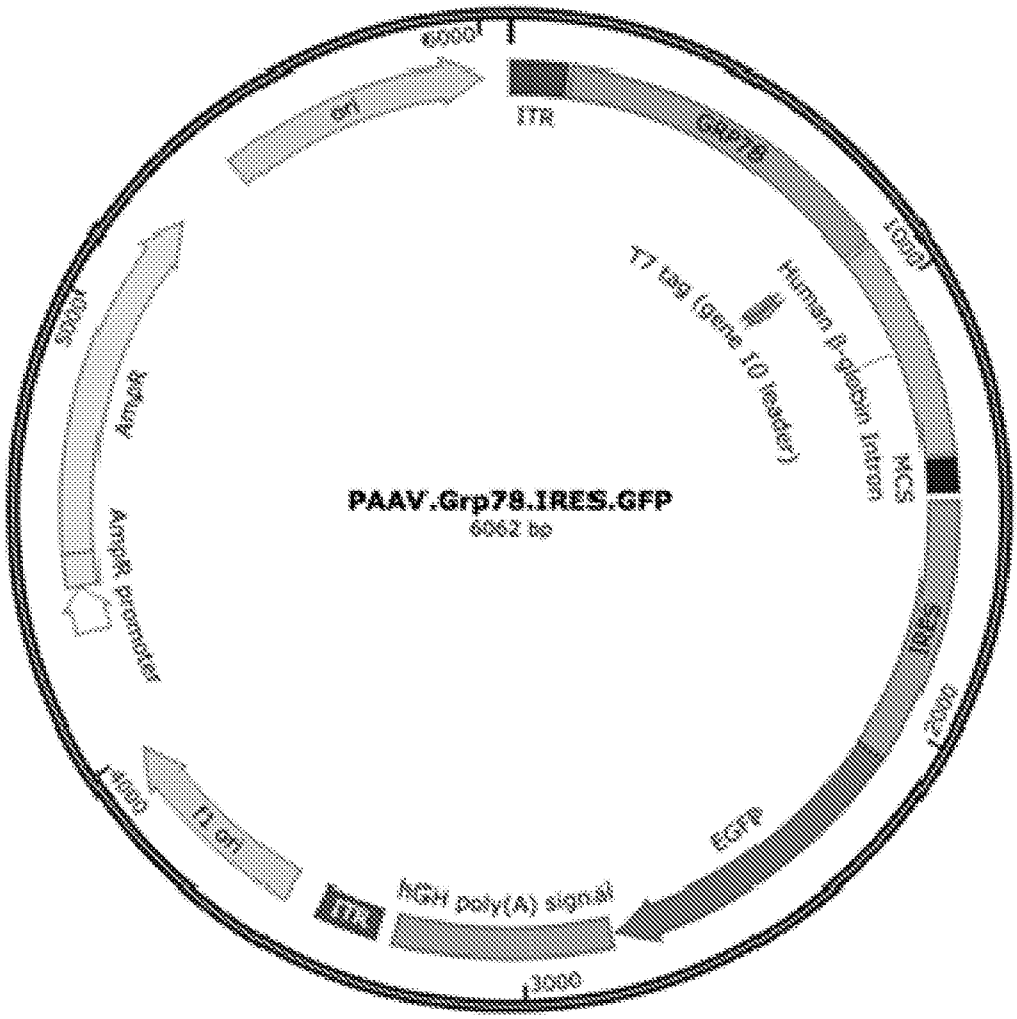
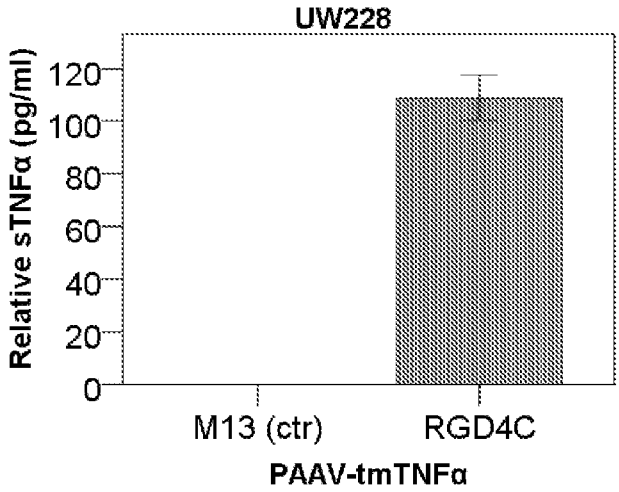


Figure 38

A.


UW228
Relative sTNFα (pg/ml)
120
100
80
60
40
20
0
M13 (ctr)
RGD4C
PAAV-tmTNFα

B.

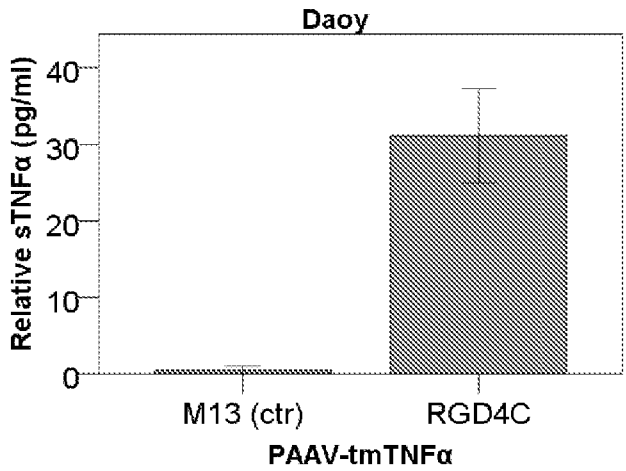

Daoy
Relative sTNFα (pg/ml)
40
30
20
10
0
M13 (ctr)
RGD4C
PAAV-tmTNFα

Figure 39

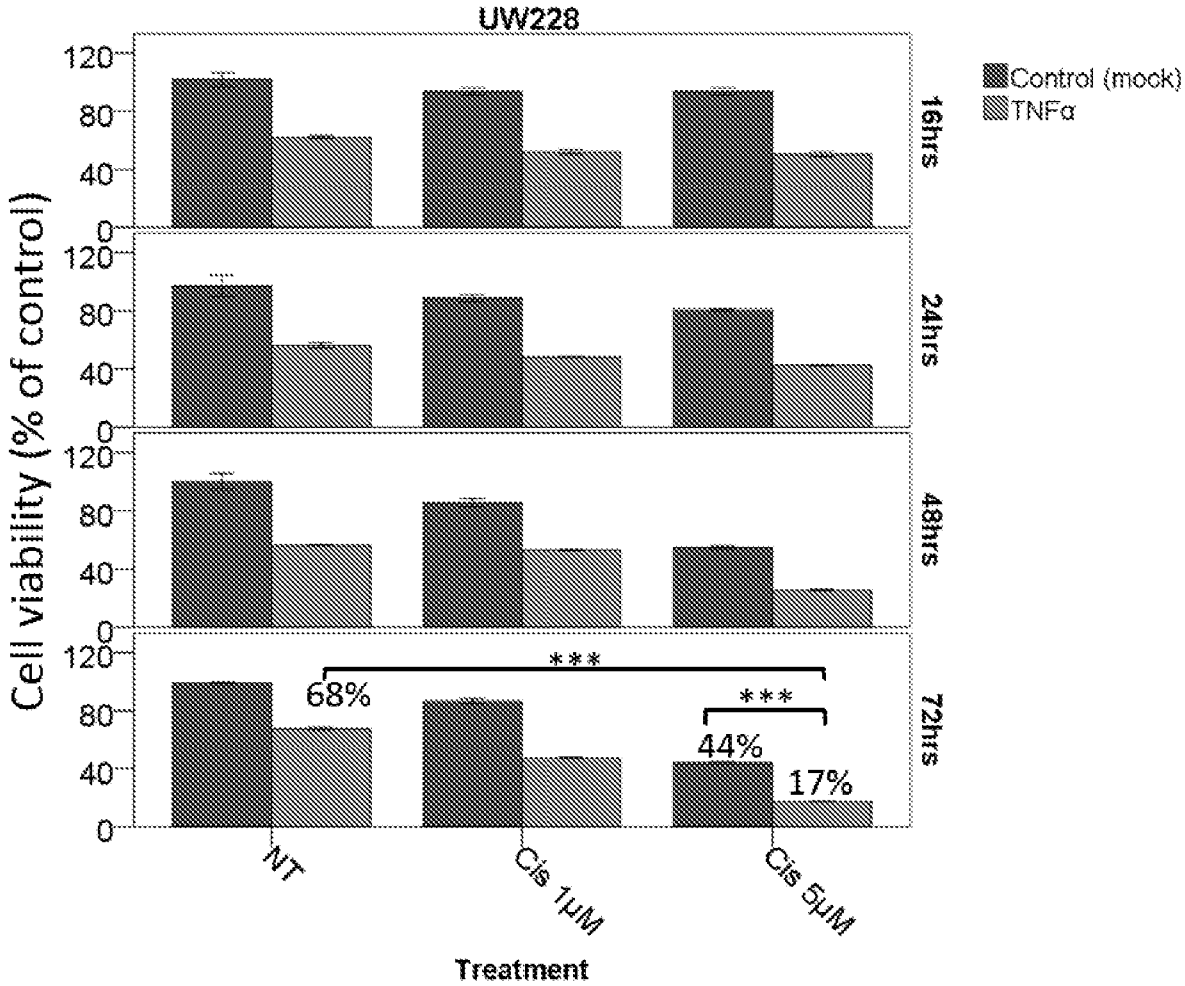

UW228
Control (mock)
TNFα
16hrs
24hrs
48hrs
72hrs
Cell viability (% of control)
120
80
40
0
***
68%
***
44%
17%
NT
Cis 1μM
Cis 5μM
Treatment

Figure 40

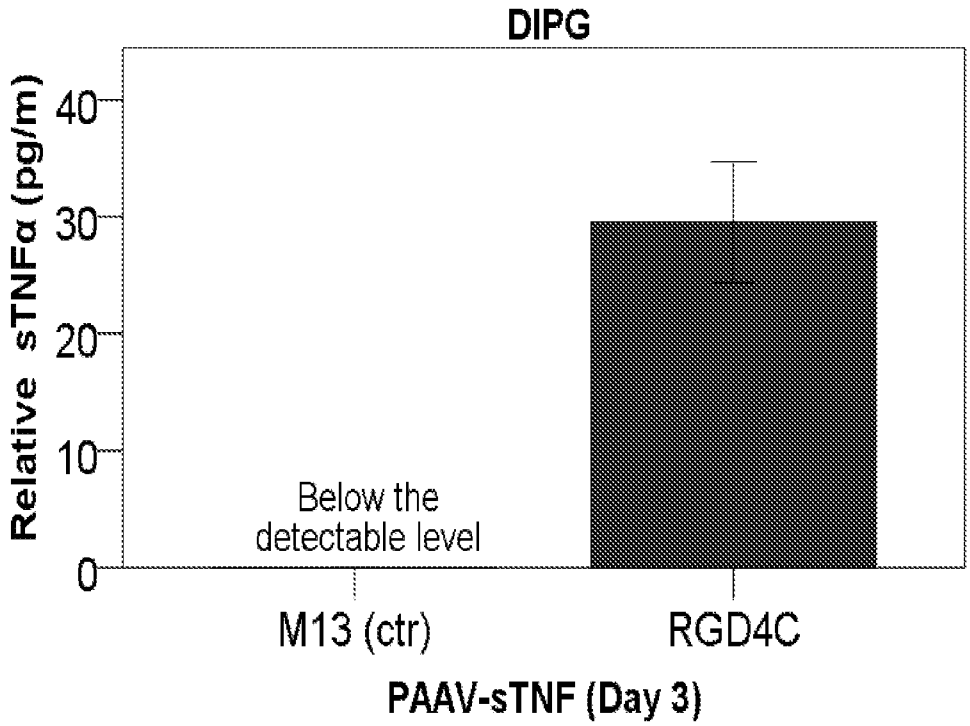

DIPG
Relative sTNFα (pg/m)
40
30
20
10
0
Below the detectable level
M13 (ctr)
RGD4C
PAAV-sTNF (Day 3)

Figure 41

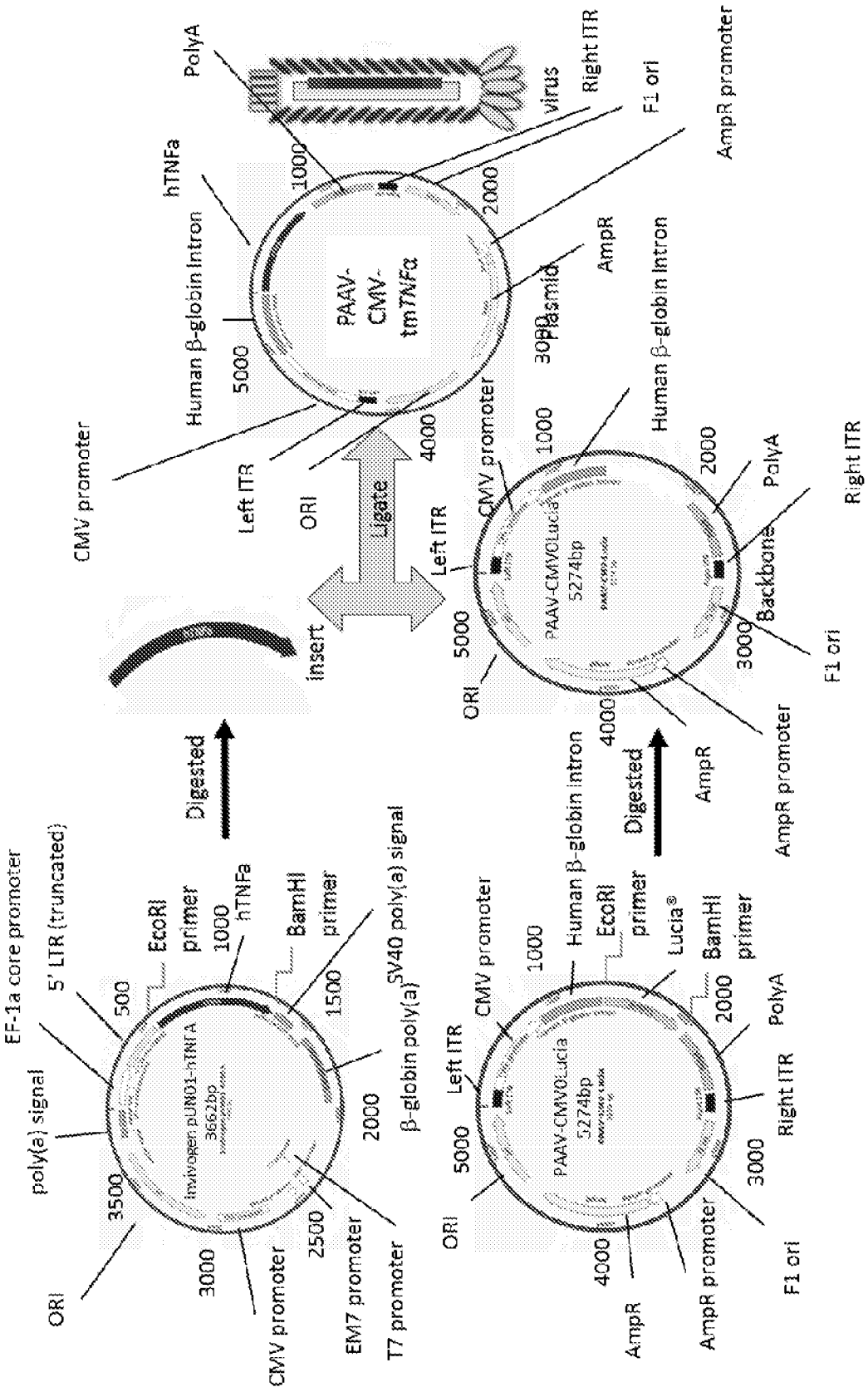

PolyA
virus
Right ITR
F1 ori
AmpR promoter
hTNFa
Human β-globin Intron
PAAV-CMV-tmTNFa
Plasmid
AmpR
CMV promoter
Left ITR
ORI
Ligate
Insert
Digested
EF-1a core promoter
5' LTR (truncated)
poly(a) signal
ORI
EcoRI primer
hTNFa
BamHI primer
SV40 poly(a) signal
β-globin poly(a)
CMV promoter
EM7 promoter
T7 promoter
Invivogen pUNO1-hTNFA
3662bp
PAAV-CMV0Lucia
5274bp
Lucia®
Backbone
AmpR
AmpR promoter
F1 ori
Right ITR
PolyA

Figure 42

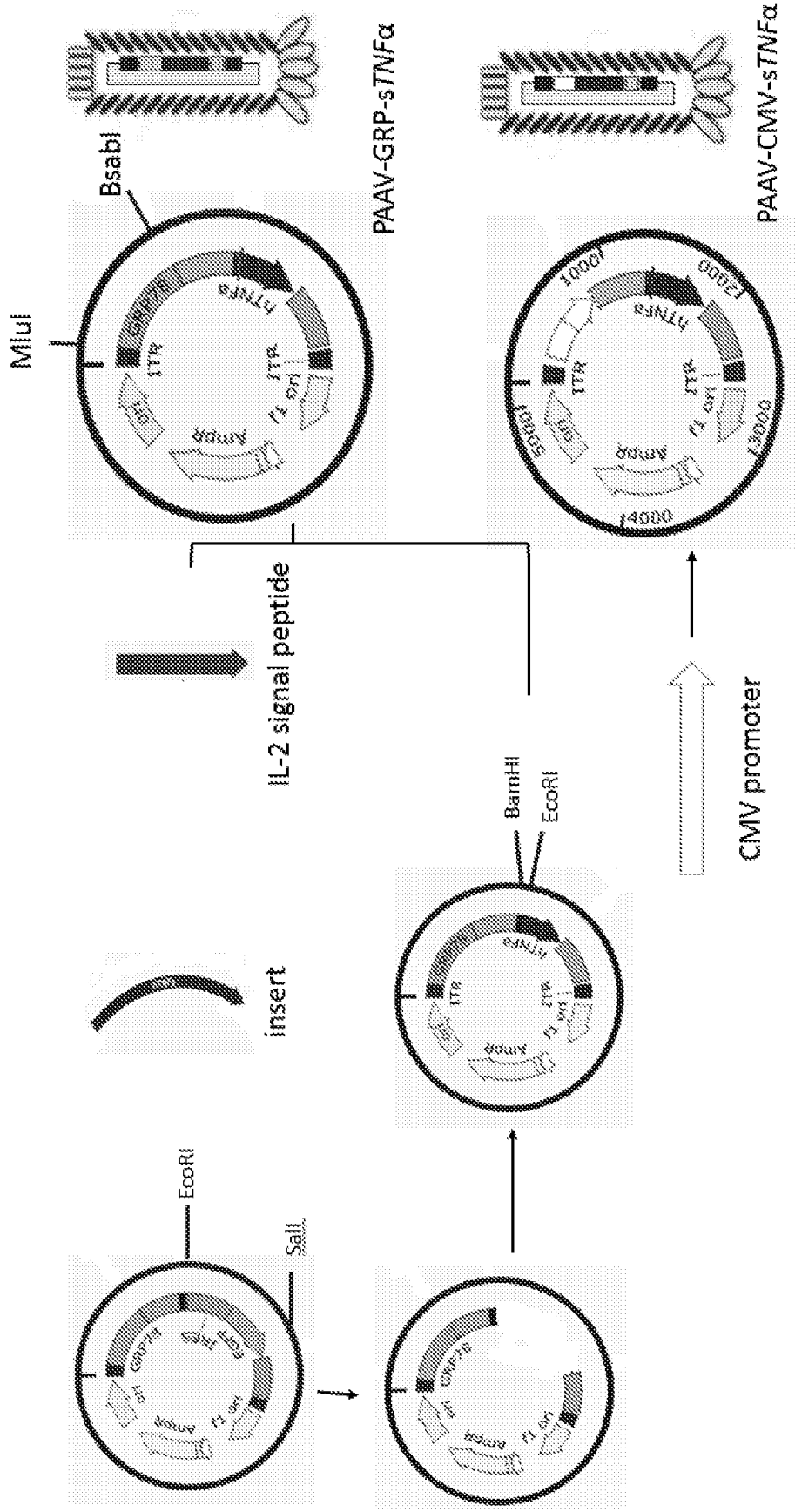

PAAV-GRP-sTNFα
PAAV-CMV-sTNFα
BsabI
MluI
IL-2 signal peptide
CMV promoter
BamHI
EcoRI
insert
EcoRI
SalI

Figure 43

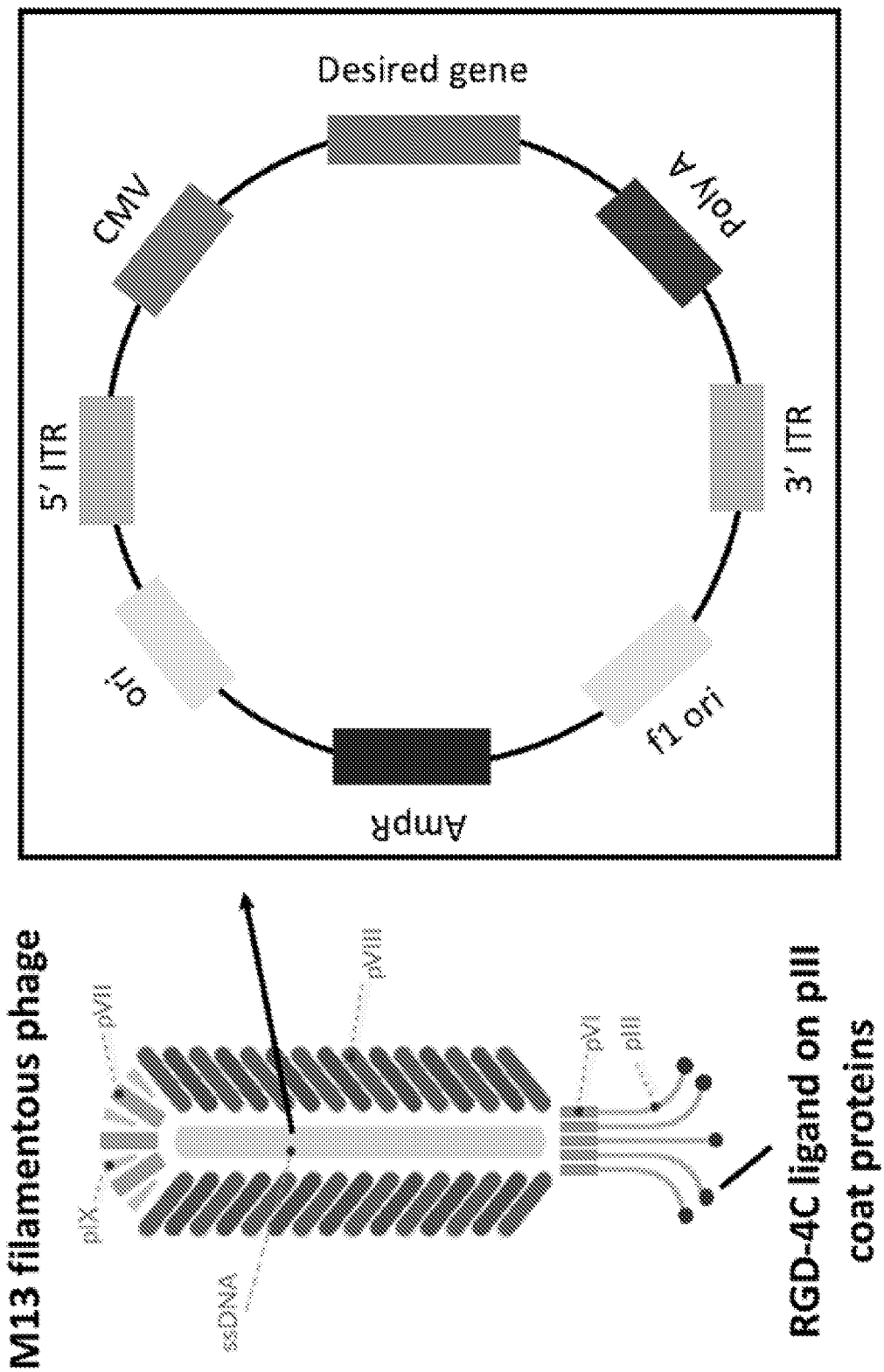

Desired gene
CMV
Poly A
5' ITR
3' ITR
ori
f1 ori
AmpR
M13 filamentous phage
pIX
pVIII
pVIII
pVI
pIII
ssDNA
RGD-4C ligand on pIII
coat proteins

Figure 44

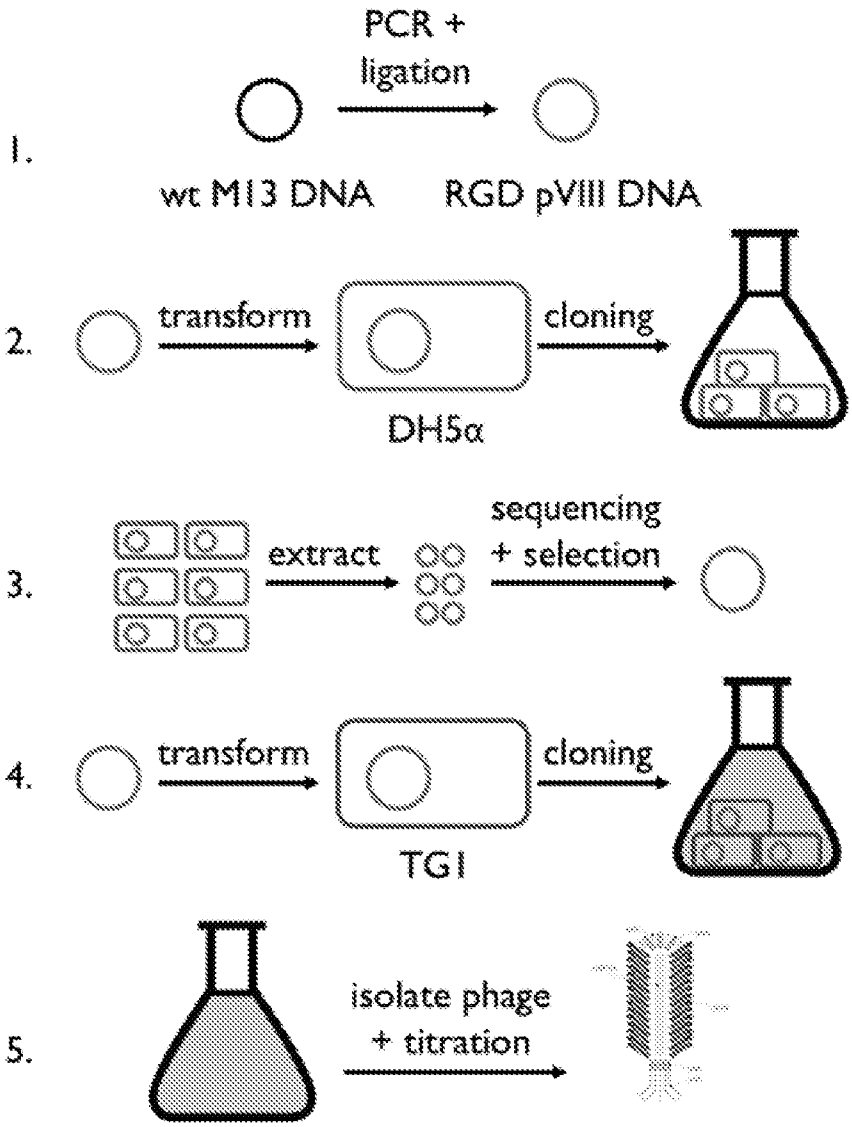

1.
PCR +
ligation
wt M13 DNA
RGD pVIII DNA
2.
transform
cloning
DH5α
3.
extract
sequencing
+ selection
4.
transform
cloning
TG1
5.
isolate phage
+ titration

Figure 45

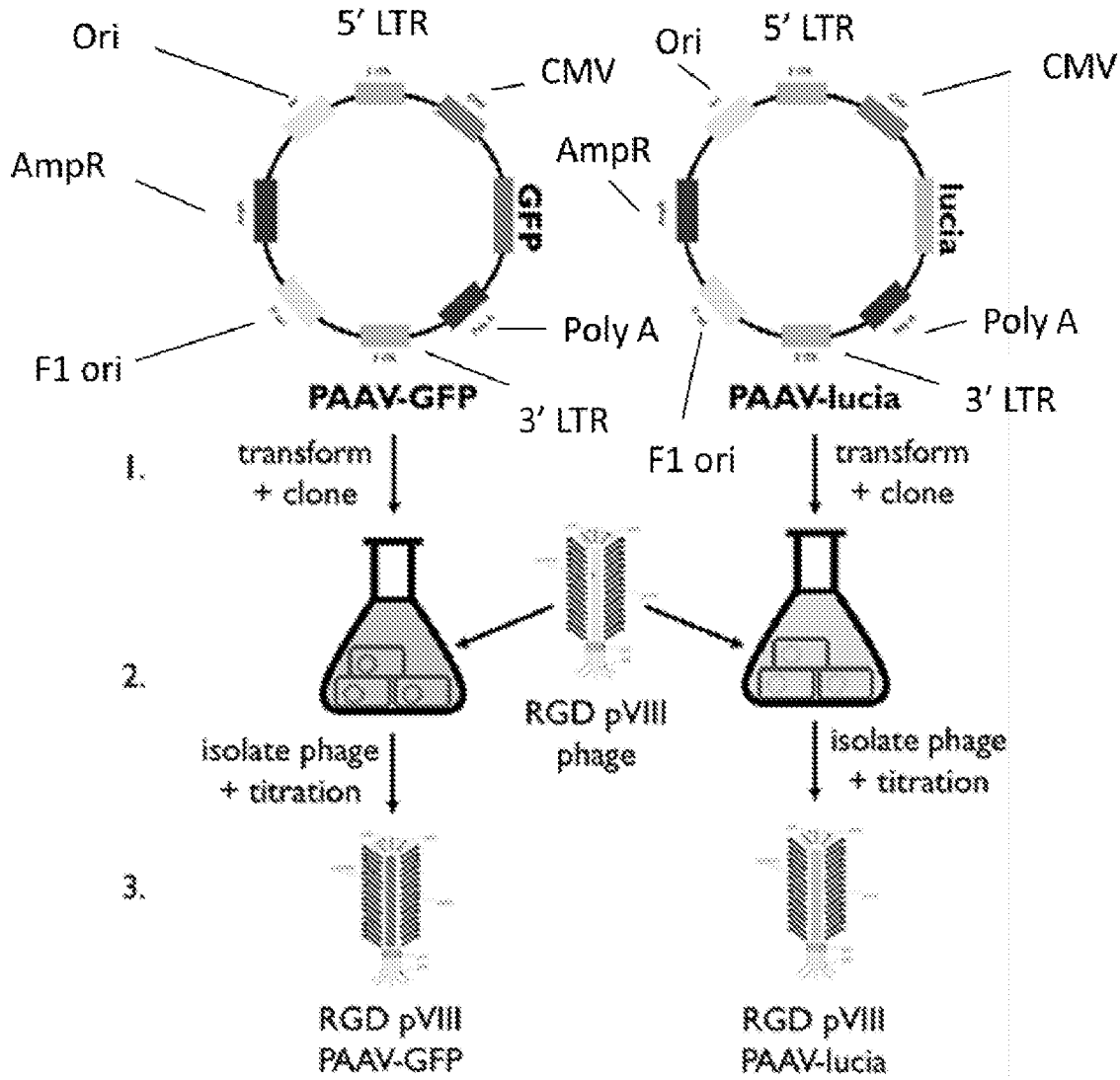

Ori
5' LTR
CMV
AmpR
GFP
Poly A
F1 ori
PAAV-GFP
3' LTR
Ori
5' LTR
CMV
AmpR
lucia
Poly A
PAAV-lucia
3' LTR
F1 ori
1.
transform
+ clone
transform
+ clone
2.
RGD pVIII
phage
isolate phage
+ titration
isolate phage
+ titration
3.
RGD pVIII
PAAV-GFP
RGD pVIII
PAAV-lucia

Figure 46

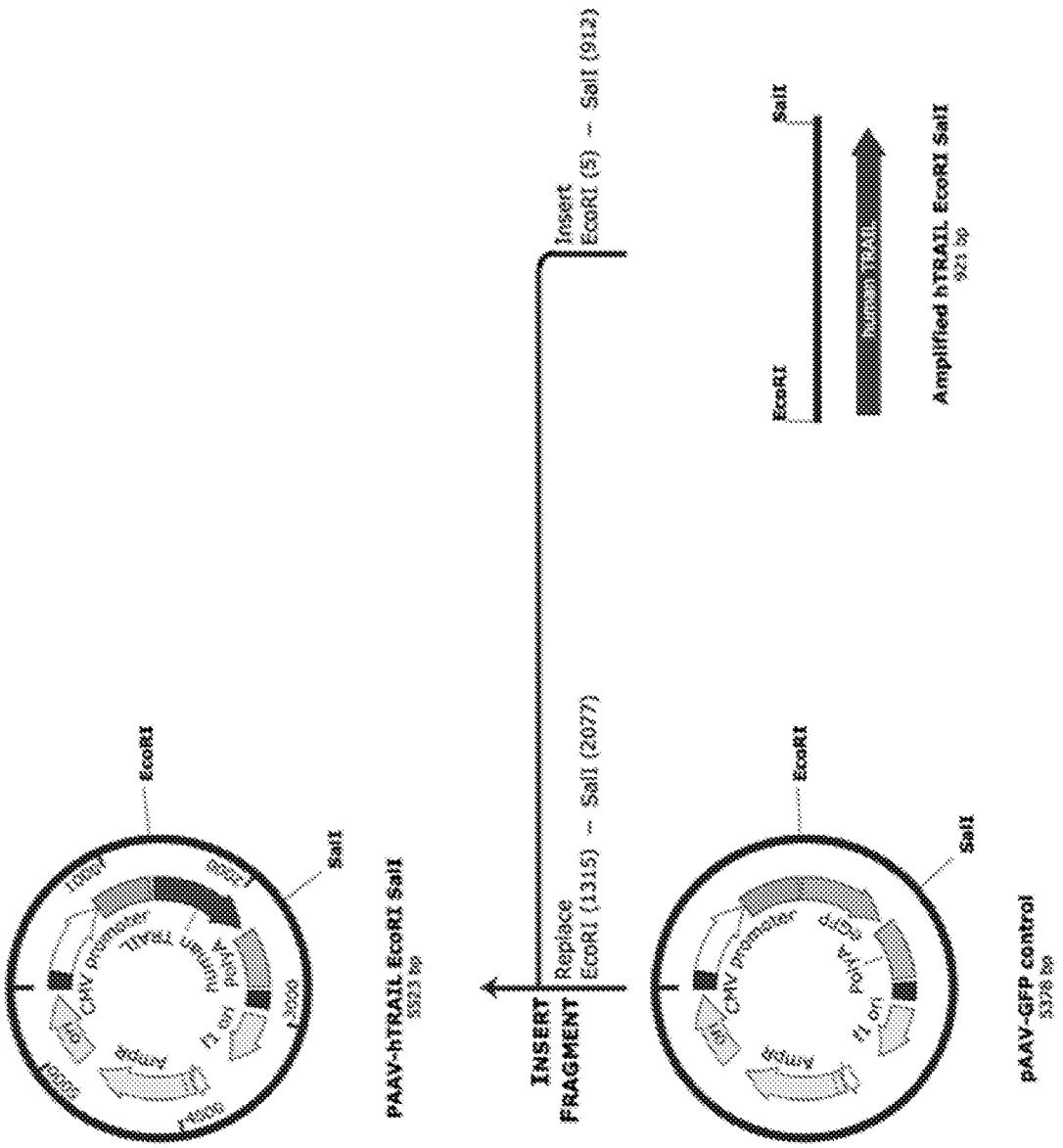
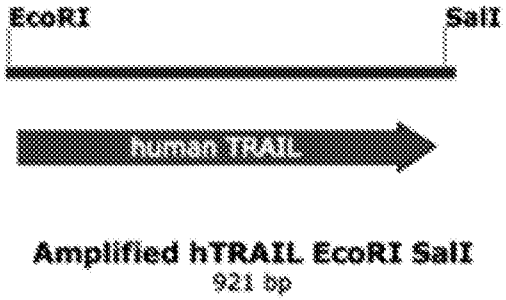
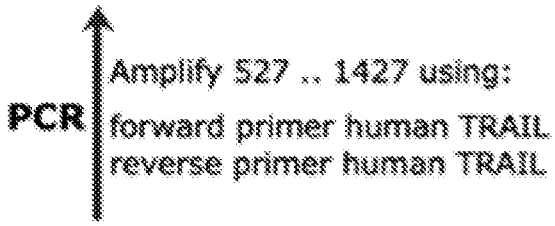
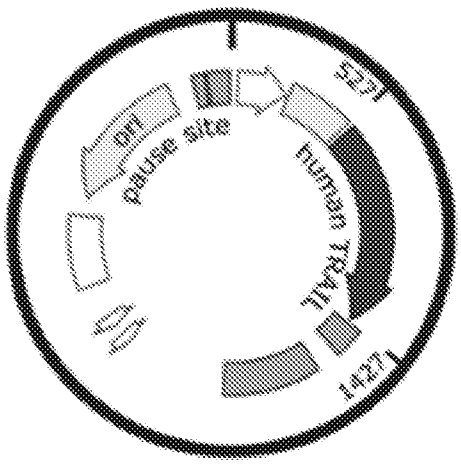

EcoRI
SalI
CMV promoter
pAAV-hTRAIL EcoRI SalI
5323 bp
INSERT FRAGMENT
Insert EcoRI (8) ~ SalI (913)
Replace EcoRI (1315) ~ SalI (2077)
EcoRI
SalI
Amplified hTRAIL EcoRI SalI
921 bp
EcoRI
SalI
CMV promoter
pAAV-GFP control
5378 bp

Figure 46 continued



Amplified hTRAIL EcoRI SalI
921 bp





pUNO1-hTRAIL
4026 bp

Figure 47

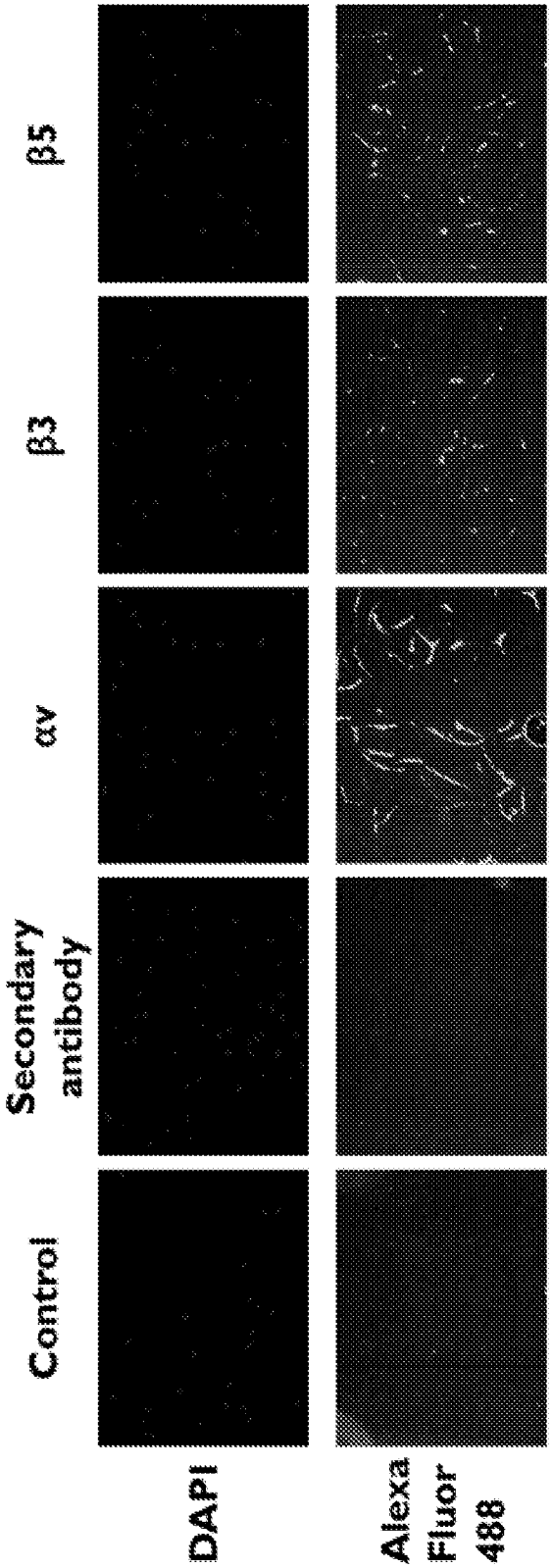


Figure 48

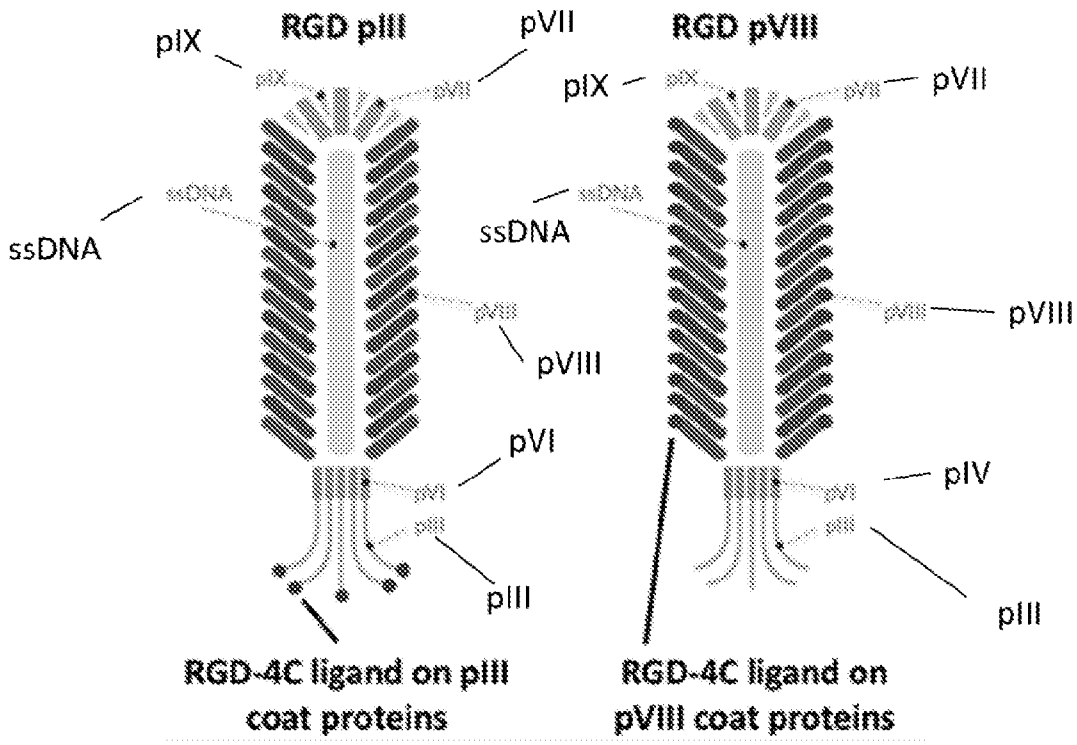

RGD pIII
pIX
pVII
ssDNA
pVIII
pVI
pIII
RGD-4C ligand on pIII coat proteins
RGD pVIII
pIX
pVII
ssDNA
pVIII
pIV
pIII
RGD-4C ligand on pVIII coat proteins

Figure 49

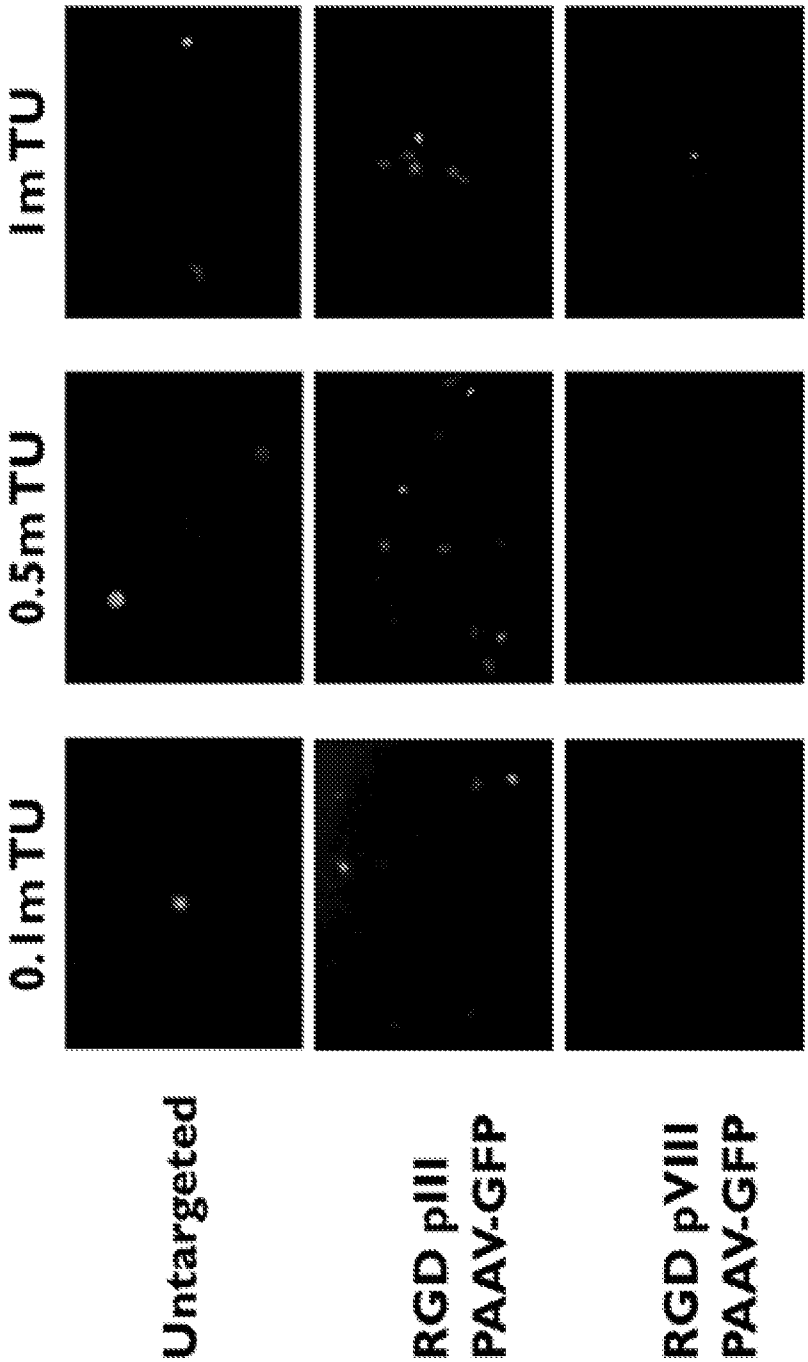

1m TU
0.5m TU
0.1m TU
Untargeted
RGD pIII PAAV-GFP
RGD pVIII PAAV-GFP

Figure 50

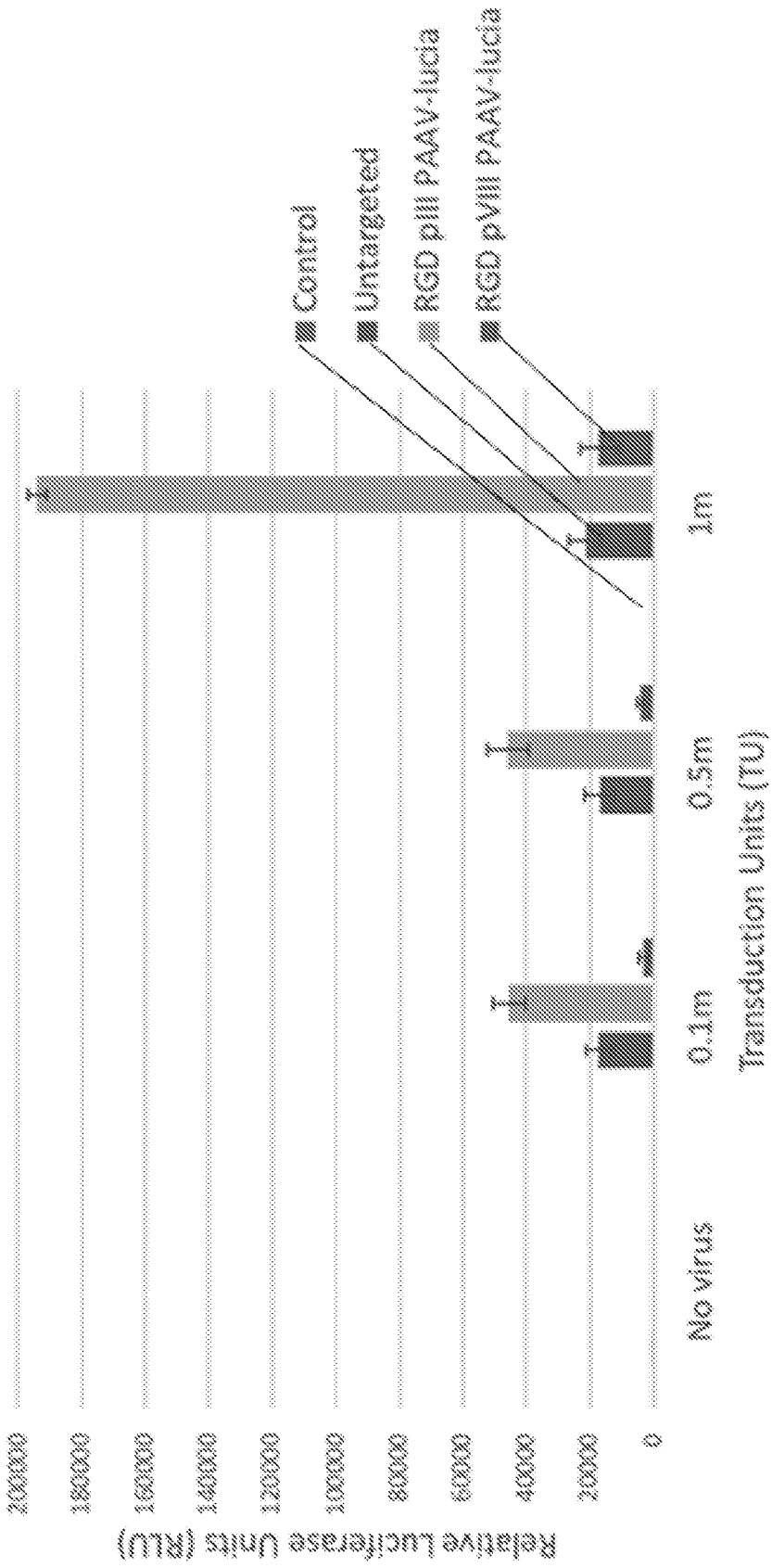

Control
Untargeted
RGD pIII PAAV-lucia
RGD pVIII PAAV-lucia
Relative Luciferase Units (RLU)
200000
180000
160000
140000
120000
100000
80000
60000
40000
20000
0
No virus
0.1m
0.5m
1m
Transduction Units (TU)

Figure 51

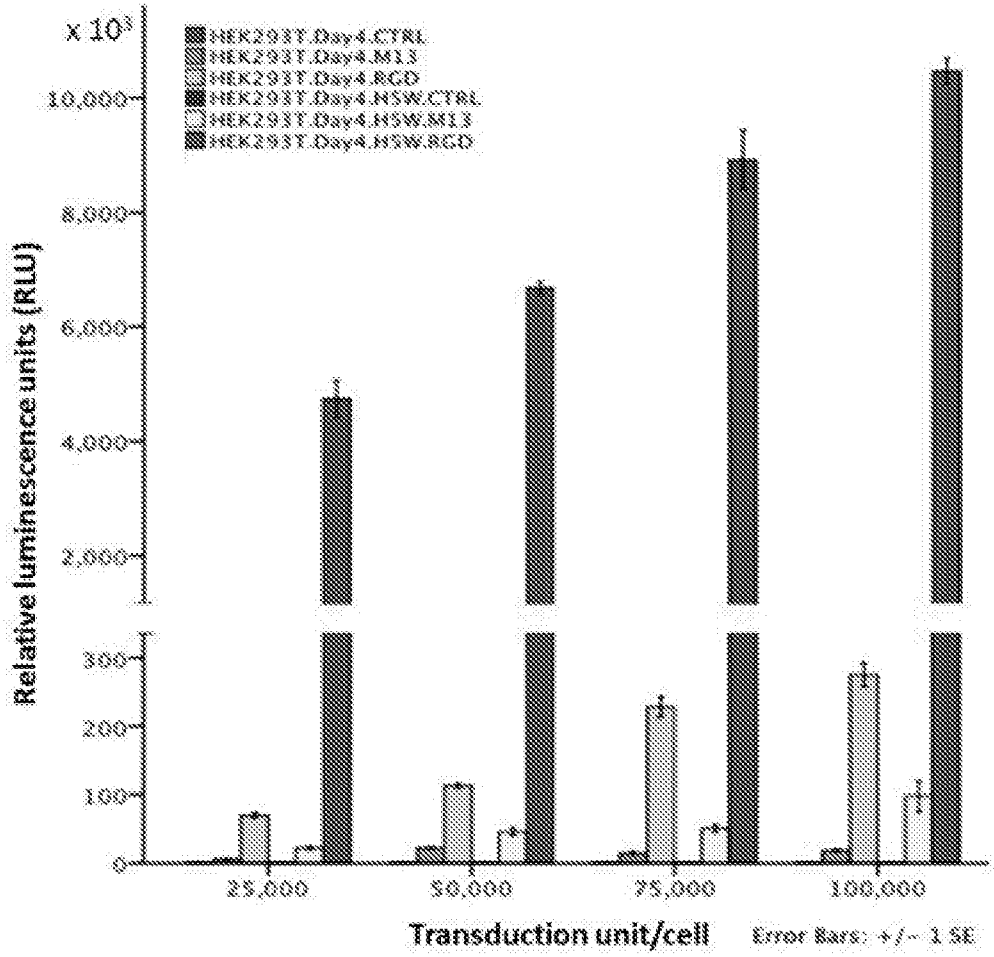

x 10³
HEK293T.Day4.CTRL
HEK293T.Day4.M13
HEK293T.Day4.RGD
HEK293T.Day4.H5W.CTRL
HEK293T.Day4.H5W.M13
HEK293T.Day4.H5W.RGD
Relative luminescence units (RLU)
10,000
8,000
6,000
4,000
2,000
300
200
100
0
25,000
50,000
75,000
100,000
Transduction unit/cell
Error Bars: +/- 1 SE

Figure 52

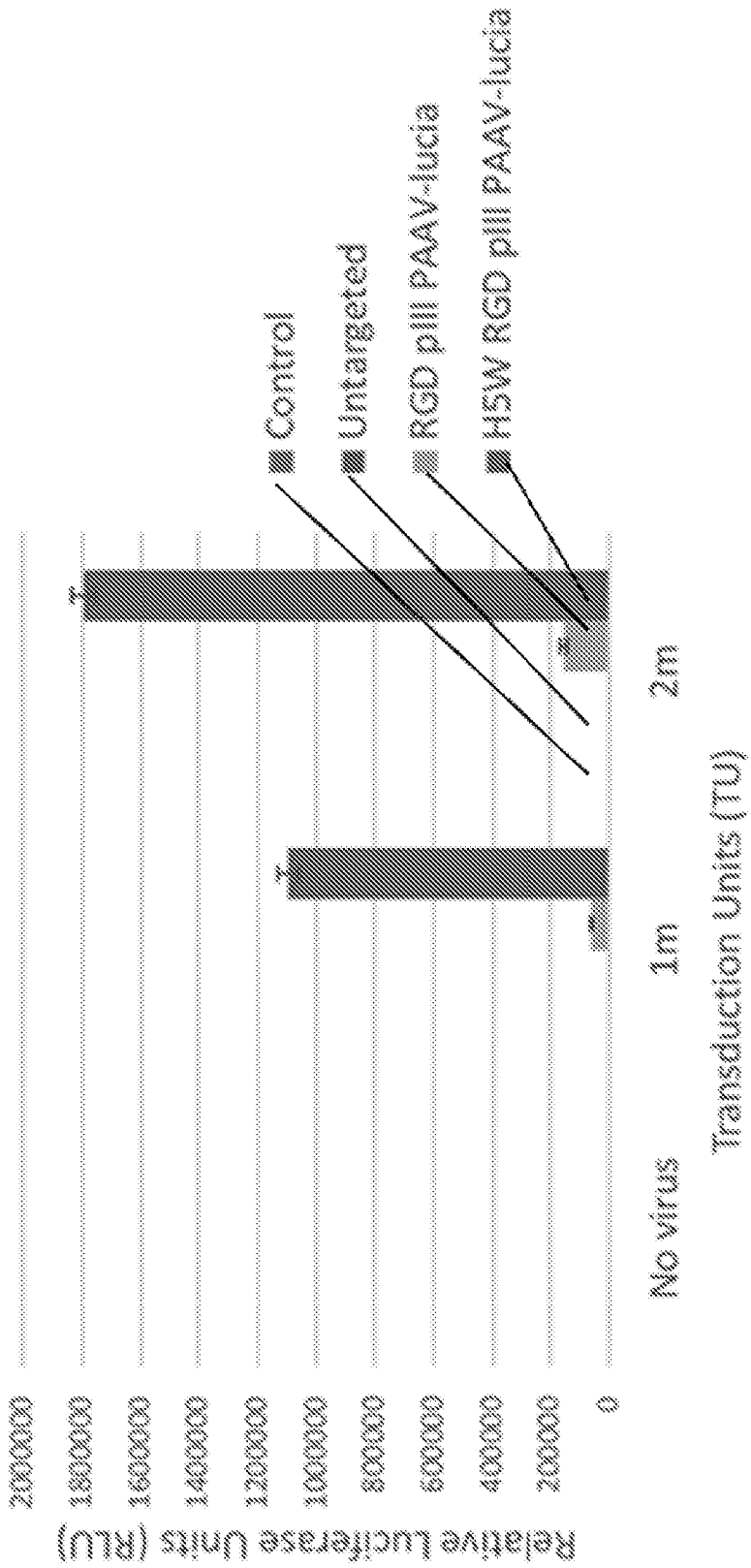

Control
Untargeted
RGD pIII PAAV-lucia
H5W RGD pIII PAAV-lucia
Relative luciferase Units (RLU)
2000000
1800000
1600000
1400000
1200000
1000000
800000
600000
400000
200000
0
No virus
1m
2m
Transduction Units (TU)

Figure 53

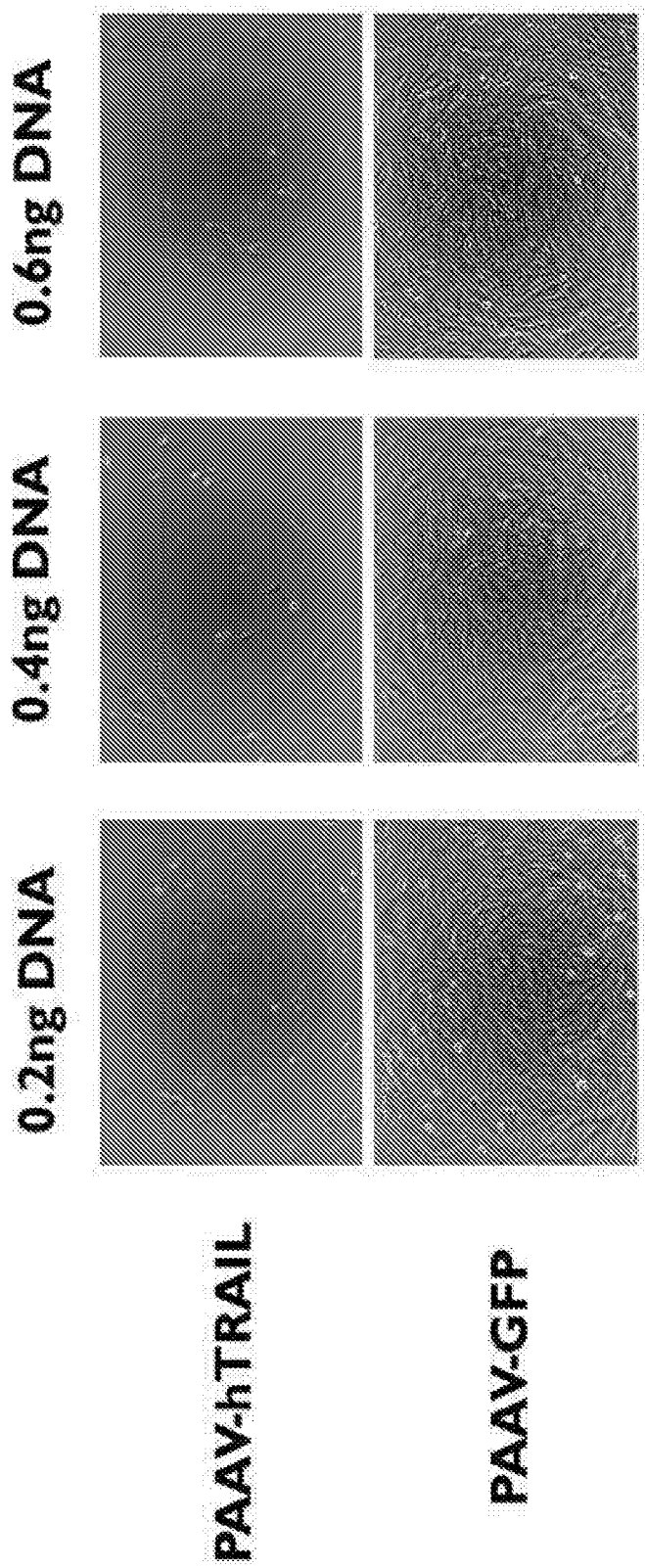


Figure 54

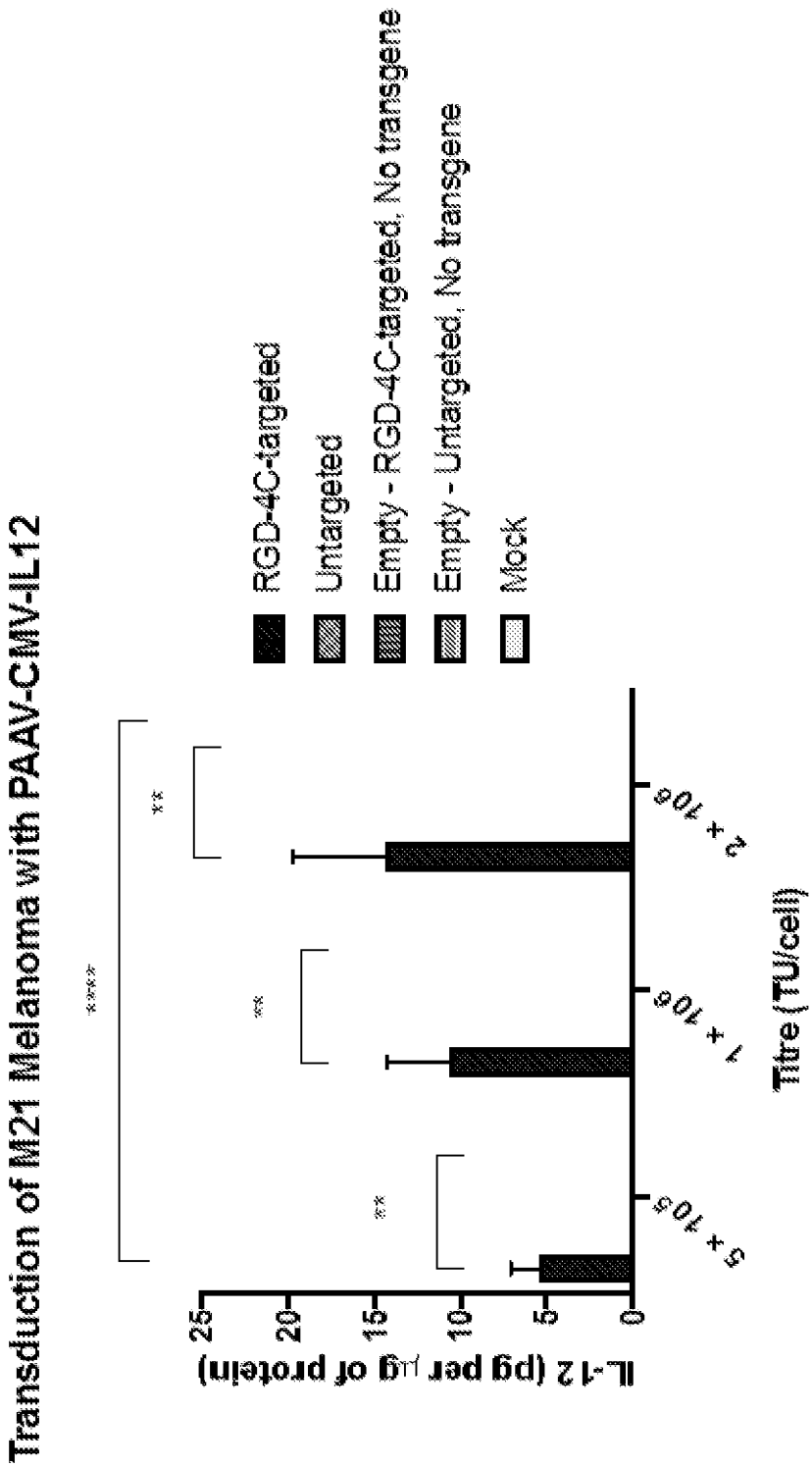

Transduction of M21 Melanoma with PAAV-CMV-IL12
RGD-4C-targeted
Untargeted
Empty - RGD-4C-targeted, No transgene
Empty - Untargeted, No transgene
Mock
IL-12 (pg per µg of protein)
25
20
15
10
5
0
****
**
**
**
5 x 10^5
1 x 10^6
2 x 10^6
Titre (TU/cell)

Figure 55

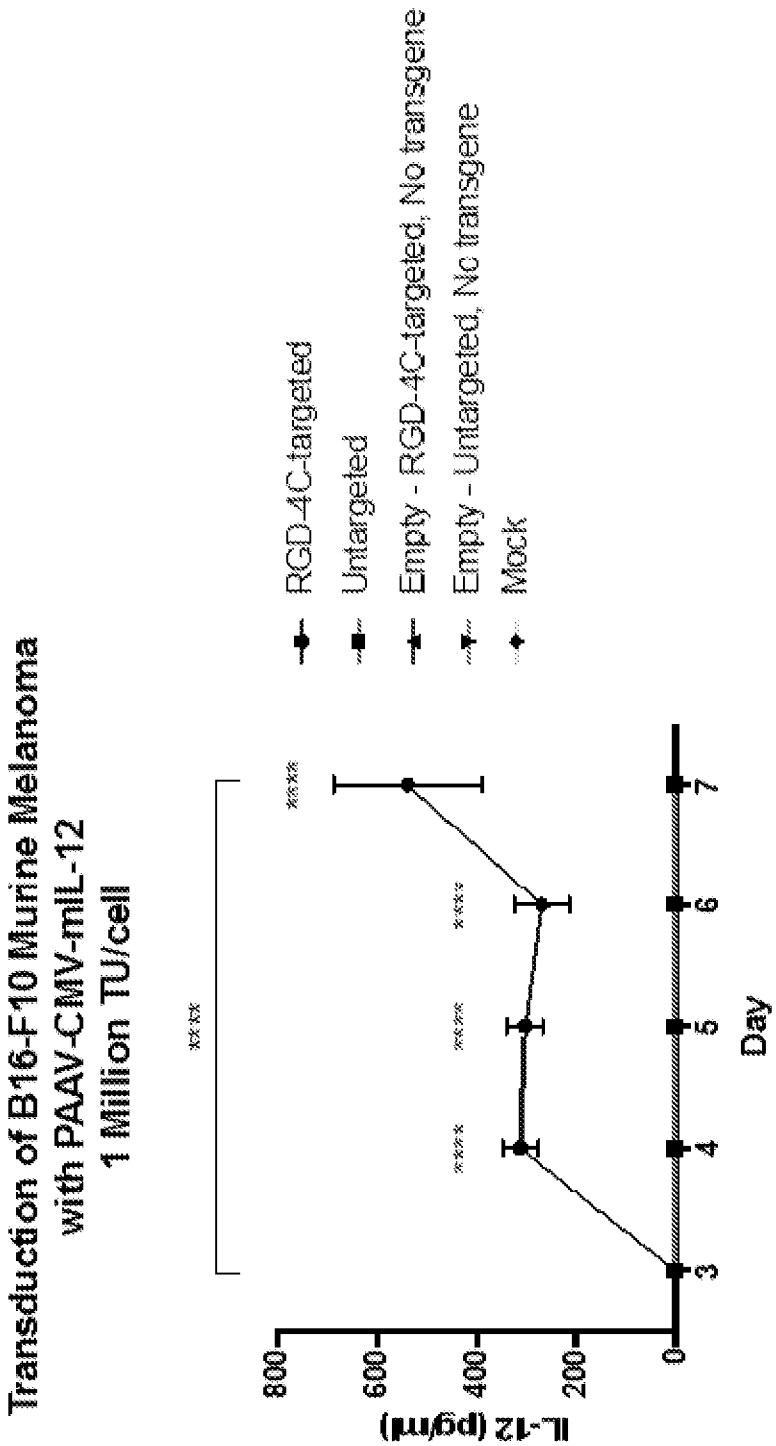

Transduction of B16-F10 Murine Melanoma
with PAAV-CMV-miL-12
1 Million TU/cell
RGD-4C-targeted
Untargeted
Empty - RGD-4C-targeted, No transgene
Empty - Untargeted, No transgene
Mock
IL-12 (pg/ml)
800
600
400
200
0
3
4
5
6
7
Day
****
****
****
****

Figure 56

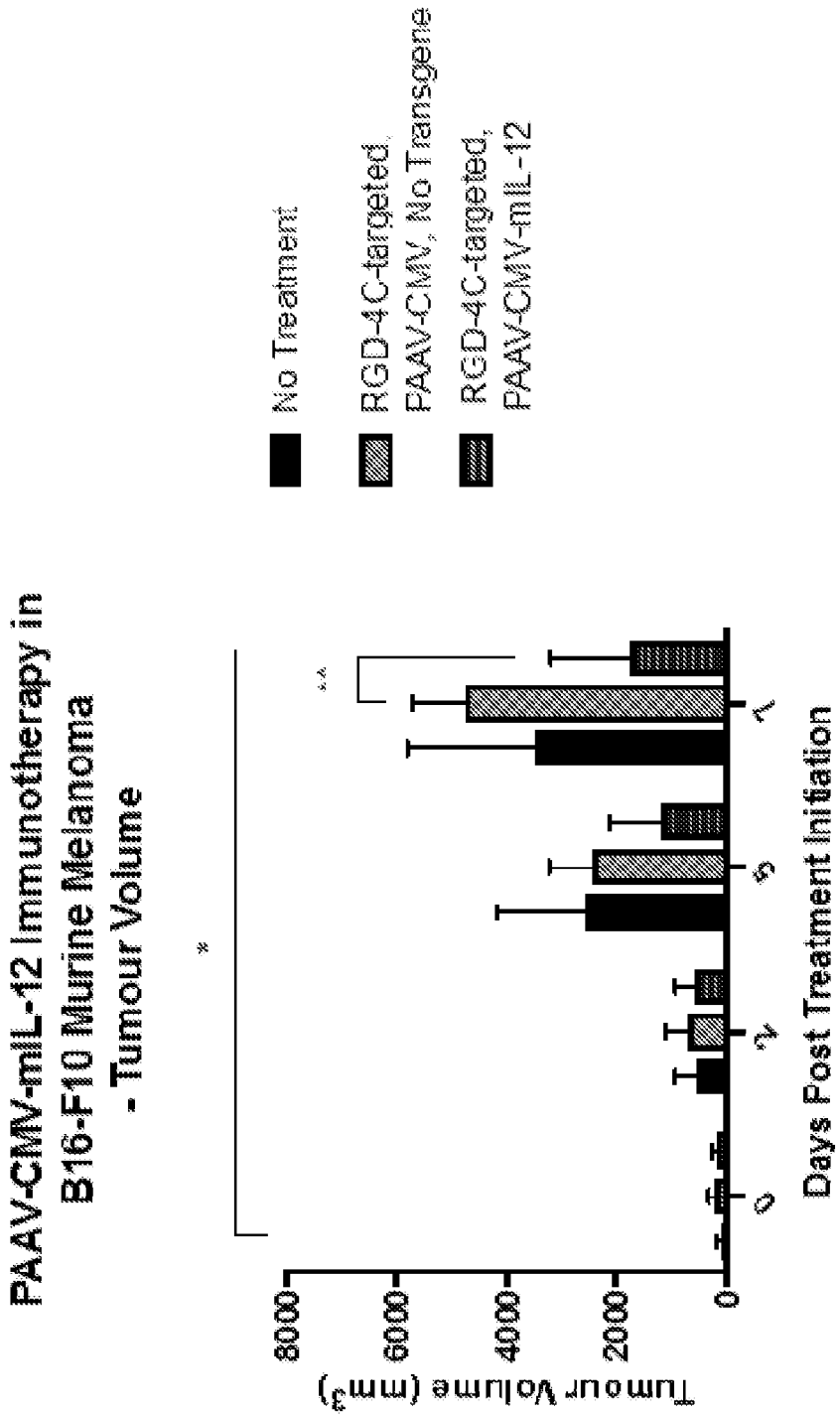

PAAV-CMV-mIL-12 Immunotherapy in
B16-F10 Murine Melanoma
- Tumour Volume
No Treatment
RGD-4C-targeted, PAAV-CMV, No Transgene
RGD-4C-targeted, PAAV-CMV-mIL-12
Tumour Volume (mm3)
8000
6000
4000
2000
0
0
2
5
7
Days Post Treatment Initiation
*
**

Figure 57

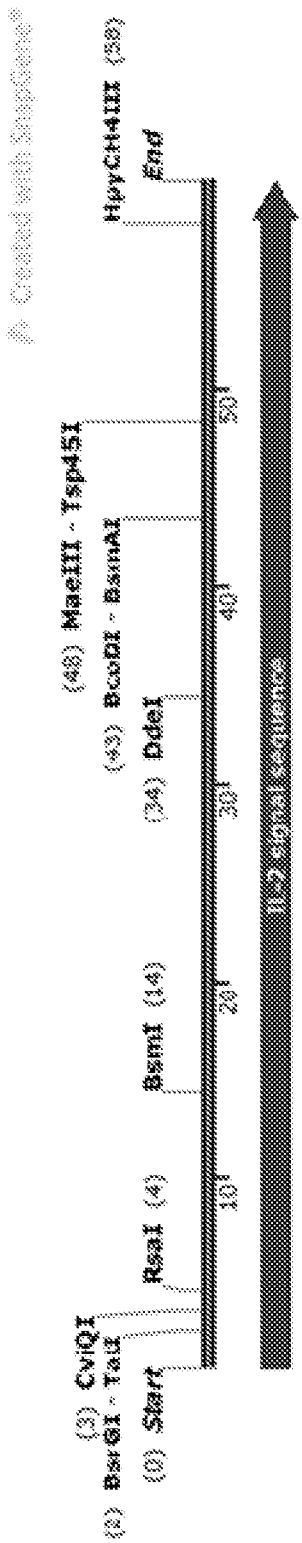

Created with SnapGene®
(2) BsrGI - TaiI
(3) CviQI
(0) Start
RsaI (4)
BsmI (14)
DdeI (34)
(49) BcoDI - BsmAI
(46) MaeIII - Tsp45I
HpyCH4III (89)
End
1
101
201
301
401
502
1.2 Chromosome

Figure 58

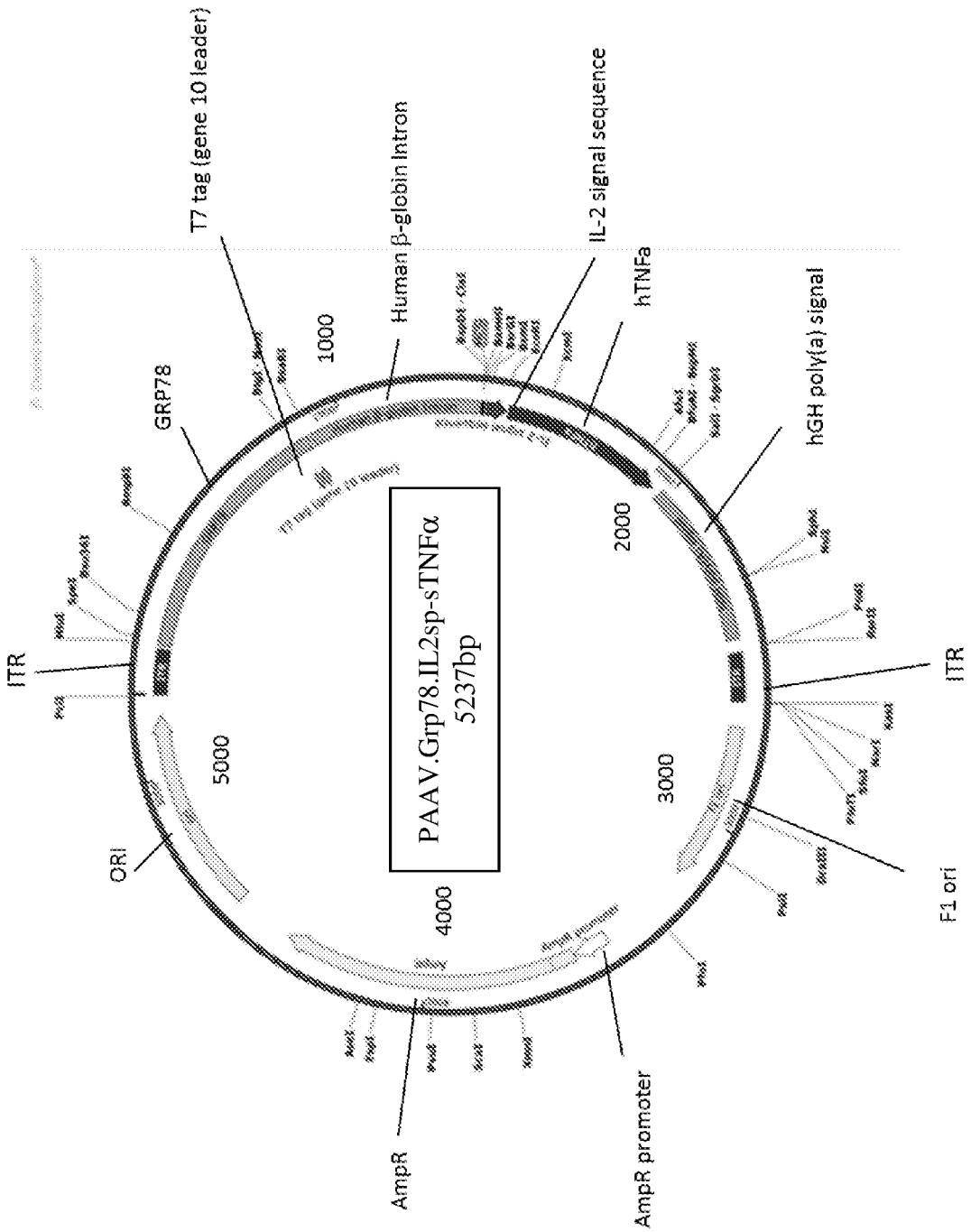

PAAV.Grp78.IL2sp-sTNFα
5237bp
T7 tag (gene 10 leader)
Human β-globin Intron
IL-2 signal sequence
hTNFa
hGH poly(a) signal
ITR
GRP78
ITR
ORI
AmpR
AmpR promoter
F1 ori
1000
2000
3000
4000
5000

Figure 59

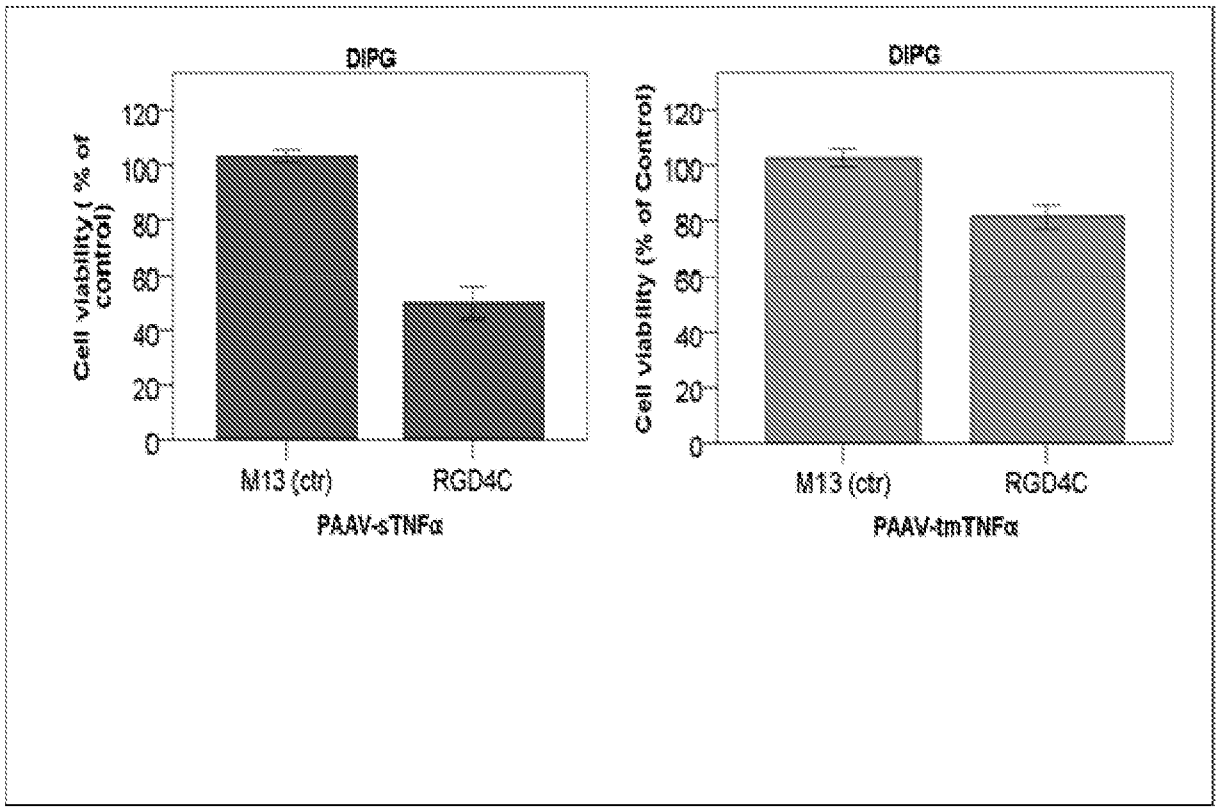
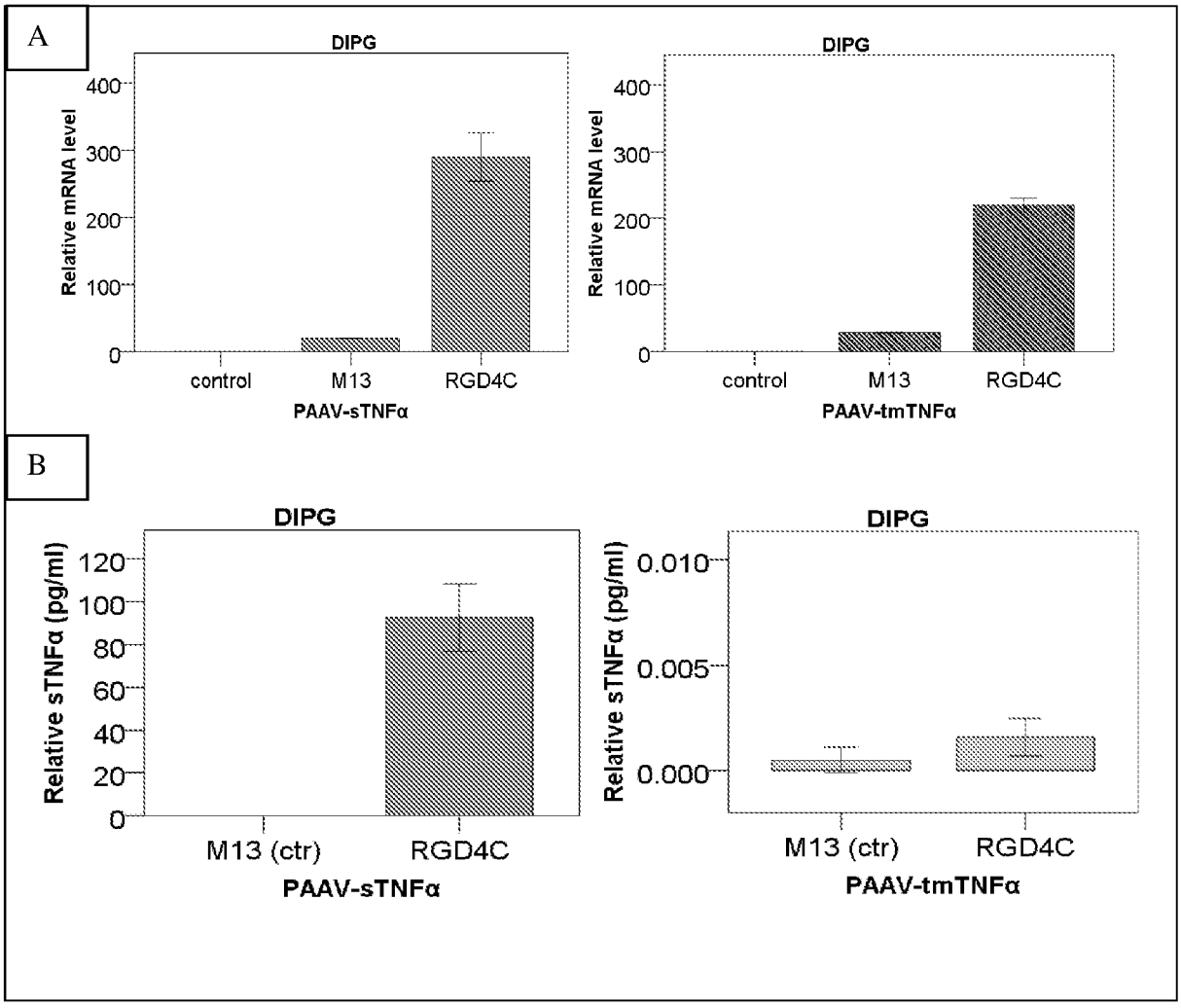

DIPG
Cell viability (% of control)
120
100
80
60
40
20
0
M13 (ctr)
RGD4C
PAAV-sTNFα
DIPG
Cell viability (% of Control)
120
100
80
60
40
20
0
M13 (ctr)
RGD4C
PAAV-tmTNFα

Figure 6o


A
DIPG
Relative mRNA level
400
300
200
100
0
control
M13
RGD4C
PAAV-sTNFα
DIPG
Relative mRNA level
400
300
200
100
0
control
M13
RGD4C
PAAV-tmTNFα
B
DIPG
Relative sTNFα (pg/ml)
120
100
80
60
40
20
0
M13 (ctr)
RGD4C
PAAV-sTNFα
DIPG
Relative sTNFα (pg/ml)
0.010
0.005
0.000
M13 (ctr)
RGD4C
PAAV-tmTNFα

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2018/051070

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K14/525 C07K14/54 C12N15/113 C12N15/86 C12N7/00
C12N7/04 A61K33/24 A61K39/00 A61K48/00
ADD.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K C07K C12N

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, Sequence Search, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y A	WO 2007/118245 A2 (UNIV TEXAS [US]; PASQUALINI RENATA [US]; ARAP WADIH [US]; GELOVANI JUR) 18 October 2007 (2007-10-18) paragraphs [0031], [0096], [0098]; figures 7,12,16-18; example 2 -----	1-5,9-38 6-8
Y	ZONGHAI LI 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 page 706, column 1, line 13 - line 16 -----	1-5,9-38
	-/--	

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

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

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

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

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

"P" document published prior to the international filing date but later than the priority date claimed

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

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
27 June 2018	11/09/2018

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Lonnoy, Olivier

Form PCT/ISA/210 (second sheet) (April 2005)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB2018/051070

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

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

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

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

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

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

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

see additional sheet

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

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

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

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

1-21(completely); 22-38(partially)

Remark on Protest

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

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

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2018/051070

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	PAUL E LUND ET AL: "Pseudovirions as Vehicles for the Delivery of siRNA", PHARMACEUTICAL RESEARCH, KLUWER ACADEMIC PUBLISHERS-PLENUM PUBLISHERS, NL, vol. 27, no. 3, 9 December 2009 (2009-12-09), pages 400-420, XP019793915, ISSN: 1573-904X figure 1	1-5,9-38
Y	Albahrani M et al: "Selective cytokine gene therapy for the treatment of paediatric brain cancer", Annual Conference of the British-Society-for-Gene-and-Cell-Therapy / Joint UK-Regenerative-Medicine-Platform Meeting; Cardiff, UK; April 19 -21, 2017 Human Gene Therapy, vol. 28, no. 8, P037, 20 April 2017 (2017-04-20), August 2017 (2017-08), page A33, XP002782597, Retrieved from the Internet: URL:https://www.liebertpub.com/doi/full/10.1089/hum.2017.29044.abstracts [retrieved on 2018-06-27] the whole document	1-5,9-38
X,P	WO 2017/077275 A1 (IMP INNOVATIONS LTD [GB]) 11 May 2017 (2017-05-11) page 6, line 34 - line 35; claim 1	1-5,9-38
A	WO 92/09690 A2 (GENENTECH INC [US]) 11 June 1992 (1992-06-11) claim 7	1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/GB2018/051070

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2007118245 A2	18-10-2007	AU 2007234698 A1	18-10-2007
		BR PI0710671 A2	16-08-2011
		CA 2649182 A1	18-10-2007
		CN 101460055 A	17-06-2009
		DK 2009990 T3	09-01-2017
		EP 2009990 A2	07-01-2009
		JP 5479087 B2	23-04-2014
		JP 2009534314 A	24-09-2009
		US 2007274908 A1	29-11-2007
		US 2010254896 A1	07-10-2010
		US 2012178903 A1	12-07-2012
		WO 2007118245 A2	18-10-2007
WO 2017077275 A1	11-05-2017	EP 3371315 A1	12-09-2018
		WO 2017077275 A1	11-05-2017
WO 9209690 A2	11-06-1992	AT 164395 T	15-04-1998
		CA 2095633 A1	04-06-1992
		CA 2405246 A1	11-06-1992
		DE 69129154 D1	30-04-1998
		DE 69129154 T2	20-08-1998
		DK 0564531 T3	28-09-1998
		EP 0564531 A1	13-10-1993
		ES 2113940 T3	16-05-1998
		GR 3026468 T3	30-06-1998
		US 5750373 A	12-05-1998
		US 5821047 A	13-10-1998
		US 5834598 A	10-11-1998
		US 6040136 A	21-03-2000
		US 2006115874 A1	01-06-2006
		US 2008038717 A1	14-02-2008
		US 2010035236 A1	11-02-2010
		WO 9209690 A2	11-06-1992

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-21(completely); 22-38(partially)

   A recombinant phagemid particle comprising at least one transgene expression cassette comprising a nucleic acid sequence encoding one or more cytokine, and comprising a genome which lacks at least 50 % of its bacteriophage genome; Said phagemid for use in a method for treating, preventing or ameliorating cancer; System and methods for producing said phagemid; Composition comprising said phagemid.

   ---

2. claims: 22-38(partially)

   A recombinant vector comprising rAAV, rep-cap, adenohelper genes, and a nucleic acid sequence encoding one or more antigen or cytokine, for use in the treatment, prevention, or amelioration of cancer; A phagemid particle comprising said vector, for said use.
