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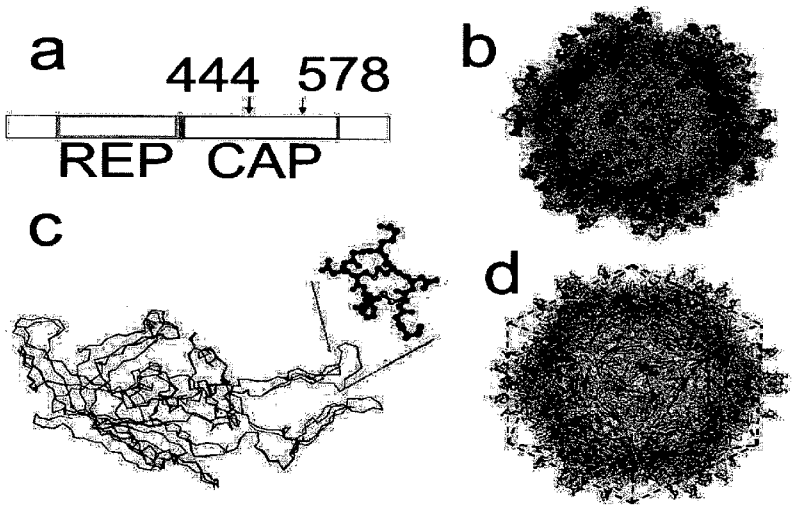
(54) Title: ADENO ASSOCIATED VIRUS SEROTYPE FIVE VECTOR FOR TARGETED GENE DELIVERY

[Fig. 1]



(57) Abstract: Disclosed herein is the use as a gene carrier of adeno-associated virus serotype 5 having high safety, among adeno-associated virus serotypes. Specifically, disclosed are a targetable, adeno- associated virus serotype 5-derived recombinant virus vector, prepared by inserting a targeting motif peptide, specifically recognizing a target molecule, into the internal amino acid residue of a capsid protein, and a method of delivering a gene in a target cell-specific manner using the vector.

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Description

ADENO ASSOCIATED VIRUS SEROTYPE FIVE VECTOR FOR TARGETED GENE DELIVERY

Technical Field

[1] The present invention relates to a targetable, adeno-associated virus serotype 5-derived recombinant virus vector, prepared by inserting a targeting motif peptide, specifically recognizing a target molecule, into the internal amino acid residue of a capsid protein, and to a method of delivering a gene in a target cell-specific manner using said vector.

Background Art

[2] Recombinant adeno-associated virus (rAAV) possesses many properties that make an attractive vector to deliver a gene of interest. rAAV, capable of infecting both dividing and non-dividing cells, offers persistent transgene expression with induction of minimal immune response [Wu, Z. et al., 2006, *Mol Ther* 14: 316-327; Warrington, K. H. et al., 2006, *Hum Genet* 119: 571-603]. Among many AAV serotypes which can be used to delivery target genes, the most widely studied vector is an adeno-associated virus serotype 2, which is now being used for clinical gene transfer for cystic fibrosis [Moss, R. B., et al., 2007, *Hum Gene Ther* 18: 726-732], hemophilia [Wu, Z., et al., 2007, *Mol Ther.*; Sabatino, D. E., et al.,2007, *Mol Ther* 15: 1677-1685; Wiwanitkit, V.,2007, *Hum Gene Ther* 18: 89-92], and canavan's disease [McPhee, S. W., et al.,2006, *J Gene Med* 8: 577-588]. Recently, the evidence regarding the potential of rAAV has been increased in cancer gene therapy [Li, C. et al., 200*Cancer gene therapy* 12: 913-925; Hacker, U. T., et al., 2005, *J Gene Med* 7: 1429-1438.].

[3] Nonetheless, due to unexpected transgene expression outside of the tissue of interest with a very low level of transduction within target tissue, the broad host range of rAAV limits its usage. Primary determinant of the cell/tissue tropism depend on the binding specificity of rAAV capsid to cellular receptor [Berns, K. I. et al., 2001, *Field's Virology*, Lippincott-Raven, Philadelphia]. Based on this fact, several research groups have genetically manipulated the rAAV capsid region to modify receptor specificity. By insertional mutagenesis of the rAAV2 capsid protein (introducing 14-amino acid targeting peptide), Girod *et al.* offered the first successful attempt for re-targeting rAAV to cells that are normally resistant to rAAV2 infection [Girod, A., et al., 1999, *Nat Med* 5: 1438]. Since then, there have been a body of reports supporting that retargeted gene delivery not only *in vivo*, but also *in vitro* occurs by modifying

rAAV2 by the similar strategies [Work, L. M., et al., 2006, Mol Ther 13: 683-693; Grifman, M., et al., 2001, Mol Ther 3: 964-975; White, K., et al., 2007, Gene therapy; Lieber, A., 2003, Nature biotechnology 21: 1011-1013]. So far, retargeting study of rAAV has been exclusively conducted using rAAV2 as a backbone.

[4] Grifman *et al* previously aligned the AAV1-5 capsid sequences to the corresponding regions of canine parvovirus to identify for possible incorporation site [Grifman, M., et al., 2001, *Mol Ther* 3: 964-975]. He pointed out that loop III and IV including Arg at amino acid 447 and 587 of rAAV2, respectively, can be freely modified without losing virus replication ability. These locations was originally identified to be modification target regions by Girol *et al.* and loop IV has been frequently utilized to alter rAAV2 tropism [Girod, A., et al., 1999, *Nat Med* 5: 1438]. In the case of rAAV1 serotype, there is an example in which an RGD-4C motif was inserted at amino acid 590 of rAAV1 capsid protein corresponding to amino acid 587 of rAAV2 capsid in order to target vascular endothelial cells and to alter tropism [MD Stachler, MD. et al., 2006, *Gene Therapy* 13:926-931]. However, there is still no study on the occurrence of genetic mutation in capsid proteins of other rAAV serotypes for tropism alteration.

[5] Along with systemic mutation study [Wu, P., et al., 2000, *J Virol* 74: 8635-864], atomic structure analysis of AAV2 strongly suggested that the primary receptor binding motif (loop IV) within amino acid 587 is located within the threefold-proximal peak [Xie, Q., et al., 2002, *Proceedings of the National Academy of Sciences of the United States of America* 99: 10405-10410]. By contrast, another loop (loop III), responsible for receptor recognition, resides on distal floor of the peak. rAAV5, which shows about 55% identity to rAAV2 capsid, requires 2,3-linked sialic acid on host cells distinct from heparin sulfate for AAV2 [Walters, R. W., et al., 2001, *The Journal of biological chemistry* 276: 20610-20616].

[6] Although both rAAV5 and canine parvovirus (CPV) capsid are known to bind to sialic acid, structural comparison of these capsids suggested that rAAV5 surface properties do not correlate well with this receptor phenotype of CPV. Model-based comparison of AAV2 and AAV5 to CPV implied that 3 amino acids (I528, N546 and M547) located on the wall in the depression at the twofold axis may be involved in the receptor binding of rAAV5 [Walters, R. W., et al., 2004, *J Virol* 78: 3361-3371]. The data of the present invention suggested that the introduction of homing peptide at amino acid 578 sharply drops TE by rAAV5, probably knocking down innate rAAV5 tropism. Thus, a region near amino acid 578 residing on threefold-proximal peak is likely responsible for rAAV5 binding to sialic acid, not the region on the inward

surface within twofold axis. However, more study has to be carried out to elucidate the detailed features for the receptor binding phenotype of rAAV5.

[7] Alpha V integrins are often over-expressed in a wide variety of tumor cells and vasculature [Bello, L., et al., 2001, *Neurosurgery* 49: 380-389; discussion 390; Brooks, P. C., et al., 1994, *Science* 264: 569-571]. Alpha V integrin-binding RGD motif (RGD-4C) was initially introduced to genetically targeting adenovirus and this mutant significantly enhanced human cancer cells both *in vitro* and *in vivo* [Kanerva, A., et al., 2004, *International journal of cancer* 110: 475-480; Niu, G., et al., 2007, *Mol Imaging Biol*]. rAAV2 exposing RGD motif was successfully generated to retarget rAAV2 [Girod, A., et al. 1999, *Nat Med* 5: 1438; Shi, W., etal., 2006, *Gynecologic oncology* 103: 1054-1062]. Sialyl Lewis X (sLeX) is found on the surfaces of various cancer cells and the sLeX binding motif was previously identified by peptide - AHWIPRYSSPAT- binding analysis [Kwon, M., et al., 2002, *J Am Chem Soc* 124: 13996-13997]. Tenascin C (TnC) is frequently over-expressed in different human tumors [Wiksten, J. P., et al., 2003, *Oncology* 64: 245-250]. TnC-homing peptide was identified by the phage display as the same strategy for sLeX.

[8] Expectation for anti-tumoral efficacy, not only potential application of rAAV, continues to grow in the cancer gene therapy field [Li, C., et al., 2005, *Cancer gene therapy* 12: 913-925; Bazan-Peregrino, M., et al., 2007, *Cancer gene therapy* 14: 117-127]. Anti-angiogenesis therapy targeting the formation of tumor vasculature has been considered as an attractive strategy in cancer treatment in both primary tumor growth and metastasis [Folkman, J. et al., 1989, *Nature* 339: 58-61]. With the accumulative evidences suggesting the requirement of consistent level in angiogenic inhibitors for anti-tumoral efficacy, rAAV has been identified as a proper vector to obtain long-term anti-tumoral effect [Davidoff, A. M., et al., 2002, *Cancer research* 62: 3077-3083]. However, systemic administration of rAAV *in vivo* results in the localization of the majority of the virus in the liver and spleen, thus limiting its application [Nathwani, A. C., et al., 2007, *Blood* 109: 1414-1421]. Baker and coworkers accomplished targeted delivery to vascular tissue by rAAV2 that possess the desired tropism [Work, L. M., et al., 2006, *Mol Ther* 13: 683-693; White, K., et al., 2007, *Gene therapy* White, S. J., et al. 2004, *Circulation* 109: 513-519].

[9] Transduction efficiency (TE) mediated by rAAV would be negatively influenced by pre-existing neutralizing antibodies. There have been several reports, suggesting antibodies against rAAV2 are highly prevalent in normal human population [Boissier, M. C., et al., 2007, *Hum Gene Ther* 18: 525-535; Blacklow, N. R., et al., 1968, *Journal of*

the National Cancer Institute 40: 319-327; Blacklow, N. R., et al., 1971, *American journal of epidemiology* 94: 359-366; Halbert, C. L., et al., 2006, *Hum Gene Ther* 17: 440-447]. Thus, the potency of rAAV2 as a gene delivery vehicle would be severely hindered for *in vivo* application, particularly in case of systemic administration of rAAV2.

[10] Recent study measuring neutralizing antibodies against AAV2, 5 and 6 indicated that the least population was sero-positive for AAV5, suggesting that rAAV5 can be a promising alternative to rAAV2 as a backbone in order to provide altered tropism by genetically manipulating its native tropism [Halbert, C. L., et al., 2006, *Hum Gene Ther* 17: 440-447]. rAAV2 is also prone to provoke cellular immunity, compared to other AAV serotypes [Vandenberghe, L. H., et al., 2006, *Nat Med* 12: 967-971; Mingozzi, F., et al., 2007, *Nat Med* 13: 419-422]. In contrast to rAAV2, normal human populations are less sero-positive to rAAV5 [Blacklow, N. R., et al., 1971, *American journal of epidemiology* 94: 359-366; Halbert, C. L., et al., 2006, *Hum Gene Ther* 17: 440-447]. While optimizing production of various rAAV serotypes, Grimm *et al.* found that rAAV5 was much more efficiently than rAAV2 [Grimm, D., et al., 2003, *Mol Ther* 7: 839-850].

Disclosure of Invention

Technical Problem

[11] It is an object of the present invention to use relatively safe serotype 5 as a gene delivery vector in place of adeno-associated virus serotype 2, which has been used as a popular gene transfer vector both *in vitro* and *in vivo*, but induces serious immune responses by neutralizing antibodies, such that the serotype 5 can target specific cells or tissues in order to more effectively use it in gene delivery.

Technical Solution

[12] To achieve the above object, in the present invention, the characteristics and functions of a capsid protein constituting the envelope of adeno-associated virus serotype 5 were modified such that the virus could target specific cells. Specifically, a vector producing a virus, which has modified surface characteristics and can recognize and bind to a target molecule, was constructed by inserting a targeting motif peptide into the capsid protein of a virus vector.

[13] Hereinafter, the present invention will be described in detail.

[14] In one aspect, the present invention relates to a targeted recombinant virus vector derived from adeno-associated virus serotype 5, in which the virus vector comprises a

nucleic acid sequence encoding a capsid protein containing a targeting motif peptide inserted in the internal amino acid residue of the capsid protein.

[15] The virus vector of the present invention is an adeno-associated virus serotype 5-derived virus vector having improved specificity and efficiency, which expresses a capsid protein having the functions and characteristics modified so as to have tropism specific for a target molecule. Thus, the virus vector can deliver a therapeutic gene or a genetic substance specifically into cells, tissues and/or organs. The vector of the present invention expresses a chimeric capsid protein, in which a targeting motif peptide is linked to a capsid protein, a viral structural protein, in the form of a single-stranded polypeptide through a peptide bond. Thus, a virus produced from the vector of the present invention has an envelope having tropism specific for targets depending on the kind and characteristics of a peptide, inserted therein, and it also has the capability to transduce cells depending on the kind and characteristics of peptide. The tropism and characteristics of the vector of the present invention can be easily manipulated according to the intended use thereof by controlling the kind of a peptide which is inserted into the vector.

[16] In the vector of the present invention, a peptide-encoding nucleic acid sequence is inserted in a capsid protein-encoding nucleic acid sequence, such that the capsid protein, recombined at the genetic level, and a motif peptide, are expressed as one polypeptide.

[17] As used herein, the term "targeting motif peptide" refers to a peptide capable of specifically recognizing and binding to a target molecule, which is present specifically in the membrane of specific cells or present in the membrane in excess. Targeting motif peptides that can be inserted into the vector of the present invention include all peptides which can impart a targeting function to virus without losing the virus-producing ability.

[18] Specifically, the targeting motif peptide of the present invention is a peptide specifically recognizing and/or binding to molecules, which are expressed in tumor cells, for example, sialic Lewis X, integrin or Tenascin C. More specifically, the targeting motif peptide specifically recognizing sialic Lewis X has an amino acid sequence of SEQ ID NO: 1 or SEQ ID NO: 2, the motif peptide specifically recognizing integrin has an amino acid sequence of SQ ID NO: 3 or SEQ ID NO: 4, and the motif peptide specifically recognizing Tenascin C has an amino acid sequence of SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7 or SEQ ID NO: 8.

[19] The size of the targeting motif peptide is limited to a size which can produce virus

without losing the viral replication ability and assembly ability, when the peptide is inserted into the vector and produced as virus. The targeting motif peptide preferably comprises 5-25 amino acids, and more preferably 7-20 amino acids. Peptide sequences having various sizes ranging from 7 to 20 amino acids could be readily inserted into a capsid protein without losing the viral replication and assembly ability, while they have particular buoyant density. There was no mutual relation between insert size and the efficiency of producing virus particles within this range. This size in the present invention is much longer than previously determined in case of rAAV2 bearing 17 amino acids [Blacklow, N. R., et al., 1968, *Journal of the National Cancer Institute* 40: 319-327].

[20] The motif peptide of the present invention is inserted at amino acid residues of a capsid protein which can be genetically modified without affecting the viral replication ability and assembly ability. The present inventors have found that both loops of amino acids 444 and 578 of adeno-associated virus serotype 5 are regions which can be genetically modified without affecting the viral replication ability and assembly ability. Specifically, the present inventors have found that: i) a recombinant adeno-associated virus serotype 5 (rAAV5) can be genetically modified within the capsid region, particularly at VP1 amino acid position 444 or 578, into which an external peptide can be inserted; ii) mutants having inserted therein the targeting motif peptide having the above-specified size have a virus production ability equal or similar to that of wild-type virus; and iii) viruses genetically modified by the insertion of the peptide can transduce human cancer cells depending on the characteristics of retargeted receptor.

[21] In addition, the vector of the present invention can be amended, modified or applied according to any conventional method known in the vector preparation field.

[22] The vector of the present invention can be used for the prevention and treatment of various diseases in the medical and veterinary fields by inserting a nucleic acid, encoding at least one gene to be delivered and expressed in cells, tissues and organs, into the vector. Examples of genes, which can be inserted into the vector of the present invention, include, but are not limited to, various physiologically active peptides, such as hormones, cytokines, enzymes, antibodies, growth factors, transcription regulatory factors, blood factors, vaccines, structural proteins, drug-sensitive genes, drug-resistant genes, ligand proteins and receptors, cell surface antigens, receptor antagonists, and derivatives thereof. In addition, they may include anti-sense nucleic acid regulating gene expression in target cells, or nucleic acid expressing siRNA.

[23] In another aspect, the present invention relates to a method of delivering a gene in a

target cell-specific manner using a targeted recombinant virus vector, which is derived from adeno-associated virus serotype 5 and contains a nucleic acid sequence encoding a capsid protein comprising a targeting motif peptide inserted into the amino acid sequence thereof.

[24] Specifically, the inventive targeted recombinant virus vector encoding at least one gene to be delivered or expressed in cells, tissues and/or organs can proliferate into viruses using adeno-associated virus replication methods known in the art, for example, using helper virus such as adenovirus, vaccinia virus or herpes virus, in consideration of purity, stability, morphology and infectivity.

[25] The adeno-associated virus serotype 5 virus can be used as a gene carrier in not only humans, but also animals having infectivity with virus, through an *ex vivo* method of transferring the virus into cells isolated from an individual and then transplanting the cells again into the individual, or an *in vivo* method of injecting the virus directly into an individual.

[26] The vector and virus of the present invention can be used to treat various diseases, including blood diseases, such as hemophilia, sickle cell anemia, and anemia caused by chemotherapy, central nervous system diseases, such as Alzheimer's disease, Parkinson's disease, multiple sclerosis, Huntington's disease, and mental disorder caused by genetic defect, metabolic diseases, such as cystic fibrosis, diabetes, growth hormone deficiency and osteoporosis, autoimmune diseases, such as multiple sclerosis, psoriasis and rheumatoid disease, and infectious diseases, such as HIV infection, influenza, herpes simplex, papillomavirus and mycobacteria.

[27] The virus of the present invention may be formulated into a suitable preparation form together with a pharmaceutically acceptable carrier and administered via various routes, for example, oral, intraperitoneal, intravenous, intramuscular, subcutaneous, intracutaneous, local, intranasal, intrapulmonary and intrarectal routes.

Advantageous Effects

[28] The adeno-associated virus serotype 5 mutants produced from the vector of the present invention showed a virus-producing ability similar to that of wild-type virus without losing the virus production ability, and showed transducing activity specific for certain cells depending on the kind and characteristics of a peptide inserted therein.

Brief Description of the Drawings

[29] FIG. 1 shows the structure of the mutant AAV5 capsid subunit and the topology of the virus surface. Specifically, FIG. 1(a) shows the schematic structure of a

pSp72-R2C5 plasmid containing AAV2 rep coding gene (REP) and VP1 capsid coding gene (CAP), FIG. 1(b) shows the surface of rAAV5-sLeX1 at aa 444 site (red) and rAAV5-sLeX2 at aa 578 (yellow) viewed as ribbons, and FIG. 1(c) shows the comparison of the backbones of AAV2 (blue) and rAAV5-RGD1 (green). In FIG. 1(c), the red loop region is an RGD-4C peptide domain inserted at aa578 site. This area is magnified and viewed as the colorful spheres and stick forms. FIG. 1(d) illustrates the cartoon of rAAV5-RGD1, which is assembled by 60 capsid subunits with icosahedral symmetry. In FIG. 1(d), all subsequent surface rendering was performed using PyMOL, and red fragments show RGD-based homing motives at aa 578 position.

[30] FIG. 2 shows transmission electron microscopy pictures of rAAV5 and rAAV5-sLeX2 viruses. Specifically, FIG. 2(a) shows wild-type rAAV5 particles, and FIG. 2(b) shows mutant rAAV5-sLeX2 particles. In FIG. 2, all the virus particles were in 10mM Tris buffer (pH7.9) containing 2 mM $MgCl_2$ and 2% sorbitol.

[31] FIG. 3 shows RGDS peptide-mediated inhibition of rAAV5-RGD1 transduction in integrin-positive cancer cells. In FIG. 3, HeLa cells, SK-Hep1 cells, and U87-MG cells were infected with rAAV5-eGFP (MOI 10, 100, and 200, respectively), or rAAV5-RGD1 (MOI 5,000, 10,000, and 5,000), respectively. These cells were incubated with RGDS or non-specific RGES peptide. Specifically, FIG. 3(a) shows the results of flow cytometry of GFP-positive cells after 48 hr, and FIG. 3(b) shows pictures of U87-MG. All (n=3, *: $p>0.05$, + : $p<0.05$).

Best Mode for Carrying Out the Invention

[32] **Example 1: Materials and methods**

[33] **1-1: Cell culture and rAAV infection**

[34] 293T and human glial cancer U251 cells were kindly provided by Dr. Jung and Dr. Lee at Harvard Medical School and University of Pittsburgh, respectively. Other human cancer cells (hepatocellular SK-Hep1, cervical HeLa, breast MDA-MB-231 and MDA-MD-435S, glioblastoma U-251, U87-MG, U118-MG, colon HCT-116) were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). The cells were incubated in Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum, L-glutamax (2 mM), penicillin (100 IU/ml) and streptomycin (50 μg/ml), in a 5% CO_2 incubator at 37 °C. For gene transfer, the cells were treated with rAAVs at various multiplicities of infection (MOI). Mock-treated cells used as a control were cells not treated with rAAVs. GFP expression was observed under an inverted fluorescent microscope (Leica DMIRB, Leica, Germany) and the pictures were taken.

[35]

[36] **1-2: Viral plasmid construction**

[37] pR2C5 and pHpa-trs-SK were obtained from Dr. High, and the adenoviral pXX6 helper plasmid was obtained from Stratagene (La jolla, CA, USA). pcDNA3.1(+)-sc39TK was provided by Donald B Kohn (University of Southern California, Keck School of Medicine, University of Los Angeles). pHpa-trs-SK was composed of the EGFP expression cassette in the self-complementary AAV2 (scAAV2) genome. The pSp72-R2C5 plasmid was constructed by subcloning of the 4.2-kb partial *Kpn*I-*Xba*I fragment of pR2C5 into the *Kpn*I-*Xba*I sites of the pSp72 vector. Then, for easy subcloning, the production of *Sna*BI site at 1210 bp to 1215 bp without replacement of codon in rAAV5 VP1-encoding sequence of pSp72-R2C5 was performed using the QuikChange Multi Site-Directed Mutagenesis Kit as described by the supplier (Stratagene, La Jolla, California), and this plasmid was named pSp72-R2C5-*Sna*BI. The 1.0-kb *Xba*I-*Sna*BI fragment of a PCR product was obtained from pSp72-R2C5 using primers of SEQ ID NO: 9 and SEQ ID NO: 10.

[38] The plasmid pGEM-T easy-Cap5 was obtained by TA cloning the 1.0 kb *Xba*I-*Sna*BI fragment of pR2C5 into the 3' T overhangs at the insertion site of pGEM-T Easy Vector (Promega, Madison, WI). For each mutant, a PCR fragment of the QuikChange Multi Site-Directed Mutagenesis was prepared using the pGEM T-easy-Cap5 plasmid as a template with each of primer pairs: one (Forward) containing more than 15 nucleotides belonging to the VP1 gene upstream of the insertion site and also some nucleotides coding for the 5' terminal end of the motive peptide, and the other (Reverse) containing the over 15 nucleotides belonging to the cap gene downstream of the insertion site and also some nucleotides coding for the 3' terminal end of the motive peptide. The following primer pairs were used: SEQ ID NO: 11 and SEQ ID NO: 12, SEQ ID NO: 13 and SEQ ID NO: 14, SEQ ID NO: 15 and SEQ ID NO: 16, SEQ ID NO: 17 and SEQ ID NO: 18, SEQ ID NO: 19 and SEQ ID NO: 20, SEQ ID NO: 21 and SEQ ID NO: 22, SEQ ID NO: 23 and SEQ ID NO: 24, and SEQ ID NO: 25 and SEQ ID NO: 26. The 1.0-kb TnC3 PCR fragment was obtained from the template pGEM-Cap5-TnC1. The PCR products were ligated, amplified into XL-Blue *E. coli* (Strategene, La Jolla, California) and sequenced.

[39] Each of pSp72-R2C5 mutant plasmids was constructed by subcloning of the 1.0-kb *Sna*BI-*Xba*I fragment containing the motive peptide-encoding sequence of each pGEM-Cap5 mutant plasmid into the *Xba*I and *Sna*BI sites of pSp72-R2C5-*Sna*BI. pSp72-scAAV2-eGFP was constructed by blunt-end subcloning of the 2.3-kb *Pvu*II-

*Pvu*II fragment of pHpa-trs-SK into the *Bgl*II and *Hin*dIII sites of the pSp72 vector, and thus it contained the full-length self-complementary AAV2 genome encoding an EGFP protein.

[40] pSp72-scAAV2-sc39TK was constructed by subcloning of the *Bam*HI-*Sal*I PCR fragment of sc39TK gene from pcDNA3.1(+)-sc39TK into the *Bam*HI-*Sal*I sites in pSp72-scAAV2-eGFP from which the corresponding fragment had been removed.

[41]

[42] **1-3: Recombinant adeno-associated virus (rAAV) production and titration**

[43] The preparation of self-complementary rAAV vectors was described in detail in Wiksten, J. P., et al., 2003, *Oncology* 64: 245-250. Briefly, 293T cells were transfected with DNA plasmids (pSp72-R2C5, pSp72-R2C5-sLeX1, -sLeX2, - RGD1, -RGD2, -TnC1 to -TnC4 for the production of the wild-type rAAV5, rAAV5-sLeX1, -sLeX2, -RGD1, -RGD2, -TnC1 to -TnC4 mutant viral preparations, respectively; pSp72-scAAV2-eGFP AAV2 genome plasmid, and the helper plasmid). Pure populations of rAAVs were prepared by two sequential steps of CsCl gradient ultracentrifugation. After dialysis against 50 mM Tris buffer (pH 7.8) containing 1 mM $MgCl_2$ and 10% sorbitol, the purified rAAVs were aliquoted and stored at -80 °C. The total number of rAAV particles was calculated by TaqMan-based real-time PCR analysis (iQ™ supermix, Bio-Rad, Hercules, CA), using primers of SEQ ID NO: 27 and SEQ ID NO: 28 and a primer of SEQ ID NO: 29 (the 5' end of the primer was linked with FAM, and the 3' end of the primer was linked with TAMRA) targeting the cytomegalovirus (CMV) promoter [Moon, M. S., et al., 2005, *Intervirology* 48: 153-160].

[44]

[45] **1-4: Flow cytometric analysis**

[46] For analyzing cell surface markers, cells were incubated with primary antibodies for αVβ3, αVβ5 conjugated with FITC (Chemicon, Temecula, CA) in DMEM media containing 10% FBS for 30 min at room temperature, and then the cells were washed three times with PBS buffer. To measure alpha-2,3-sialic acids on the cell surface, the cells were stained with primary MAL II lectin conjugated with biotin (Vector laboratories, Burlingame, CA) in DMEM media containing 10% FBS, for 30 min at room temperature, and then the secondary streptavidin-FITC (Vector laboratories, Burlingame, CA) were incubated for 30 min at room temperature after washing with PBS three times.

[47] To evaluate the transduction efficiencies (TEs) and the means of fluorescence intensities (MFIs), cells transduced by scAAV bearing the GFP gene and immune-

complexed cells were harvested, washed in PBS by centrifugation, and then fixed with ice-cold PBS containing 4% paraformaldehyde. The proportions of fluorescence-positive cells and MFI were determined using a flow cytometer and the CellQuest Plus program (FACSCalibur, Becton Dickinson, San Jose, CA). The percentage of GFP positive cells was defined as the fraction beyond the region of 99.9% of un-infected control cells.

[48]

[49] **1-5: RGD peptide competition assay**

[50] HeLa. SK-Hep1 and U87-MG cells were cultured in 48-well plates for 24 hr before treatment with the peptide. The cells were incubated in fresh media supplemented with 200, 100, 50, 20, 10 μM active RGDS (Sigma, Steinheim, Germany), and inactive RGES peptide (Sigma, Sigma, Steinheim, Germany) with 2% FBS for 30 min at room temperature. Then, the cells were treated with rAAV5-GFP or mutants containing GFP expressing cassette (virus titers at approximate 50% transduction) for 4 hr. To maximize the GFP expression in the cells, adenovirus type 5 (PFU 2 or 5 per cell) were added with rAAV5 vectors. The cells were washed three times with PBS. 48 hours after the transduction, the cells each from the treated and untreated groups were pelleted and resuspended in 200 $\mu\ell$ PBS or 200 $\mu\ell$ PBS containing 4% paraformaldehyde. GFP-positive cells were measured by a flow cytometer.

[51]

[52] **1-6: RT-PCR for TnC**

[53] RNA was isolated from HCT-116, U251, U87-MG, and U118-MG using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction. For the detection of the TnC alternative splicing form expression, RT-PCR was performed using a TNfn 5 forward primer of SEQ ID NO: 30 and a TNfn A1 reverse primer of SEQ ID NO: 31, and for the detection of the TNC C-terminal form expression, RT-PCR was performed using a TNfbg forward primer of SEQ ID NO: 32 and a TNfbg reverse primer of SEQ ID NO: 33. In each of the RT-PCR reactions, 2μg of RNA was used and the reverse transcription reaction was carried out for 1 hr at 37 °C with reverse transcriptase (1U/$\mu\ell$; Qiagen, Crawley, West Sussex, UK).

Mode for the Invention

[54] **Example 2: Construction and production of rAAV5s bearing genetically modified capsids**

[55] To re-target rAAV5, the present inventors hired 8 different peptide sequences derived from 3 homing motives as shown in Table 1. The present inventors attempted

to insert these epitopes at VP1 amino acid 444 or 578 position (FIG. 1b and 1d), based on previous studies: i) construction of genetically modified rAAV2 by inserting various peptide motives (FIG. 1c) [Huttner, N. A., et al., 2003, *Gene therapy* 10: 2139-2147], ii) analysis of AAV5 cryo-structure [Walters, R. W., et al., 2004, *J Virol* 78: 3361-3371], and iii) the alignment of the VP1 region of AAV serotypes performed in vector NTI. The structural simulation of rAAV5 capsid revealed that both the amino acid positions would be protruded from other domains and flexible, by which insertion of target peptide might be endured (Fig. 1a). Expected locations of insertion sites on the virus surface were also shown by computer-modeling (FIG. 1b; red: 444, and yellow: 578).

[56] The present inventors first analyzed the mutants for their ability to amplify virus particles and the data showed that virus production was mostly as efficient as that of wild-type rAAV5 (Table 1). Virus production rate ranged from 7.3 x 10^{10} to 2.8 x 10^{11} virus genome copies per ml. After treatment with nuclease benzonaze, virus particle remained intact without losing its transducing activity (data not shown). This indicates that the efficient virus assembly occurred and the resultant virus particles are intact as much as the parental type. The buoyant densities of each virus under $CsCl_2$ gradient were within a narrow range from 1.380 to 1.385 g/ml, slightly lighter than that of the wild-type (1.394±0.007 g/ml). Infectious viruses were exclusively detected in these major peaks (data not shown). Transmission electron microscopy implied that the mutant maintained the structural integrity (FIG. 2). The average diameters of both the rAAV5 and the sLeX2 mutant were similar as 22.2±1.3 and 23.0±1.5 nm, respectively. Taken together, the data implies that genetic modification of rAAV5 by inserting short peptide is possible at VP1 amino acid positions 444 and 578.

[57] Table 1

[Table 1]

Motives	Viruses	Sites	Homing peptide	Aminoacidlength	Titer (VG/ml)	Relative titer	Buoyang density (g/ml)
	Wildtype	-	-	-	$1.3x10^{11}$	1.0	1.394±0.007
sLeX	sLeX1	444	AHWIPRYSSPAT	12	$7.3x10^{10}$	0.6	ND
	sLeX2	578	G-AHWIPRYSSPAT-GG	15	$9.8x10^{10}$	0.8	1.385
RGD	RGD1	578	G-CDCRGDCFC-	10	$1.2x10^{11}$	0.9	1.330
	RGD2	578	G-CDCRGDCFC-GLS	13	$1.0x10^{11}$	0.8	ND
TnC	TnC1	578	GCDCG-FHKHK-SCFC	14	$1.1x10^{11}$	0.9	ND
	TnC2	578	G-FHKHKS	7	$1.9x10^{11}$	1.4	ND
	TnC3	578	GCDCG-FHKHKSPALSPV-CFC	20	$2.4x10^{11}$	1.9	ND
	TnC4	578	G-FHKHKSPALSPV	13	$2.0x10^{11}$	1.5	ND

VG: viral genome. Genome copies were determined by real-time PCR as described in Materials and Methods. Relative titer shows the ratio of the mutant virus titer to wild rAAV5 titer. Buoyant density of $CsCl_2$ gradients menas the $CsCl_2$ density of the major peak of rAAV5 mutants vectors purified and fractioned.

[58] <Comparison of virus particle-producing ability between

[59] wild-type mutant and mutants>

[60]

[61] **Example 3: Transduction of human cancer cells by rAAV5 expressing RGD-binding motif**

[62] The tropism of RGD rAAV5 to integrins (αVβ3 or αVβ5) was evaluated by infecting various human cancer cells with either rAAV5 or RGD viruses encoding GFP transgene and examining transduction efficiency (TE). Transducing characteristics by RGD1 virus was superior to that by RGD2, so the rest of study was carried out by RGD1 (data not shown). At MOI=5000, a majority of both Hela and U87-MG were transduced and TE were estimated as 67.51±1.71 % and 62.90±7.01 %, respectively (Table 2). MDA-MB-435S and SK-Hep1 were also transduced, while MDA-MB-231 was poorly transduced. Flow cytometric analysis implied that these cells were all positive for 2,3-sialic acid known as the major host receptor of rAAV5, subsequently resulting in high TE. By contrast, these cells greatly differed in the profile of αVβ3 or αVβ5 integrin expression. Nevertheless, the cells being transduced by RGD virus expressed one or both types or integrins, whereas MDA-MB-231 was negative for both integrins, supporting its non-permissiveness to RGD virus. To determine whether the RGD-4C peptide of RGD1 virus would bind to integrins, the present inventors performed a competition assay by incubating the RGD1 virus with the RGDS peptide prior to introducing to cells (FIG. 3). Gene transfer by RGD1 virus was significantly inhibited with the RGDS peptide depending on the peptide concentration, not with non-specific sham RGES peptide (data not shown). TEs in Hela, and SK-Hep1, U87-MG cells decreased to 63.28±5.37 %, 85.08±8.92 %, and 52.60±16.83 %, at 200, 200, 50 μM, respectively, compared to those without treatment with the RGDS peptide. Non-specific peptide RGES did not reduce TE by RGD1 virus. As expected, the TE of WT virus was interrupted by neither peptide. Taken together, the data demonstrates that the RGD1 virus infects human cancer cells in an integrin-dependent manner.

[63] Table 2

[Table 2]

Cells	FC analysis			TE		Ralative TE (% of WT)	RGD Competition* (%)
	αVβ3	αVβ5	α2,3-siallic acid	RGD1 (MOI5,000)	WT (MOI100)		
HeLa	-	+	++	67.51±1.71	93.98±3.26	0.23	63.28±5.37
MDA-MB-231	-	-	+	6.54±3.44	52.13±17.87	0.01	ND
MDA-MB-435S	+++	+++	+	20.12±6.66	33.88±11.60	0.50	ND
SK-Hep1	-	++	+	14.05±6.19	52.31±11.12	0.13	85.08±8.92
U87-MG	+	-	+	62.90±7.01	86.69±5.68	0.53	52.60±16.83↑

↑Competition assy of rAAV5-RGD1 with 50μM of PGDS peptide in U87-MG cells.
*Relative inhibition(%) of rAAV5-RGD1 infection with 200μM of PGDS peptide.

[64] <Analysis of transduction of rAAV-RGD1 and rAAV viruses

[65] and integrin expression in human cancer cells>

[66]

[67] **Example 4: Transduction of human cancer cells by rAAV5-TnC variants**

[68] To examine the transducing potential of 4 different TnC viruses via tenascin C, TE was measured on TnC-positive or -negative human cancer cells. TnC-positive cells were transduced by TnC3 and TnC4. However, among TnC viruses, TnC4 viruse can transduce the cells, much better than the other viruses (data not shown). As shown in Table 3, TnC-negative HCT-116 could not be transduced by TnC viruses, while they can be efficiently transduced by WT virus. U87-MG cells experienced the highest TEs by TnC4 virus. Thus, along with the data regarding RGD virus, these facts suggest that the newly inserted peptides on the loop 3 of rAAV5 VP1 can guide the mutant viruses to recognize corresponding surface receptors.

[69] Table 3

[Table 3]

	TE		
	TnC expression	TnC4 (MOI 10,000)	scAAV5 TE(MOI 1,000)
U-251	+	+	+++
U87-MG	+	++	+++
U118-MG	+	+	++
HCT-116	-	-	++

[70] <Transduction of tenascin C-positive and tenascin C-negative

[71] cancer cells with TnC4 virus>

Industrial Applicability

[72] As described above, the adeno-associated virus serotype 5-derived targetable vector of the present invention has excellent safety and transducing activity and can be widely used to prevent and treat various diseases, including blood diseases, central nervous system diseases, metabolic diseases, autoimmune diseases, infectious diseases and tumors, in the medical and veterinary fields.

Claims

[1] A targeted recombinant virus vector derived from adeno-associated virus serotype 5, in which the virus vector comprises a nucleic acid sequence encoding a capsid protein containing a targeting motif peptide inserted in the internal amino acid residue of the capsid protein.

[2] The targeted recombinant virus vector of Claim 1, wherein the amino acid residue is amino acid residue 444 or 578 of the capsid protein.

[3] The targeted recombinant virus vector of Claim 1, wherein the targeting motif peptide has an amino acid length of 5-25 amino acids.

[4] The targeted recombinant virus vector of Claim 1, wherein the targeting motif peptide recognizes a molecule present in a tumor cell membrane.

[5] The targeted recombinant virus vector of Claim 4, wherein the molecule present in the tumor cell membrane is sialyl Lewis X, integrin or tenascin C.

[6] The targeted recombinant virus vector of Claim 5, wherein the targeting motif peptide specifically recognizing sialyl Lewis X has an amino acid sequence of SEQ ID NO: 1 or SEQ ID NO: 2.

[7] The targeted recombinant virus vector of Claim 5, wherein the motif peptide specifically recognizing integrin has an amino acid sequence of SEQ ID NO: 3 or SEQ ID NO: 4.

[8] The targeted recombinant virus vector of Claim 5, wherein the motif peptide specifically recognizing tenascin C has an amino acid sequence of SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7 and SEQ ID NO: 8.

[9] A method of delivering a gene in a target cell-specific manner using a targeted recombinant virus vector derived from adeno-associated virus serotype 5, in which the virus vector comprises a nucleic acid sequence encoding a capsid protein containing a targeting motif peptide inserted in the internal amino acid residue of the capsid protein.

[10] The method of Claim 9, wherein the amino acid residue is amino acid residue 444 or 578 of the capsid protein.

[11] The method of Claim 9, wherein the targeting motif peptide has an amino acid length of 5-25 amino acids.

[12] The method of Claim 9, wherein the targeting motif peptide recognizes a molecule present in a tumor cell membrane.

[13] The method of Claim 12, wherein the molecule present in the tumor cell

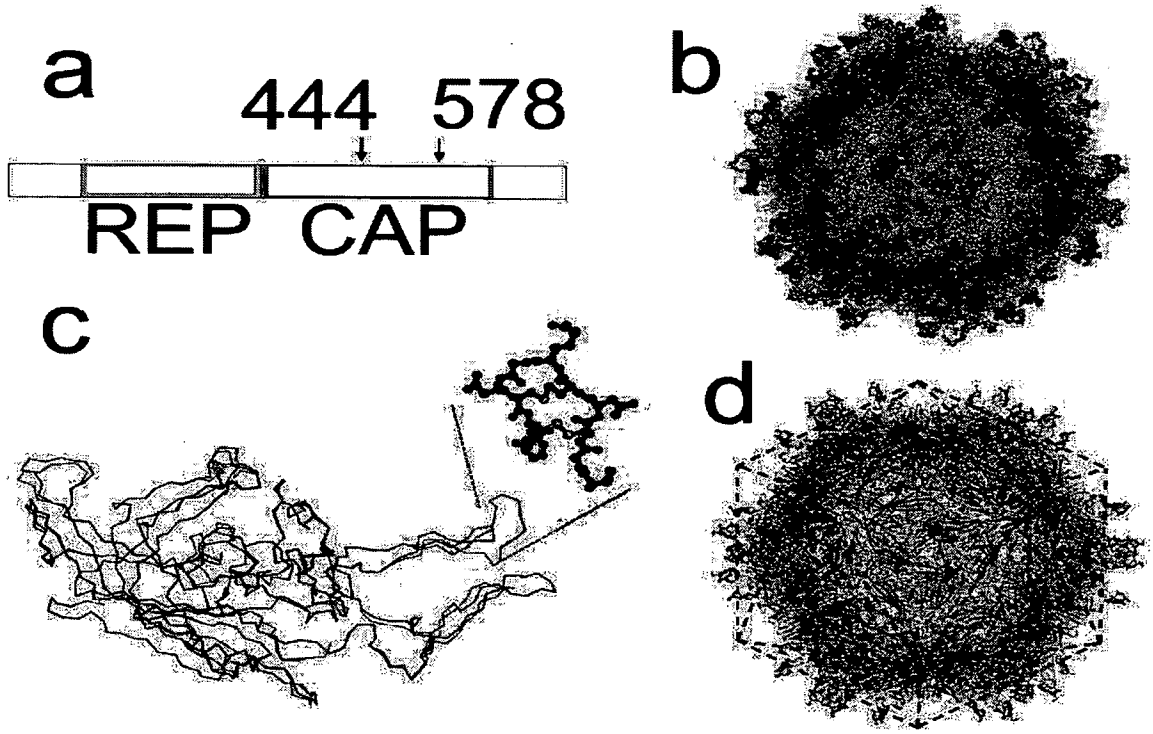
membrane is sialyl Lewis X, integrin or tenascin C.

[14] The method of Claim 13, wherein the targeting motif peptide specifically recognizing sialyl Lewis X has an amino acid sequence of SEQ ID NO: 1 or SEQ ID NO: 2.

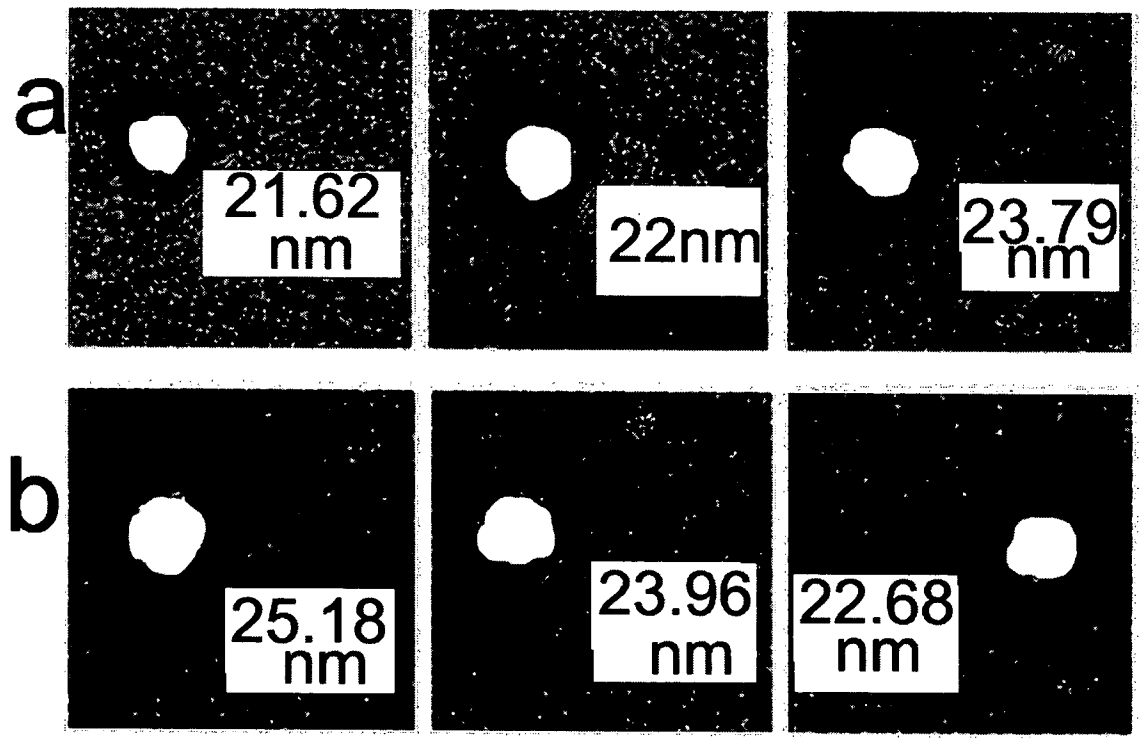
[15] The method of Claim 13, wherein the motif peptide specifically recognizing integrin has an amino acid sequence of SEQ ID NO: 3 or SEQ ID NO: 4.

[16] The method of Claim 13, wherein the motif peptide specifically recognizing tenascin C has an amino acid sequence of SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7 or SEQ ID NO: 8.

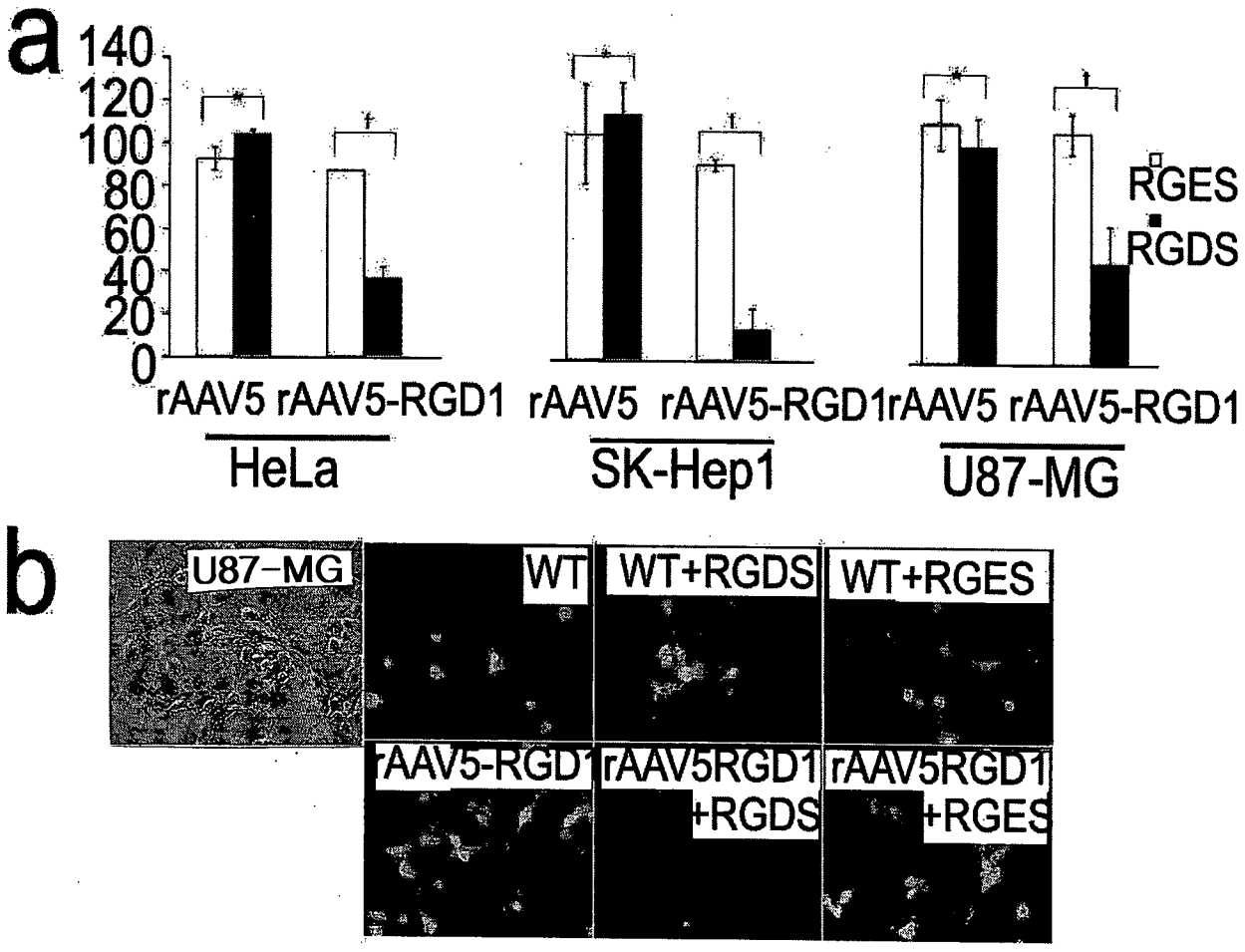
[Fig. 1]


a
444 578
REP CAP
b
c
d

[Fig. 2]


a
21.62 nm
22nm
23.79 nm
b
25.18 nm
23.96 nm
22.68 nm

[Fig. 3]


a
140
120
100
80
60
40
20
0
RGES
RGDS
rAAV5 rAAV5-RGD1 rAAV5 rAAV5-RGD1 rAAV5 rAAV5-RGD1
HeLa
SK-Hep1
U87-MG
b
U87-MG
WT
WT+RGDS
WT+RGES
rAAV5-RGD1
rAAV5RGD1 +RGDS
rAAV5RGD1 +RGES

INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR2008/004476

A. CLASSIFICATION OF SUBJECT MATTER

C12N 15/66(2006.01)i

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)

IPC: C12N 15/66

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)

Pubmed, Esp@snet, Delphion

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- A	Grifman, M. et al. 'Incorporation of Tumor-targeting peptides into recombinant adeno-associated virus capsids.' Molecular Therapy. Vol. 3(6), pp. 964-975 (June 2001) See abstract; Fig.1.	1, 3-5, 9, 11-13 --- 2, 6-8, 10, 14-16
A	Kwon, M. et al. 'Mimicry of tandem repeat peptides against cell surface carbohydrates.' Journal of the American Chemical Society. Vol. 124(47), pp. 13996-13997 (27 November 2002)	1-16
A	Ireson, C. R. and Kelland, L. R. 'Discovery and development of anticancer aptamers.' Molecular Cancer Therapy. Vol. 5(12), pp. 2957-2962 (December 2006)	1-16
A	Holig, P. et al. 'Novel RGD lipopeptides for the targeting of liposomes to integrin-expressing endothelial and melanoma cells.' Protein Engineering, Design & Selection. Vol. 17(5), pp. 433-441 (21 July 2004)	1-16
A	Davidson, B. L. et al. 'Recombinant adeno-associated virus type 2, 4, and 5 vectors: transduction of variant cell types and regions in the mammalian central nervous system.' Proceedings of the National Academy of Sciences, USA. Vol. 97(7), pp. 3428-3432 (28 March 2000)	1-16

☐ Further documents are listed in the continuation of Box C.

☐ See patent family annex.

* Special categories of cited documents:

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

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
25 FEBRUARY 2009 (25.02.2009)	**25 FEBRUARY 2009 (25.02.2009)**

Name and mailing address of the ISA/KR

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Facsimile No. 82-42-472-7140

Authorized officer

KIM, JI YUN

Telephone No. 82-42-481-8288

INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR2008/004476

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item1.b of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of :

   a. type of material
   - [x] a sequence listing
   - [ ] table(s) related to the sequence listing

   b. format of material
   - [x] on paper
   - [x] in electronic form

   c. time of filing/furnishing
   - [x] contained in the international application as filed
   - [x] filed together with the international application in electronic form
   - [ ] furnished subsequently to this Authority for the purposes of search

2. [ ] In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments: