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(54) Title: TUMOR-TARGETING SYNTHETIC ADENOVIRUSES AND USES THEREOF

(57) Abstract: Synthetic adenoviruses with liver detargeting mutations and expressing an adenovirus type 34 (Ad34) fiber protein, or a chimeric fiber protein with an Ad34 knob domain, are described. The synthetic adenoviruses traffic to sites of tumors. Use of the synthetic adenoviruses for delivering diagnostic or therapeutic transgenes to tumors are also described.

TUMOR-TARGETING SYNTHETIC ADENOVIRUSES AND USES THEREOF

CROSS REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Application No. 62/433,140, filed
5 December 12, 2016, which is herein incorporated by reference in its entirety.

FIELD

This disclosure concerns synthetic adenoviruses having chimeric fiber proteins and liver detargeting mutations that traffic to sites of tumors. This disclosure further concerns use of the
10 synthetic adenoviruses to express diagnostic or therapeutic transgenes in tumors.

BACKGROUND

Adenovirus (Ad) is a natural multi-gene expression vehicle. Certain coding regions of the virus, such as the E1, E3 and E4 regions, are either not necessary for replication in culture or can be
15 complemented with available cell lines. Therefore, each of these regions can be replaced with non-viral genes to drive the expression of multiple transgenes from a single virus. There are 68 different human adenovirus serotypes, each of which has different properties. Ad5 has been the predominant Ad vector used in basic research, gene therapy and oncolytic virus therapy. However, Ad5 has a limited tropism and only infects epithelial cells that have the coxsackie adenovirus
20 receptor (CAR) receptor for viral uptake. Furthermore, when injected intravenously, Ad5 binds to blood factors that cause it to be sequestered in the liver where it can trigger potentially limiting inflammation and toxicity. Thus, a need remains for modified adenovirus vectors capable of infecting specific cell types following intravenous administration.

25
SUMMARY

Described herein is the finding that a liver-detargeted synthetic adenovirus expressing a fiber protein with an adenovirus type 34 (Ad34) knob domain is capable of homing to sites of tumors. The synthetic adenoviruses can be used to deliver and express diagnostic or therapeutic transgenes in tumor cells, including tumor stromal cells.

30 Provided herein is a method of expressing a transgene in tumor cells of a subject. In some embodiments, the method includes administering to the subject a synthetic adenovirus that includes the transgene, a native or modified capsid that detargets the synthetic adenovirus from the liver, and an Ad34 fiber protein or a chimeric fiber protein comprising an adenovirus type 5 (Ad5) shaft

domain and an Ad34 knob domain. The transgene can be, for example, a diagnostic transgene or a therapeutic transgene.

Also provided herein is a method of diagnosing a subject as having a tumor. In some embodiments, the method includes administering to the subject a synthetic adenovirus that includes a diagnostic transgene, a native or modified capsid that detargets the synthetic adenovirus from the liver, and an Ad34 fiber protein or a chimeric fiber protein comprising an Ad5 shaft domain and an Ad34 knob domain. In some examples, the diagnostic transgene is a positron emission tomography (PET) reporter gene. In other examples, the diagnostic transgene encodes a fluorescent protein or an enzyme.

Further provided herein is a method of treating a tumor in a subject. In some embodiments, the method includes administering to the subject a synthetic adenovirus that includes a therapeutic transgene, a native or modified capsid that detargets the synthetic adenovirus from the liver, and an Ad34 fiber protein or a chimeric fiber protein comprising an Ad5 shaft domain and an Ad34 knob domain. In some examples, the therapeutic transgene encodes an anti-cancer agent or an agent that disrupts or kills tumor stromal cells.

Synthetic adenovirus genomes having at least 95% sequence identity to SEQ ID NO: 2 or SEQ ID NO: 5 are also provided by the present disclosure.

The foregoing and other objects and features of the disclosure will become more apparent from the following detailed description, which proceeds with reference to the accompanying figures.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGS. 1A-1B. AdSyn-CO176 homes to pancreatic tumors. (FIG. 1A) Cre-LoxP $Kras^{G12D}$/p53 pancreatic tumor model overview. Mice designated as “Kras; p53/p53” encode the $Kras^{G12D}$ oncogene downstream of the sequence encoding LoxP-stop codon-LoxP. The stop codon blocks the expression of $Kras^{G12D}$ in the absence of Cre recombinase. However, in the presence of Cre recombinase, the stop codon is removed and allows for expression of the $Kras^{G12D}$ oncogene. In these same mice, both alleles of the p53 gene are flanked by LoxP sites (LoxP-p53-LoxP). Mice designated “p53/p53; Cre” have both alleles of the p53 gene flanked by LoxP sites (LoxP-p53-LoxP) and also express the Cre recombinase transgene driven by the pancreatic and duodenal homeobox 1 (Pdx1) promoter. Pdx1 is a gene that is expressed specifically in the pancreatic cells, and thus both copies of p53 are deleted in the pancreatic cells. Breeding between the strains gives

rise to offspring in which the Pdx1 promoter-driven Cre mediates the deletion of both alleles of the tumor suppressor p53 and activation of the mutant $Kras^{G12D}$ in pancreatic cells. Homozygous mice designated "Kras; p53/p53; Cre" develop pancreatic tumors in 5-7 weeks. (FIG. 1B) AdSyn-CO176, a synthetic virus with a chimeric fiber protein comprising the Ad34 knob domain, was
5 injected intravenously into Kras; p53/p53 and Kras; p53/p53; Cre mice. Seventy-two hours after the injection of virus, tissues were collected, incubated for 5 minutes with luciferin, and then scanned for 5 minutes using the IVIS™ imaging system. The Kras; p53/p53 mouse had a normal pancreas, and the luciferase signal was mainly from the spleen. The Kras; p53/p53; Cre mouse had pancreatic tumors, and the signal was mainly from the pancreatic tumor.

10 **FIGS. 2A-2B. AdSyn-CO176 infected pancreatic tumor after the tail vein injection in a Cre-mediated genetic manipulation heterozygous model.** (FIG. 2A) Kras; p53/p53 mice are as described for FIG. 1A. Mice designated as "p53/+; Cre" mice have one wild type p53 allele and one p53 allele flanked by LoxP sites (LoxP-p53-LoxP). Breeding between these two strains gives rise to offspring in which the Pdx1 promoter-driven Cre recombinase mediates the deletion of a
15 single allele of the tumor suppressor p53 and activation of the mutant $Kras^{G12D}$ in pancreatic cells. These heterozygous mice, designated "Kras; p53/+; Cre," develop tumors later in life (at 4-9 months of age) due to the fact that they have one wild type allele of p53. This wild type allele must be lost or mutated in order for pancreatic tumors to develop. (FIG. 2B) AdSyn-CO176 was injected intravenously into p53/+; Cre and Kras; p53/+; Cre mice (4 months old). Seventy-two hours after
20 the injection of virus, tissues were collected, incubated with luciferin for 5 minutes, and then scanned for 1 minute using the IVIS™ imaging system. The p53/+; Cre mouse had a normal pancreas, and the signal was mainly from spleen. The Kras; p53/+; Cre mouse at 4 months of age had a pancreatic tumor, and the signal was mainly from the tumor and liver.

FIGS. 3A-3C. AdSyn-CO176 can infect and diagnose a pancreatic tumor at an early
25 **stage after tail vein injection.** The heterozygous Kras; p53/+; Cre mice develop pancreatic tumors in 4-9 months. To test whether AdSyn-CO176 can infect pancreatic tumors at a very early stage of tumor development (before tumors are visible), AdSyn-176 was injected into Kras; p53/+; Cre mice at 2 months of age and luciferase expression was measured. (FIG. 3A) AdSyn-CO176 was injected intravenously into Kras; p53/+; Cre mice at 2 months of age. Seventy-two hours after the injection
30 of virus, tissues were collected, incubated with luciferin for 5 minutes, and scanned for 4 minutes using the IVIS™ imaging system. The pancreas of Kras; p53/+; Cre mouse at 2 months old looked normal but luciferase signal was found in this tissue. (FIG. 3B) H&E staining showing the typical histology of normal pancreas (a) and pancreatic tumor (b). (FIG. 3C) H&E staining of the pancreas

from a Kras; p53/+; Cre mouse at 2 months of age showing that a small part of the pancreas was developing the tumor (as shown in the polygon). Most of the pancreas appeared normal. This result indicates that AdSyn-CO176 can infect pancreatic tumors at a very early stage.

FIGS. 4A-4B. AdSyn-CO176 infects stromal cells in the pancreatic tumor. In pancreatic tumors, only 10% of the cells are cancer cells; the remaining 90% are stroma cells. As determined by immunohistochemistry (IHC) and immunofluorescence (IF) staining, the cells that were infected by AdSyn-CO176 were stromal cells. (FIG. 4A) IHC staining of a pancreatic tumor infected with AdSyn-CO176. CK19 is a marker of tumor cells while smooth muscle actin (SMA) is a marker of stromal cells. The staining of GFP, which was expressed from AdSyn-CO176, overlapped with SMA staining, indicating that AdSyn-CO176 targets stromal cells. (FIG. 4B) IF staining of the pancreatic tumor infected by AdSyn-CO176. GFP staining overlapped with SMA staining, confirming AdSyn-CO176 infection of stromal cells.

FIGS. 5A-5D. AdSyn-CO176 infected glioblastoma after tail vein injection. Synthetic adenovirus AdSyn-CO176 was injected into mice with glioblastoma by tail vein and the luciferase signal was found in the glioblastoma. (FIG. 5A) Schematic of a Cre-mediated genetic manipulation glioblastoma model. Lentiviruses were injected directly into the brain of GFAP-Cre mice. Glial fibrillary acidic protein (GFAP) promoter-driven Cre recombinase cleaves out RFP from the lentivirus-encoded gene and induces the expression of $HRas^{V12}$ and GFP primarily in astrocytes. Expression of lentivirus-encoded U6-p53 shRNA knocks down the expression of p53 in the brain cells that take up the virus. The expression of $HRas^{V12}$ and the knock down of p53 induces tumorigenesis in the brain from 1 week after the injection. GFP signal is used to indicate the formation of glioblastoma. (FIG. 5B) Saline, AdSyn-CO171, or AdSyn-CO176 were injected via intravenous (IV) administration into GFAP-Cre mice that had received the tumor-inducing lentiviruses 4 weeks earlier. Forty-eight hours after the injection of virus, mice were scanned for 1 minute using the IVIS™ imaging system 5 minutes after the intraperitoneal injection of luciferin. The luciferase signal was detected in AdSyn-CO176-infected mice (arrow), while no signal was detected in saline-treated or AdSyn-CO171-injected mice. (FIG. 5C) Wild type mice (normal brain) and GFAP-Cre mice with the injection of lentiviruses (develop brain tumors) were injected with AdSyn-CO171 or AdSyn-CO176. Brain tissues were collected 72 hours after the injection of synthetic adenoviruses, incubated with luciferin for 5 minutes, and scanned for 5 minutes using the IVIS™ imaging system. Only the GFAP-Cre mouse injected with the tumor-inducing lentiviruses showed a luciferase signal from AdSyn-CO176. This demonstrates that AdSyn-CO176 will traffic to the brain tissue only when a tumor is present. (FIG. 5D) Brain tissues were also scanned for the

GFP signal. The GFP signal is used to identify the glioblastoma. Both of the GFAP-Cre mice that received the tumor-inducing lentiviruses had the GFP signal in the brain, while no GFP was detected in mice that did not receive lentivirus. The GFP signal overlapped with the luciferase signal perfectly in the GFAP-Cre mouse that received the tumor-inducing lentiviruses and AdSyn-CO176.

FIGS. 6A-6B. Trafficking of AdSyn-CO176 to glioblastoma is driven by the tumor, not injury. The injection of the tumor-inducing lentiviruses causes temporary injury to the brain at the injection site. Although the synthetic adenoviruses (AdSyn-CO171 and AdSyn-CO176) were injected 4 weeks after the initial injection of lentiviruses, it was still unclear whether trafficking of AdSyn-CO176 to the glioblastoma was driven by the tumor or by the injection site injury. To answer this question, GFAP-Cre mice were injected with synthetic adenovirus 4 weeks after either no injection, sham-injection or injection with tumor-inducing lentivirus. (FIG. 6A) GFAP-Cre mice were injected with either Hanks' balanced salt solution (HBSS) or tumor-inducing lentiviruses. After 4 weeks, AdSyn-CO171 was injected intravenously. There was no luciferase signal from AdSyn-CO171 in the brain in either group of mice. (FIG. 6B) GFAP-Cre mice were injected with HBSS or tumor-inducing lentiviruses, or received no injection. After 4 weeks, mice were injected intravenously with AdSyn-CO176. The luciferase signal was detected only in the brain of the mouse that received the tumor-inducing lentiviruses, while the mouse that received HBSS or no injection produced no signal. These results demonstrate that the specificity of AdSyn-CO176 is driven by the tumor and not the injection site injury.

FIGS. 7A-7B. AdSyn-CO176 can traffic to human glioblastoma xenograft tumors. (FIG. 7A) Schematic of the human glioblastoma xenograft model. Human glioblastoma U87 cells that expresses the tdTomato fluorescent protein (U87-tdTomato) were injected intracranially into NOD scid gamma (NSG) mice to generate glioblastoma tumors. (FIG. 7B) AdSyn-CO171 and AdSyn-CO176 were injected intravenously into NSG mice by tail vein injection 4 weeks after they received the intracranial injection of U87-tdTomato. Forty-eight hours after the injection of viruses, the tissues indicated in the panel were collected, incubated with luciferin for five minutes and then scanned for 1 minute using the IVIS™ imaging system. Only the AdSyn-CO176 injected mice showed luciferase signal in the brain, and this signal completely overlapped with tdTomato expression.

FIGS. 8A-8C. Administration of a synthetic adenovirus with a therapeutic transgene. (FIG. 8A) Schematic of the KPCL (Kras^{G12D}; p53 knockout; Pdx1-Cre; firefly Luciferase) mouse model. KPCL mice are similar to homozygous "Kras; p53/p53; Cre" mice, which specifically

express $Kras^{G12D}$ in the pancreas and have the p53 gene knocked out only in the pancreas. However, KPCL mice also specifically express firefly luciferase in the pancreas. The development of tumors in KPCL mice is also similar to the "Kras; p53/p53; Cre" mice. (FIG. 8B) Table showing average survival of KPCL mice for each treatment (at least 4 mice per treatment group).
5 AdSyn-CO987 is a synthetic adenovirus based upon AdSyn-CO176. The herpes simplex virus-1 thymidine kinase (TK)/ganciclovir (GCV) suicide gene was cloned into AdSyn-CO176 to replace the firefly luciferase/GFP gene. A Renilla luciferase was also inserted just after TK in the genome of AdSyn-CO176. Control virus AdSyn-CO989 was generated by cloning TK-P2A-renilla luciferase into AdSyn-CO171 to replace the original firefly luciferase/GFP gene. KPCL mice were
10 injected intravenously via the tail vein with 1 x 10^6 plaque forming units (PFU) of the indicated viruses at 5-6 weeks of age. Two days later, the mice were injected intraperitoneally (i.p.) or intravenously (i.v.) with GCV. Three control groups were used: AdSyn-CO989+GCV; AdSyn-CO987 followed with saline injection (AdSyn-CO987+saline); and GCV injection only (i.p. or i.v.). Treatment with AdSyn-CO987+GCV extended mouse survival compared with controls.
15 (FIG. 8C) Images of representative mice showing firefly luciferase signal. The firefly luciferase signal (expressed by tumors) was analyzed during treatment to monitor tumor growth. The treatment for mouse Z619R was AdSyn-CO987+saline, which served as the control. Mice Z601R and Z607R were treated with AdSyn-CO987+GCV (i.p.). While the strength of the firefly luciferase signal increased in the control mouse Z619R (indicating an increase in tumor size), the
20 signal decreased in mice Z601R and Z607R (indicating a reduction in tumor size).

FIGS. 9A-9B. Histology of tumors in mice treated with AdSyn-CO987. (FIG. 9A) Images of H&E staining of pancreatic tumors. Mice Z655, 1806, Z619 and Z621 were all control mice. Mouse Z655 was treated with i.p. injected GCV only; mouse 1806 was treated with i.v. injected GCV only; mouse Z619 was treated with AdSyn-CO987+saline; and mouse Z621 received
25 no treatment. Mouse Z656 received treatment with AdSyn-CO987+GCV i.v. Compared to the controls, the tumor from Z656 had more regions of necrosis (as indicated by the arrowheads). (FIG. 9B) Representative regions of necrosis in the tumor of Z656 with magnification. The regions are also indicated in FIG. 9A.

30

SEQUENCE LISTING

The nucleic and amino acid sequences listed in the accompanying sequence listing are shown using standard letter abbreviations for nucleotide bases, and three letter code for amino acids, as defined in 37 C.F.R. 1.822. Only one strand of each nucleic acid sequence is shown, but

the complementary strand is understood as included by any reference to the displayed strand. The Sequence Listing is submitted as an ASCII text file, created on November 30, 2017, 215 KB, which is incorporated by reference herein. In the accompanying sequence listing:

SEQ ID NO: 1 is the nucleotide sequence of synthetic adenovirus AdSyn-CO171.

5 **SEQ ID NO: 2** is the nucleotide sequence of synthetic adenovirus AdSyn-CO176.

SEQ ID NO: 3 is the amino acid sequence of Ad5 hexon.

SEQ ID NO: 4 is the amino acid sequence of Ad5 hexon E451Q.

SEQ ID NO: 5 is the nucleotide sequence of synthetic adenovirus AdSyn-CO987.

SEQ ID NO: 6 is the nucleotide sequence of synthetic adenovirus AdSyn-CO989.

10

DETAILED DESCRIPTION

I. Abbreviations

Ad adenovirus

CAR coxsackie adenovirus receptor

15 CEA carcinoembryonic antigen

EGF epidermal growth factor

EGFR epidermal growth factor receptor

FLT3 Fms-related tyrosine kinase 3

GCV ganciclovir

20 GFAP glial fibrillary acidic protein

GFP green fluorescent protein

GM-CSF granulocyte macrophage colony stimulating factor

H&E hematoxylin and eosin

HSV herpes simplex virus

25 ICAM intercellular adhesion molecule

IF immunofluorescence

IHC immunohistochemistry

IL interleukin

IRES internal ribosomal entry site

30 i.p. intraperitoneal

i.v. intravenous

KPCL $Kras^{G12D}$; p53 knockout; Pdx1-Cre; firefly Luciferase

LFA lymphocyte function-associated antigen

miR	microRNA
MUC1	mucin 1
NOD	non-obese diabetic
NSG	NOD scid gamma
PD-1	programmed cell death protein 1
PDGF	platelet derived growth factor
PET	positron emission tomography
PFU	plaque forming unit
shRNA	short hairpin RNA
SMA	smooth muscle actin
TGF	transforming growth factor
TK	thymidine kinase
TNF	tumor necrosis factor
UTR	untranslated region
VEGF	vascular endothelial growth factor
WT	wild-type

II. Terms and Methods

Unless otherwise noted, technical terms are used according to conventional usage. Definitions of common terms in molecular biology may be found in Benjamin Lewin, *Genes V*, published by Oxford University Press, 1994 (ISBN 0-19-854287-9); Kendrew *et al.* (eds.), *The Encyclopedia of Molecular Biology*, published by Blackwell Science Ltd., 1994 (ISBN 0-632-02182-9); and Robert A. Meyers (ed.), *Molecular Biology and Biotechnology: a Comprehensive Desk Reference*, published by VCH Publishers, Inc., 1995 (ISBN 1-56081-569-8).

In order to facilitate review of the various embodiments of the disclosure, the following explanations of specific terms are provided:

Adenovirus: A non-enveloped virus with a linear, double-stranded DNA genome and an icosahedral capsid. There are currently 68 known serotypes of human adenovirus, which are divided into seven species (species A, B, C, D, E, F and G). Different serotypes of adenovirus are associated with different types of disease, with some serotypes causing respiratory disease (primarily species B and C), conjunctivitis (species B and D) and/or gastroenteritis (species F and G).

Administration: To provide or give a subject an agent, such as a therapeutic agent (*e.g.* a recombinant virus), by any effective route. Exemplary routes of administration include, but are not limited to, injection (such as subcutaneous, intramuscular, intradermal, intraperitoneal, intratumoral and intravenous), oral, intraductal, sublingual, rectal, transdermal, intranasal, vaginal and inhalation routes.

Chimeric: Composed of at least two parts having different origins. In the context of the present disclosure, a "**chimeric adenovirus**" is an adenovirus having genetic material and/or proteins derived from at least two different serotypes (such as from Ad5 and a second serotype of adenovirus). In this context, a "**capsid-swapped**" adenovirus refers to a chimeric adenovirus in which the capsid proteins are derived from one serotype of adenovirus and the remaining proteins are derived from another adenovirus serotype. Similarly, a "**chimeric fiber**" is a fiber protein having amino acid sequence derived from at least two different serotypes of adenovirus. For example, a chimeric fiber can be composed of a fiber shaft from Ad5 and a fiber knob from a second serotype of adenovirus.

Contacting: Placement in direct physical association; includes both in solid and liquid form.

Degenerate variant: In the context of the present disclosure, a "degenerate variant" refers to a polynucleotide encoding a peptide that includes a sequence that is degenerate as a result of the genetic code. There are 20 natural amino acids, most of which are specified by more than one codon. Therefore, all degenerate nucleotide sequences encoding a peptide are included as long as the amino acid sequence of the peptide encoded by the nucleotide sequence is unchanged.

Detargeted: In the context of the present disclosure, a "detargeted" adenovirus is a recombinant or synthetic adenovirus comprising one or more modifications that alter tropism of the virus such that is no longer infects, or no longer substantially infects, a particular cell or tissue type. In some embodiments, the recombinant or synthetic adenovirus comprises a capsid mutation, such as a mutation in the hexon protein (for example, E451Q). In some embodiments, the recombinant or synthetic adenovirus comprises a native capsid from an adenovirus that naturally does not infect, or does not substantially infect, a particular cell or tissue type. In some embodiments herein, the recombinant or synthetic adenovirus is liver detargeted and/or spleen detargeted.

E1A: The adenovirus early region 1A (E1A) gene and polypeptides expressed from the gene. The E1A protein plays a role in viral genome replication by driving cells into the cell cycle. As used herein, the term "E1A protein" refers to the proteins expressed from the E1A gene and the term includes E1A proteins produced by any adenovirus serotype.

Fiber: The adenovirus fiber protein is a trimeric protein that mediates binding to cell surface receptors. The fiber protein is comprised of a long N-terminal shaft and globular C-terminal knob.

Fusion protein: A protein containing amino acid sequence from at least two different (heterologous) proteins or peptides. Fusion proteins can be generated, for example, by expression of a nucleic acid sequence engineered from nucleic acid sequences encoding at least a portion of two different (heterologous) proteins. To create a fusion protein, the nucleic acid sequences must be in the same reading frame and contain no internal stop codons. Fusion proteins, particularly short fusion proteins, can also be generated by chemical synthesis.

Glioblastoma: A fast-growing type of central nervous system tumor that forms from glial tissue of the brain and spinal cord. Glioblastoma usually occurs in adults and affects the brain more often than the spinal cord. Glioblastoma is the most common and most aggressive cancer that initiates in the brain. Glioblastoma is also known as glioblastoma multiforme (GBM) and grade IV astrocytoma.

Heterologous: A heterologous protein or gene refers to a protein or gene derived from a different source or species.

Hexon: A major adenovirus capsid protein. An exemplary hexon sequence from Ad5 is set forth herein as SEQ ID NO: 3. A mutant hexon sequence comprising an E451Q substitution is set forth herein as SEQ ID NO: 4.

Isolated: An "isolated" biological component (such as a nucleic acid molecule, protein, virus or cell) has been substantially separated or purified away from other biological components in the cell or tissue of the organism, or the organism itself, in which the component naturally occurs, such as other chromosomal and extra-chromosomal DNA and RNA, proteins and cells. Nucleic acid molecules and proteins that have been "isolated" include those purified by standard purification methods. The term also embraces nucleic acid molecules and proteins prepared by recombinant expression in a host cell as well as chemically synthesized nucleic acid molecules and proteins.

MicroRNA (miRNA or miR): A single-stranded RNA molecule that regulates gene expression in plants, animals and viruses. A gene encoding a microRNA is transcribed to form a primary transcript microRNA (pri-miRNA), which is processed to form a short stem-loop molecule, termed a precursor microRNA (pre-miRNA), followed by endonucleolytic cleavage to form the mature microRNA. Mature microRNAs are approximately 21-23 nucleotides in length and are partially complementary to the 3'UTR of one or more target messenger RNAs (mRNAs).

MicroRNAs modulate gene expression by promoting cleavage of target mRNAs or by blocking translation of the cellular transcript. In the context of the present disclosure, a "**liver-specific microRNA**" is a microRNA that is preferentially expressed in the liver, such as a microRNA that is expressed only in the liver, or a microRNA that is expressed significantly more in the liver as compared to other organs or tissue types. In some embodiments, the microRNA is miR-122. In the context of the present disclosure, a "**spleen-specific microRNA**" is a microRNA that is preferentially expressed in the spleen, such as a microRNA that is expressed only in the spleen, or a microRNA that is expressed significantly more in the spleen as compared to other organs or tissue types. In some embodiments, the microRNA is miR-142-3p.

Modification: A change in the sequence of a nucleic acid or protein sequence. For example, amino acid sequence modifications include, for example, substitutions, insertions and deletions, or combinations thereof. Insertions include amino and/or carboxyl terminal fusions as well as intrasequence insertions of single or multiple amino acid residues. Deletions are characterized by the removal of one or more amino acid residues from the protein sequence. In some embodiments herein, the modification (such as a substitution, insertion or deletion) results in a change in function, such as a reduction or enhancement of a particular activity of a protein. As used herein, "Δ" or "delta" refer to a deletion. Substitutional modifications are those in which at least one residue has been removed and a different residue inserted in its place. Amino acid substitutions are typically of single residues, but can occur at a number of different locations at once. Substitutions, deletions, insertions or any combination thereof may be combined to arrive at a final mutant sequence. These modifications can be prepared by modification of nucleotides in the DNA encoding the protein, thereby producing DNA encoding the modification. Techniques for making insertion, deletion and substitution mutations at predetermined sites in DNA having a known sequence are well known in the art. A "**modified**" protein, nucleic acid or virus is one that has one or more modifications as outlined above.

Operably linked: A first nucleic acid sequence is operably linked with a second nucleic acid sequence when the first nucleic acid sequence is placed in a functional relationship with the second nucleic acid sequence. For instance, a promoter is operably linked to a coding sequence if the promoter affects the transcription or expression of the coding sequence. Generally, operably linked DNA sequences are contiguous and, where necessary to join two protein-coding regions, in the same reading frame.

Pancreatic cancer: Cancer that begins in the tissues of the pancreas. Pancreatic cancer typically spreads rapidly and is seldom detected at early stages, leading to a poor prognosis for

most diagnosed patients. The most common type of pancreatic cancer is pancreatic adenocarcinoma, which accounts for approximately 85% of pancreatic cancer cases.

Pharmaceutically acceptable carrier: The pharmaceutically acceptable carriers (vehicles) useful in this disclosure are conventional. *Remington's Pharmaceutical Sciences*, by E. W. Martin, Mack Publishing Co., Easton, PA, 15th Edition (1975), describes compositions and formulations suitable for pharmaceutical delivery of one or more therapeutic compounds, molecules or agents (*e.g.* a synthetic virus disclosed herein).

In general, the nature of the carrier will depend on the particular mode of administration being employed. For instance, parenteral formulations usually comprise injectable fluids that include pharmaceutically and physiologically acceptable fluids such as water, physiological saline, balanced salt solutions, aqueous dextrose, glycerol or the like as a vehicle. For solid compositions (for example, powder, pill, tablet, or capsule forms), conventional non-toxic solid carriers can include, for example, pharmaceutical grades of mannitol, lactose, starch, or magnesium stearate. In addition to biologically-neutral carriers, pharmaceutical compositions to be administered can contain minor amounts of non-toxic auxiliary substances, such as wetting or emulsifying agents, preservatives, and pH buffering agents and the like, for example sodium acetate or sorbitan monolaurate.

Polypeptide, peptide or protein: A polymer in which the monomers are amino acid residues which are joined together through amide bonds. When the amino acids are alpha-amino acids, either the L-optical isomer or the D-optical isomer can be used. The terms "polypeptide," "peptide" and "protein" are used interchangeably herein. These terms apply to amino acid polymers in which one or more amino acid residue is an artificial chemical mimetic of a corresponding naturally occurring amino acid, as well as to naturally occurring amino acid polymers and non-naturally occurring amino acid polymers. The term "residue" or "amino acid residue" includes reference to an amino acid that is incorporated into a protein, polypeptide, or peptide.

A conservative substitution in a polypeptide is a substitution of one amino acid residue in a protein sequence for a different amino acid residue having similar biochemical properties. Typically, conservative substitutions have little to no impact on the activity of a resulting polypeptide. For example, a protein or peptide including one or more conservative substitutions (for example no more than 1, 2, 3, 4 or 5 substitutions) retains the structure and function of the wild-type protein or peptide. A polypeptide can be produced to contain one or more conservative substitutions by manipulating the nucleotide sequence that encodes that polypeptide using, for

example, standard procedures such as site-directed mutagenesis or PCR. In one example, such variants can be readily selected by testing antibody cross-reactivity or its ability to induce an immune response. Examples of conservative substitutions are shown below.

Original Residue	Conservative Substitutions
Ala	Ser
Arg	Lys
Asn	Gln, His
Asp	Glu
Cys	Ser
Gln	Asn
Glu	Asp
His	Asn; Gln
Ile	Leu, Val
Leu	Ile; Val
Lys	Arg; Gln; Glu
Met	Leu; Ile
Phe	Met; Leu; Tyr
Ser	Thr
Thr	Ser
Trp	Tyr
Tyr	Trp; Phe
Val	Ile; Leu

Conservative substitutions generally maintain (a) the structure of the polypeptide backbone in the area of the substitution, for example, as a sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the side chain.

The substitutions which in general are expected to produce the greatest changes in protein properties will be non-conservative, for instance changes in which (a) a hydrophilic residue, for example, seryl or threonyl, is substituted for (or by) a hydrophobic residue, for example, leucyl, isoleucyl, phenylalanyl, valyl or alanyl; (b) a cysteine or proline is substituted for (or by) any other residue; (c) a residue having an electropositive side chain, for example, lysyl, arginyl, or histadyl, is substituted for (or by) an electronegative residue, for example, glutamyl or aspartyl; or (d) a residue

having a bulky side chain, for example, phenylalanine, is substituted for (or by) one not having a side chain, for example, glycine.

Positron emission tomography (PET): An imaging technique that is used to observe metabolic processes in the body. PET detects pairs of gamma rays emitted indirectly by a positron-emitting radionuclide, which is introduced into the body on a biologically active molecule. PET reporter genes encode molecules (such as receptors or enzymes) that provide a target for PET probes, which can then be detected by imaging. PET reporter genes are generally classified into three different groups: (1) reporter genes encoding enzymes that phosphorylate specific PET reporter probes, leading to their intracellular entrapment; (2) reporter genes encoding protein receptors that can be bound by specific PET reporter probes; and (3) reporter genes encoding protein transporters that transport a radionuclide reporter probe into cells expressing the reporter gene (Yaghoubi *et al.*, *Theranostics* 2(4):374-391, 2012). As used herein, a "**PET reporter gene**" includes any gene that encodes a protein capable of interacting with a PET reporter probe in a manner allowing for detection of the probe by molecular imaging. Exemplary PET reporter genes include, but are not limited to, herpes simplex virus (HSV) thymidine kinase (TK) and mutant forms thereof, varicella zoster virus (VSV) TK, human mitochondrial TK and mutants thereof, mutants of deoxycytidine kinase, dopamine 2 receptors mutants, human estrogen receptor α ligand binding domain (hERL), human somatostain receptor subtype 2 (hSSTr2), recombinant human carcinoembryonic antigen (CEA), engineered antibody fragments, humanized membrane anchored anti-polyethylene glycol (PEG), sodium iodide symporter (NIS), and human norepinephrine transporter (hNET) (see Yaghoubi *et al.* (2012) for a review of PET reporter genes and corresponding reporter probes).

Preventing, treating or ameliorating a disease: "Preventing" a disease refers to inhibiting the full development of a disease. "Treating" refers to a therapeutic intervention that ameliorates a sign or symptom of a disease or pathological condition after it has begun to develop. "Ameliorating" refers to the reduction in the number or severity of signs or symptoms of a disease.

Promoter: A region of DNA that directs/initiates transcription of a nucleic acid (*e.g.* a gene). A promoter includes necessary nucleic acid sequences near the start site of transcription. Typically, promoters are located near the genes they transcribe. A promoter also optionally includes distal enhancer or repressor elements which can be located as much as several thousand base pairs from the start site of transcription. A "constitutive promoter" is a promoter that is continuously active and is not subject to regulation by external signals or molecules. In contrast, the activity of an "inducible promoter" is regulated by an external signal or molecule (for example,

a transcription factor or tetracycline). A "tissue-specific promoter" is a promoter that is substantially active only in a particular tissue or tissues.

Protein IX (pIX): A minor component of the adenovirus capsid that associates with the hexon protein

5 **Purified:** The term "purified" does not require absolute purity; rather, it is intended as a relative term. Thus, for example, a purified peptide, protein, virus, or other active compound is one that is isolated in whole or in part from naturally associated proteins and other contaminants. In certain embodiments, the term "substantially purified" refers to a peptide, protein, virus or other active compound that has been isolated from a cell, cell culture medium, or other crude preparation
10 and subjected to fractionation to remove various components of the initial preparation, such as proteins, cellular debris, and other components.

Recombinant: A recombinant nucleic acid molecule, protein or virus is one that has a sequence that is not naturally occurring or has a sequence that is made by an artificial combination of two otherwise separated segments of sequence. This artificial combination can be accomplished
15 by chemical synthesis or by the artificial manipulation of isolated segments of nucleic acid molecules, such as by genetic engineering techniques. The term "recombinant" also includes nucleic acids, proteins and viruses that have been altered solely by addition, substitution, or deletion of a portion of the natural nucleic acid molecule, protein or virus.

Sequence identity: The identity or similarity between two or more nucleic acid sequences,
20 or two or more amino acid sequences, is expressed in terms of the identity or similarity between the sequences. Sequence identity can be measured in terms of percentage identity; the higher the percentage, the more identical the sequences are. Sequence similarity can be measured in terms of percentage similarity (which takes into account conservative amino acid substitutions); the higher the percentage, the more similar the sequences are. Homologs or orthologs of nucleic acid or amino acid
25 sequences possess a relatively high degree of sequence identity/similarity when aligned using standard methods. This homology is more significant when the orthologous proteins or cDNAs are derived from species which are more closely related (such as human and mouse sequences), compared to species more distantly related (such as human and *C. elegans* sequences).

Methods of alignment of sequences for comparison are well known in the art. Various
30 programs and alignment algorithms are described in: Smith & Waterman, *Adv. Appl. Math.* 2:482, 1981; Needleman & Wunsch, *J. Mol. Biol.* 48:443, 1970; Pearson & Lipman, *Proc. Natl. Acad. Sci. USA* 85:2444, 1988; Higgins & Sharp, *Gene*, 73:237-44, 1988; Higgins & Sharp, *CABIOS* 5:151-3, 1989; Corpet *et al.*, *Nuc. Acids Res.* 16:10881-90, 1988; Huang *et al. Computer Appls. in the*

Biosciences 8, 155-65, 1992; and Pearson *et al.*, *Meth. Mol. Bio.* 24:307-31, 1994. Altschul *et al.*, *J. Mol. Biol.* 215:403-10, 1990, presents a detailed consideration of sequence alignment methods and homology calculations.

The NCBI Basic Local Alignment Search Tool (BLAST) (Altschul *et al.*, *J. Mol. Biol.* 215:403-10, 1990) is available from several sources, including the National Center for Biological Information (NCBI) and on the internet, for use in connection with the sequence analysis programs blastp, blastn, blastx, tblastn and tblastx. Additional information can be found at the NCBI web site.

Serotype: A group of closely related microorganisms (such as viruses) distinguished by a characteristic set of antigens.

Stroma: The supportive tissues of an epithelial organ or tumor, consisting of connective tissues and blood vessels. **Stromal cells** are the cells that make up the stroma, primarily fibroblasts and pericytes. **Tumor stroma** is predominantly made up of fibroblasts, extracellular matrix, immune cells, vasculature and basement membrane (Bremnes *et al.*, *J Thorac Oncol* 6:209-217, 2011). **Tumor stromal cells** are known to play a significant role in cancer growth and progression.

Subject: Living multi-cellular vertebrate organisms, a category that includes human and non-human mammals.

Synthetic: Produced by artificial means in a laboratory, for example a synthetic nucleic acid or protein can be chemically synthesized in a laboratory.

Therapeutic agent: A chemical compound, small molecule, recombinant virus or other composition, such as an antisense compound, antibody, peptide or nucleic acid molecule capable of inducing a desired therapeutic or prophylactic effect when properly administered to a subject.

Therapeutically effective amount: A quantity of a specified pharmaceutical or therapeutic agent (*e.g.* a recombinant virus) sufficient to achieve a desired effect in a subject, or in a cell, being treated with the agent. The effective amount of the agent can be dependent on several factors, including, but not limited to the subject or cells being treated, and the manner of administration of the therapeutic composition.

Transgene: A gene that has been inserted into the genome of a different organism (such as a virus). Transgenes can also be referred to as heterologous genes. As used herein, a "**diagnostic transgene**" refers to any transgene encoding a detectable product, such as, but not limited to, a fluorescent protein, an enzyme or a PET reporter. As used herein a "**therapeutic transgene**" refers to any transgene encoding product with a therapeutic application. In the context of the present disclosure, a therapeutic transgene can be, for example, an anti-cancer agent or an agent that disrupts or kills cells of the tumor stroma.

Uexon: An open reading frame located on the *l* strand (leftward transcription) between the early E3 region and the fiber gene (Tollefson *et al.*, *J Virol* 81(23):12918-12926).

Vector: A nucleic acid molecule allowing insertion of foreign nucleic acid without disrupting the ability of the vector to replicate and/or integrate in a host cell. A vector can include nucleic acid sequences that permit it to replicate in a host cell, such as an origin of replication. A vector can also include one or more selectable marker genes and other genetic elements. An expression vector is a vector that contains the necessary regulatory sequences to allow transcription and translation of inserted gene or genes.

III. Overview of Several Embodiments

It is disclosed herein that a liver-detargeted synthetic adenovirus expressing a fiber protein with an Ad34 knob domain is capable of homing to sites of tumors. The synthetic adenoviruses can be used, for example, to deliver and express diagnostic or therapeutic transgenes in tumor cells, including tumor stromal cells.

Provided herein is a method of expressing a transgene in tumor cells of a subject. In some embodiments, the method includes administering to the subject a synthetic adenovirus that includes the transgene, a native or modified capsid that detargets the synthetic adenovirus from the liver, and an Ad34 fiber protein or a chimeric fiber protein comprising an Ad5 shaft domain and an Ad34 knob domain.

In some embodiments, the transgene is a diagnostic transgene. In some examples, the diagnostic transgene encodes a fluorescent protein, such as, but not limited to a green fluorescent protein (GFP), a yellow fluorescent protein (YFP), a cyan fluorescent protein (CFP), a red fluorescent protein (RFP), a blue fluorescent protein (BFP), or an orange fluorescent protein (for example, mOrange). In other examples, the diagnostic transgene encodes an enzyme, such as a luciferase. In yet other examples, the diagnostic transgene comprises a PET reporter gene.

In other embodiments, the transgene is a therapeutic transgene. In some examples, the therapeutic transgene encodes an anti-cancer agent. In specific examples, the anti-cancer agent is a pro-inflammatory molecule or cytokine, such as granulocyte macrophage colony stimulating factor (GM-CSF), CD40 ligand (CD40L), Fms-related tyrosine kinase 3 (FLT3) ligand, interleukin (IL)-1b, IL-2, IL-4, IL-6, IL-12, tumor necrosis factor (TNF)-α, an interferon, a chemokine, B7-1, intercellular adhesion molecule (ICAM)-1, lymphocyte function-associated antigen (LFA)-3, transforming growth factor (TGF)-β, platelet derived growth factor (PDGF) or epidermal growth factor (EGF). In other specific examples, the anti-cancer agent is an anti-angiogenic factor, such as

an inhibitor of vascular endothelial growth factor (VEGF). In other specific examples, the anti-cancer agent is an inhibitor (such as a siRNA or shRNA inhibitor) of KRas. In other specific examples, the anti-cancer agent is an inhibitor of cytotoxic T lymphocyte-associated molecule (CTLA)-4, programmed cell death protein 1 (PD-1), programmed death-ligand 1 (PD-L1),
5 carcinoembryonic antigen (CEA) or mucin 1 (MUC1). In some examples, the therapeutic transgene encodes an agent that disrupts or kills tumor stromal cells. In specific examples, the agent is Rexin-G, herpes simplex virus (HSV) thymidine kinase (TK), p53, TNF-α, Fas/FasL, or diphtheria toxin A.

Also provided herein is a method of diagnosing a subject as having a tumor. In some
10 embodiments, the method includes administering to the subject a synthetic adenovirus that includes a diagnostic transgene, a native or modified capsid that detargets the synthetic adenovirus from the liver, and an Ad34 fiber protein or a chimeric fiber protein comprising an Ad5 shaft domain and an Ad34 knob domain.

In some embodiments, the diagnostic transgene is a PET reporter gene. In some examples,
15 the PET reporter gene is a viral or human thymidine kinase (or mutant form thereof), a mutant of deoxycytidine kinase, a dopamine 2 receptor mutant, a human estrogen receptor α ligand binding domain (hERL), a human somatostain receptor subtype 2 (hSSTr2), a recombinant human CEA, an engineered antibody fragment, a humanized membrane anchored anti-polyethylene glycol (PEG), a sodium iodide symporter (NIS), or a human norepinephrine transporter (hNET).

20 In other embodiments, the diagnostic transgene encodes a fluorescent protein. In some examples, the fluorescent protein comprises a GFP, YFP, CFP, RFP, BFP, or orange fluorescent protein.

In other embodiments, the diagnostic transgene encodes an enzyme. In one example, the enzyme is a luciferase.

25 Further provided herein is a method of treating a tumor in a subject. In some embodiments, the method includes administering to the subject a synthetic adenovirus that includes a therapeutic transgene, a native or modified capsid that detargets the synthetic adenovirus from the liver, and an Ad34 fiber protein or a chimeric fiber protein comprising an Ad5 shaft domain and an Ad34 knob domain.

30 In some embodiments, the therapeutic transgene encodes an anti-cancer agent. In some examples, the anti-cancer agent is a pro-inflammatory molecule or cytokine, such as GM-CSF, CD40L, FLT3, IL-1b, IL-2, IL-4, IL-6, IL-12, TNF-α, an interferon, a chemokine, B7-1, ICAM-1, LFA-3, TGF-β, PDGF or EGF. In other examples, the anti-cancer agent is an anti-angiogenic

factor, such as an inhibitor of VEGF. In other examples, the anti-cancer agent is an inhibitor (such as a siRNA or shRNA inhibitor) of KRas. In other examples, the anti-cancer agent is an inhibitor of CTLA-4, PD-1, CEA or MUC1. In other embodiments, the therapeutic transgene encodes an agent that disrupts or kills tumor stromal cells. In some examples, the agent is Rexin-G, HSV-TK, p53, TNF-α, Fas/FasL, or diphtheria toxin A.

In some embodiments of the methods disclosed herein, the synthetic adenovirus includes a modified capsid that detargets the virus from the liver. In some examples, the synthetic adenovirus comprises a modified hexon protein, such as an E451Q mutation (set forth herein as SEQ ID NO: 4). In other embodiments, the synthetic adenovirus has a native (unmodified) capsid that detargets the synthetic adenovirus from the liver (for example, a capsid from an adenovirus serotype that naturally does not infect the liver).

In some embodiments of the methods disclosed herein, the synthetic adenovirus further includes one or more binding sites, such as two or three binding sites, for a liver-specific microRNA. In some examples, the liver-specific microRNA is miR-122. In some examples, the one or more binding sites are in the 3'UTR of the transgene.

In some embodiments of the methods disclosed herein, the synthetic adenovirus further includes one or more binding sites, such as two or three binding sites, for a spleen-specific microRNA. In some examples, the spleen-specific microRNA is miR142-3p. In some examples, the one or more binding sites are in the 3'UTR of the transgene.

In some embodiments of the methods disclosed herein, the transgene is regulated by a tissue-specific promoter, such as a promoter active in the pancreas or the cells of the central nervous system. In other embodiments, the transgene is regulated by a tumor-specific promoter.

In some embodiments of the methods disclosed herein, the synthetic adenovirus is generated from an Ad5 vector genome. In some examples, the synthetic adenovirus comprises Ad5 capsid proteins and a chimeric fiber protein comprising an Ad5 shaft domain and an Ad34 knob domain.

In some embodiments of the methods disclosed herein, the tumor is a pancreatic tumor. In other embodiments, the tumor is a glioblastoma. In other embodiments, the tumor is a breast cancer, prostate cancer, gastrointestinal cancer, bone cancer or melanoma tumor.

In some embodiments of the methods disclosed herein, the tumor is characterized by a loss of p53 tumor suppressor activity. In some examples, the tumor exhibits mutations in p53. In some examples, the tumor exhibits loss of a wild-type p53 allele.

In some embodiments of the methods disclosed herein, the tumor is characterized by mutations in a Ras gene, such as KRas, HRas or NRas. In some embodiments of the methods disclosed herein, the tumor is characterized by alterations or mutations in neurofibromatosis type 1 (NF1), epidermal growth factor receptor (EGFR), BRCA1, BRCA2 or HER2.

5 In some embodiments of the methods disclosed herein, the genome of the synthetic adenovirus comprises a nucleotide sequence at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% identical to SEQ ID NO: 2. In some examples, the genome of the synthetic adenovirus comprises or consists of the nucleotide sequence of SEQ ID NO: 2.

10 In other embodiments of the methods disclosed herein, the genome of the synthetic adenovirus comprises a nucleotide sequence at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% identical to SEQ ID NO: 5. In some examples, the genome of the synthetic adenovirus comprises or consists of the nucleotide sequence of SEQ ID NO: 5.

15 Further provided herein are synthetic adenovirus genomes having at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98% or at least 99% identical to SEQ ID NO: 2 or SEQ ID NO: 5. In some examples, the genome of the synthetic adenovirus comprises or consists of the nucleotide sequence of SEQ ID NO: 2 or SEQ ID NO: 5.

20 IV. Synthetic Adenoviruses

The Adsembly, AdSLICr and RapAD technologies enable the modular design and production of adenoviruses with unique capabilities (see PCT Publication Nos. WO2012/024351 and WO2013/138505, which are herein incorporated by reference in their entirety). The ability to design custom viruses with novel functions and properties opens up the potential to expand the 25 utility of adenovirus as a vehicle to deliver therapeutic proteins by persuading the host to produce proteins *in situ*. This provides the unique capability to use human proteins that are difficult to manufacture for therapeutic purposes, and enable flexible delivery of almost any protein to diseased tissues.

The specific modifications disclosed herein are described with reference to the adenovirus 5 30 (Ad5) genome sequence, but may be used with any adenovirus serotype. Adenovirus is a natural multi-gene expression vehicle. The E1, E3, and E4 regions are either not necessary for replication in culture or can be complemented with available cell lines. Each of these regions has independent

promoter elements that can be replaced with cellular promoters if necessary to drive the expression of multiple gene products via alternative splicing.

As disclosed herein, to create Ad5 expression vectors for *in vivo* use and transgene delivery, the E1A/E1B genes were deleted and replaced with at least one transgene. In some embodiments, the transgene is an EF1α driven luciferase-GFP fusion.

The synthetic adenoviruses disclosed herein may further include modifications that detarget the virus from the liver. Ad5 hexon can bind to Factor X in the blood, which can lead to its absorption by Kuppfer cells in the liver that prevent systemic dissemination and limiting inflammation. To overcome this, synthetic adenoviruses were engineered to include additional genomic modifications in the E1 and core modules that prevent adenovirus uptake and transgene expression in the liver, as described further below.

A. Ad34 fiber and chimeric fiber proteins for retargeting

While the fiber proteins of Ad5 and many other serotypes have been shown to bind to the coxsackie adenovirus receptor (CAR) for cellular attachment, other serotypes have been shown to use CD46 (Gaggar *et al.*, *Nat Med* 9:1408-1412, 2003), desmoglein 2 (Wang *et al.*, *Nat Med* 17:96-104, 2011), sialic acid (Nilsson *et al.*, *Nat Med* 17:105-109, 2011), or others (Arnberg, *Trends Pharmacol Sci* 33:442-448, 2012). The receptor usage of many serotypes has not been thoroughly examined and CD46 is not thought to be expressed in mature mice. Since the globular knob at the C-terminus of the fiber protein is typically responsible for receptor binding, a chimera was created by replacing the Ad5 fiber knob with that from Ad34 (see Example 1 below). The synthetic virus included an E1 module containing an E1A/E1B deletion and a luciferase-GFP fusion driven by an EF1α promoter. The synthetic adenovirus also included a liver detargeting modification in the hexon protein (E451Q) and binding sites in the 3'UTR of the transgene for a microRNA that is specifically expressed in the liver (miR-122) to prevent off-target expression of the transgene.

The data disclosed herein demonstrate the ability to combine modified parts from other serotypes in order to improve Ad5-based vectors. In this case allowing for rapid assembly of viruses that are optimized for entry into tumor cells.

B. Liver detargeting modifications

Natural Ad5 vectors will only infect the lungs (via inhalation) or liver (via intravenous administration). Ad5 hexon binds to Factor X in the blood, which leads its absorption by Kuppfer cells in the liver, preventing systemic dissemination and inducing virus-limiting inflammation. To

overcome this and enable intravenous delivery of viruses that could travel to sites of tumors systemically, synthetic adenoviruses were engineered to include additional genomic modifications in the E1 and core modules that prevent uptake and expression in the liver.

To prevent virus uptake and sequestration in the liver through Ad5 hexon binding to Factor X, viruses were engineered with an additional mutation in hexon (E451Q) that prevents liver uptake. For example, AdSyn-CO171 does not accumulate in the liver and instead is able to target other organs, such as the spleen and lymph nodes. Thus, in some embodiments herein, the synthetic adenovirus comprises a modified hexon protein with an E451Q substitution.

To prevent off-target transgene expression in the liver, viruses were engineered to include binding sites in the 3' untranslated region (UTR) of the transgene for microRNAs that are specifically expressed in the liver. In particular embodiments, miR122 was selected as the liver-specific microRNA as its expression and binding sites are conserved in both human and mouse liver cells. In some examples, two micro-RNA binding sites for liver-specific miR122 were inserted in the 3'UTR of the transgene to prevent any residual transgene expression in the liver.

It is disclosed herein that a synthetic adenovirus with miR-122 binding sites and hexon mutation does not accumulate in the liver and instead is able to target tumors. In some embodiments, the one or more binding sites for the liver-specific microRNA are located in the 3'-UTR of the transgene. In some examples, the liver-specific microRNA is miR-122, miR-30 or miR-192.

Other mutations to the adenovirus hexon gene are contemplated herein to prevent adenovirus accumulation in the liver. For example, a synthetic adenovirus could be detargeted from the liver by replacing the nine hypervariable regions of hexon with those from different serotypes.

In some examples, the recombinant adenovirus comprises a hexon protein comprising or consisting of the amino acid sequence of SEQ ID NO: 3 or SEQ ID NO: 4.

C. Capsid swaps for evading neutralizing antibodies

The majority of the human population already has antibodies that recognize Ad5, the serotype most frequently used in research and therapeutic applications. Moreover, once a particular adenovirus serotype is used in a patient, new antibodies that recognize the viral capsid will be generated, making repeated administration of the same vector problematic. Therefore, the present disclosure further contemplates exploiting natural adenovirus modularity to create chimeric viruses capable of evading existing neutralizing antibodies. For example, the recombinant adenoviruses

disclosed herein may further have complete 'capsid' module swaps (almost 60% of genome), which render them 'invisible' to pre-existing antibodies and enables repeated inoculations.

In some embodiments, the E1, E3 and E4 regions of the genome are derived from a first adenovirus serotype and the E2B, L1, L2, L3, E2A and L4 regions of the genome are derived from a second adenovirus serotype, such as Ad34. In some examples, the E1 region of the first adenovirus serotype is modified to encode a pIX protein from the second adenovirus serotype; and/or the E3 region of the first adenovirus serotype is modified to encode Uexon and fiber proteins from the second adenovirus serotype. In particular examples, the first adenovirus serotype is Ad5 and the second adenovirus serotype is Ad34.

D. Expression of transgenes for diagnostic and therapeutic applications

It is disclosed herein that recombinant adenoviruses comprising a chimeric fiber protein having an Ad34 knob domain and liver detargeting mutations are capable of targeting tumors. It is further disclosed that the recombinant adenoviruses are capable of expressing transgenes in tumor tissue, such as in tumor stromal cells. In one example, the transgene is a reporter, such as a luciferase-GFP reporter that enables detection of virus-transduced cells. In another example, the transgene is a therapeutic transgene, such as an anti-cancer molecule. The present disclosure provides synthetic adenoviruses that include diagnostic or therapeutic transgenes for the diagnosis and treatment of tumors.

Provided herein is a method of diagnosing a subject as having a tumor by administering to the subject a synthetic adenovirus that includes a diagnostic transgene, a native or modified capsid that detargets the synthetic adenovirus from the liver, and an Ad34 fiber protein or a chimeric fiber protein comprising an Ad5 shaft domain and an Ad34 knob domain. The diagnostic transgene can be, for example, a PET reporter gene, a fluorescent protein or an enzyme.

Also provided herein is a method of treating a tumor in a subject by administering to the subject a synthetic adenovirus that includes a therapeutic transgene, a native or modified capsid that detargets the synthetic adenovirus from the liver, and an Ad34 fiber protein or a chimeric fiber protein comprising an Ad5 shaft domain and an Ad34 knob domain. The therapeutic transgene can encode, for example, an anti-cancer agent or an agent that disrupts or kills tumor stromal cells.

In some embodiments, the transgene is inserted into the E1 or E3 region. Appropriate transgene insertion sites are well known in the art (see, for example, PCT Publication No. WO2012/024351).

The transgene, such as a gene encoding a fluorescent protein, is operably linked to a promoter. In some embodiments, the promoter is a heterologous promoter. In some examples, the promoter is the EF1α promoter. The selection of promoter is within the capabilities of one of skill in the art. In some cases, the promoter is an inducible promoter or a tissue-specific promoter. An
5 exemplary tissue-specific promoter for expression in pancreatic tissue is Pdx1.

In some cases a single promoter is used to regulate expression of multiple genes, which can be achieved by use of an internal ribosomal entry site (IRES) or 2A peptide.

V. Pharmaceutical Compositions and Administration Thereof

10 Provided herein are compositions comprising a synthetic adenovirus (or one or more nucleic acids or vectors encoding the recombinant adenovirus). The compositions are, optionally, suitable for formulation and administration *in vitro* or *in vivo*. Optionally, the compositions comprise one or more of the recombinant adenovirus and a pharmaceutically acceptable carrier. Suitable carriers and their formulations are described in *Remington: The Science and Practice of Pharmacy, 22nd*
15 *Edition*, Loyd V. Allen *et al.*, editors, Pharmaceutical Press (2012). Pharmaceutically acceptable carriers include materials that are not biologically or otherwise undesirable, *i.e.*, the material is administered to a subject without causing undesirable biological effects or interacting in a deleterious manner with the other components of the pharmaceutical composition in which it is contained. If administered to a subject, the carrier is optionally selected to minimize degradation of
20 the active ingredient and to minimize adverse side effects in the subject.

The recombinant viruses (or one or more nucleic acids or vectors encoding the recombinant adenovirus) are administered in accord with known methods, such as intravenous administration, *e.g.*, as a bolus or by continuous infusion over a period of time, by intramuscular, intraperitoneal, intracerobrospinal, subcutaneous, intra-articular, intrasynovial, intrathecal, oral, topical,
25 intratumoral or inhalation routes. The administration may be local or systemic. The compositions can be administered via any of several routes of administration, including topically, orally, parenterally, intravenously, intra-articularly, intraperitoneally, intramuscularly, subcutaneously, intracavity, transdermally, intrahepatically, intracranially, nebulization/inhalation, or by installation via bronchoscopy. Thus, the compositions are administered in a number of ways depending on
30 whether local or systemic treatment is desired, and on the area to be treated.

In some embodiments, the compositions for administration will include a recombinant adenovirus (or recombinant genome) as described herein dissolved in a pharmaceutically acceptable carrier, preferably an aqueous carrier. A variety of aqueous carriers can be used, *e.g.*,

buffered saline and the like. These solutions are sterile and generally free of undesirable matter. These compositions may be sterilized by conventional, well known sterilization techniques. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions such as pH adjusting and buffering agents, toxicity adjusting agents and the like, for example, sodium acetate, sodium chloride, potassium chloride, calcium chloride, sodium lactate and the like. The concentration of active agent in these formulations can vary widely, and will be selected primarily based on fluid volumes, viscosities, body weight and the like in accordance with the particular mode of administration selected and the subject's needs.

Pharmaceutical formulations, particularly, of the recombinant viruses can be prepared by mixing the recombinant adenovirus (or one or more nucleic acids encoding the recombinant adenovirus) having the desired degree of purity with optional pharmaceutically acceptable carriers, excipients or stabilizers. Such formulations can be lyophilized formulations or aqueous solutions.

Acceptable carriers, excipients, or stabilizers are nontoxic to recipients at the dosages and concentrations used. Acceptable carriers, excipients or stabilizers can be acetate, phosphate, citrate, and other organic acids; antioxidants (*e.g.*, ascorbic acid) preservatives, low molecular weight polypeptides; proteins, such as serum albumin or gelatin, or hydrophilic polymers such as polyvinylpyllolidone; and amino acids, monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating agents; and ionic and non-ionic surfactants (*e.g.*, polysorbate); salt-forming counter-ions such as sodium; metal complexes (*e.g.* Zn-protein complexes); and/or non-ionic surfactants. The recombinant adenovirus (or one or more nucleic acids encoding the recombinant adenovirus) can be formulated at any appropriate concentration of infectious units.

Formulations suitable for oral administration can consist of (a) liquid solutions, such as an effective amount of the recombinant adenovirus suspended in diluents, such as water, saline or PEG 400; (b) capsules, sachets or tablets, each containing a predetermined amount of the active ingredient, as liquids, solids, granules or gelatin; (c) suspensions in an appropriate liquid; and (d) suitable emulsions. Tablet forms can include one or more of lactose, sucrose, mannitol, sorbitol, calcium phosphates, corn starch, potato starch, microcrystalline cellulose, gelatin, colloidal silicon dioxide, talc, magnesium stearate, stearic acid, and other excipients, colorants, fillers, binders, diluents, buffering agents, moistening agents, preservatives, flavoring agents, dyes, disintegrating agents, and pharmaceutically compatible carriers. Lozenge forms can comprise the active ingredient in a flavor, e.g., sucrose, as well as pastilles comprising the active ingredient in an inert

base, such as gelatin and glycerin or sucrose and acacia emulsions, gels, and the like containing, in addition to the active ingredient, carriers known in the art.

The recombinant adenovirus (or one or more nucleic acids encoding the recombinant adenovirus), alone or in combination with other suitable components, can be made into aerosol formulations (*i.e.*, they can be "nebulized") to be administered via inhalation. Aerosol formulations can be placed into pressurized acceptable propellants, such as dichlorodifluoromethane, propane, nitrogen, and the like.

Formulations suitable for parenteral administration, such as, for example, by intraarticular (in the joints), intravenous, intramuscular, intratumoral, intradermal, intraperitoneal, and subcutaneous routes, include aqueous and non-aqueous, isotonic sterile injection solutions, which can contain antioxidants, buffers, bacteriostats, and solutes that render the formulation isotonic with the blood of the intended recipient, and aqueous and non-aqueous sterile suspensions that can include suspending agents, solubilizers, thickening agents, stabilizers, and preservatives. In the provided methods, compositions can be administered, for example, by intravenous infusion, orally, topically, intraperitoneally, intravesically intratumorally, or intrathecally. Parenteral administration, intratumoral administration, and intravenous administration are the preferred methods of administration. The formulations of compounds can be presented in unit-dose or multi-dose sealed containers, such as ampules and vials.

Injection solutions and suspensions can be prepared from sterile powders, granules, and tablets of the kind previously described. The pharmaceutical preparation is preferably in unit dosage form. In such form the preparation is subdivided into unit doses containing appropriate quantities of the active component. Thus, the pharmaceutical compositions can be administered in a variety of unit dosage forms depending upon the method of administration. For example, unit dosage forms suitable for oral administration include, but are not limited to, powder, tablets, pills, capsules and lozenges.

In some embodiments, the compositions include at least two different recombinant adenoviruses, such as recombinant adenoviruses that encode different transgenes. In some examples, the composition includes two, three, four, five or six different recombinant adenoviruses.

In therapeutic applications, recombinant adenoviruses or compositions thereof are administered to a subject in an effective amount or dose. Single or multiple administrations of the compositions may be administered as needed. A “patient” or “subject” includes both humans and other animals, particularly mammals. Thus, the methods are applicable to both human therapy and veterinary applications.

An effective amount of a recombinant adenovirus is determined on an individual basis and is based, at least in part, on the particular recombinant adenovirus used; the individual's size, age, gender and general health. For example, for administration to a human, at least 10^3 plaque forming units (PFU) of a recombinant virus is used, such as at least 10^4, at least 10^5, at least 10^6, at least 10^7, at least 10^8, at least 10^9, at least 10^{10}, at least 10^{11}, or at least 10^{12} PFU, for example approximately 10^3 to 10^{12} PFU of a recombinant virus is used, depending on the type, size and number of proliferating cells or neoplasms present. The effective amount can be from about 1.0 pfu/kg body weight to about 10^{15} pfu/kg body weight (*e.g.*, from about 10^2 pfu/kg body weight to about 10^{13} pfu/kg body weight). A recombinant adenovirus is administered in a single dose or in multiple doses (*e.g.*, two, three, four, six, or more doses). Multiple doses can be administered concurrently or consecutively (*e.g.*, over a period of days or weeks).

In some embodiments, the provided methods include administering to the subject one or more therapeutic agents, such as one or more agents for the treatment of cancer, such as pancreatic cancer or glioblastoma.

Administration of the synthetic adenoviruses disclosed herein that harbor a therapeutic transgene can be accompanied by administration of other anti-cancer agents or therapeutic treatments (such as surgical resection of a tumor). Any suitable anti-cancer agent can be administered in combination with the recombinant viruses disclosed herein. Exemplary anti-cancer agents include, but are not limited to, chemotherapeutic agents, such as, for example, mitotic inhibitors, alkylating agents, anti-metabolites, intercalating antibiotics, growth factor inhibitors, cell cycle inhibitors, enzymes, topoisomerase inhibitors, anti-survival agents, biological response modifiers, anti-hormones (*e.g.* anti-androgens), CDK inhibitors and anti-angiogenesis agents. Other anti-cancer treatments include radiation therapy and other antibodies that specifically target cancer cells (*e.g.*, biologics).

Non-limiting examples of alkylating agents include nitrogen mustards (such as mechlorethamine, cyclophosphamide, melphalan, uracil mustard or chlorambucil), alkyl sulfonates (such as busulfan), nitrosoureas (such as carmustine, lomustine, semustine, streptozocin, or dacarbazine).

Non-limiting examples of antimetabolites include folic acid analogs (such as methotrexate), pyrimidine analogs (such as 5-FU or cytarabine), and purine analogs, such as mercaptopurine or thioguanine.

Non-limiting examples of natural products include vinca alkaloids (such as vinblastine, vincristine, or vindesine), epipodophyllotoxins (such as etoposide or teniposide), antibiotics (such

as dactinomycin, daunorubicin, doxorubicin, bleomycin, plicamycin, or mitomycin C), and enzymes (such as L-asparaginase).

Non-limiting examples of miscellaneous agents include platinum coordination complexes (such as cis-diamine-dichloroplatinum II also known as cisplatin), substituted ureas (such as
5 hydroxyurea), methyl hydrazine derivatives (such as procarbazine), and adrenocrotical suppressants (such as mitotane and aminoglutethimide).

Non-limiting examples of hormones and antagonists include adrenocorticosteroids (such as prednisone), progestins (such as hydroxyprogesterone caproate, medroxyprogesterone acetate, and magestrol acetate), estrogens (such as diethylstilbestrol and ethinyl estradiol), antiestrogens (such
10 as tamoxifen), and androgens (such as testerone proprionate and fluoxymesterone). Examples of the most commonly used chemotherapy drugs include Adriamycin, Alkeran, Ara-C, BiCNU, Busulfan, CCNU, Carboplatinum, Cisplatinum, Cytoxan, Daunorubicin, DTIC, 5-FU, Fludarabine, Hydrea, Idarubicin, Ifosfamide, Methotrexate, Mithramycin, Mitomycin, Mitoxantrone, Nitrogen Mustard, Taxol (or other taxanes, such as docetaxel), Velban, Vincristine, VP-16, while some more
15 newer drugs include Gemcitabine (Gemzar), Herceptin, Irinotecan (Camptosar, CPT-11), Leustatin, Navelbine, Rituxan STI-571, Taxotere, Topotecan (Hycamtin), Xeloda (Capecitabine), Zevelin and calcitriol.

Non-limiting examples of immunomodulators that can be used include AS-101 (Wyeth-Ayerst Labs.), bropirimine (Upjohn), gamma interferon (Genentech), GM-CSF (granulocyte
20 macrophage colony stimulating factor; Genetics Institute), IL-2 (Cetus or Hoffman-LaRoche), human immune globulin (Cutter Biological), IMREG (from Imreg of New Orleans, La.), SK&F 106528, and TNF (tumor necrosis factor; Genentech).

Another common treatment for some types of cancer is surgical treatment, for example surgical resection of the cancer or a portion of it. Another example of a treatment is radiotherapy,
25 for example administration of radioactive material or energy (such as external beam therapy) to the tumor site to help eradicate the tumor or shrink it prior to surgical resection.

CDK (Cyclin-dependent kinase) inhibitors are agents that inhibit the function of CDKs. Non-limiting examples of CDK inhibitors for use in the provided methods include AG-024322, AT7519, AZD5438, flavopiridol, indisulam, P1446A-05, PD-0332991, and P276-00 (see *e.g.*,
30 Lapenna *et al.*, *Nature Reviews*, 8:547-566 , 2009). Other CDK inhibitors include LY2835219, Palbociclib, LEE011 (Novartis), pan-CDK inhibitor AT7519, seliciclib, CYC065, butyrolactone I, hymenialdisine, SU9516, CINK4, PD0183812 or fascaplysin.

In some examples, the CDK inhibitor is a broad-range inhibitor (such as flavopiridol, olomoucine, roscovitine, kenpaullone, SNS-032, AT7519, AG-024322, (S)-Roscovitine or R547). In other examples, the CDK inhibitor is a specific inhibitor (such as fascaplysin, ryuvidine, purvalanol A, NU2058, BML-259, SU 9516, PD0332991 or P-276–00).

5 The choice of agent and dosage can be determined readily by one of skill in the art based on the given disease being treated. Combinations of agents or compositions can be administered either concomitantly (*e.g.*, as a mixture), separately but simultaneously (*e.g.*, via separate intravenous lines) or sequentially (*e.g.*, one agent is administered first followed by administration of the second agent). Thus, the term combination is used to refer to concomitant, simultaneous or sequential

10 administration of two or more agents or compositions.

The following examples are provided to illustrate certain particular features and/or embodiments. These examples should not be construed to limit the disclosure to the particular features or embodiments described.

15

EXAMPLES

Example 1: A synthetic adenovirus expressing an Ad5/Ad34 chimeric fiber protein and liver detargeting modifications

While the fiber proteins of Ad5 and many other serotypes have been shown to bind to CAR

20 for cellular attachment, other serotypes have been shown to use CD46 (Gaggar *et al.*, *Nat Med* 9:1408-1412, 2003), desmoglein 2 (Wang *et al.*, *Nat Med* 17:96-104, 2011), sialic acid (Nilsson *et al.*, *Nat Med* 17:105-109, 2011), or others (Arnberg, *Trends Pharmacol Sci* 33:442-448, 2012). The receptor usage of many serotypes has not been thoroughly examined and CD46 is not thought to be expressed in mature mice.

25 Adsembly/AdSLIC (see PCT Publication No. WO 2012/024351, incorporated herein by reference) was used to generate a synthetic adenovirus having a chimeric fiber protein. Since the globular knob at the C-terminus of the fiber protein is typically responsible for receptor binding, a virus with a chimeric fiber protein was created by replacing the Ad5 fiber knob with fiber knob from Ad34 (AdSyn-CO176). The control virus (AdSyn-CO171) contains an Ad5 fiber protein (*i.e.*

30 both the shaft and knob domains are from Ad5). Both viruses were created with the same E1 module containing an E1A/E1B deletion and a luciferase-GFP fusion driven by an EF1α promoter (Table 1). The recombinant viruses also include liver detargeting modifications. Natural Ad5 vectors will only infect the lungs (via inhalation) or liver (via intravenous administration). Ad5

hexon binds to Factor X in the blood, which leads to its absorption by Kuppfer cells in the liver, preventing systemic dissemination and inducing limited inflammation. To overcome this and allow for systemic administration to alternative cell types, the synthetic adenoviruses were engineered to include additional genomic modifications in the E1 and core regions that prevent uptake and
5 expression in the liver. Specifically, both viruses include binding sites in the 3'UTR of the transgene for a microRNA that is specifically expressed in the liver (miR-122) and an E451Q mutation in hexon.

Table 1. Adenoviruses with Chimeric Fiber Proteins and Liver Detargeting Modifications

Virus Name	SEQ ID NO:	E1	L3	L5	E4
AdSyn-CO171	1	ΔE1 +EF1α-luciferase-miR122	hexon E451Q	wt	wt
AdSyn-CO176	2	ΔE1 +EF1α-luciferase-miR122	hexon E451Q	Ad34 knob Ad5 shaft fiber chimera	wt

10

Example 2: A synthetic adenovirus expressing the Ad34 knob domain exhibits tropism to tumor stroma

This example describes the finding that AdSyn-CO176, expressing a chimeric fiber protein
15 with an Ad34 knob domain, specifically traffics to tumor stroma.

Pancreatic tumor models

Shown in FIG. 1A is a schematic overview of the Cre-LoxP $Kras^{G12D}$/p53 pancreatic tumor model. Mice designated as “Kras; p53/p53” encode the $Kras^{G12D}$ oncogene downstream of the
20 sequence encoding LoxP-stop codon-LoxP. The stop codon blocks the expression of mutant Kras ($Kras^{G12D}$) in the absence of Cre recombinase. However, in the presence of Cre recombinase, the stop codon is removed and allows for expression of the $Kras^{G12D}$ oncogene. In these same mice, both alleles of the p53 gene are flanked by LoxP sites (LoxP-p53-LoxP). Mice designated “p53/p53; Cre” also have both alleles of the p53 gene flanked by LoxP (LoxP-p53-LoxP), and they
25 express the Cre recombinase transgene driven by the pancreatic and duodenal homeobox 1 (Pdx1)

promoter. Pdx1 is a gene that is expressed specifically in the pancreatic cells, and thus both copies of p53 are deleted in the pancreatic cells. Breeding between the strains gives rise to offspring in which the Pdx1 promoter-driven Cre mediates the deletion of both alleles of the tumor suppressor p53 and activation of the mutant $Kras^{G12D}$ in pancreatic cells. Homozygous mice designated "Kras;
5 p53/p53; Cre" develop pancreatic tumors in 5-7 weeks. AdSyn-CO176 was injected intravenously into Kras; p53/p53 and Kras; p53/p53; Cre mice. Seventy-two hours after the injection of virus, tissues were collected, incubated for 5 minutes with luciferin, and then scanned for 5 minutes using the IVIS™ imaging system. As shown in FIG. 1B, the Kras; p53/p53 mouse had a normal pancreas, and the luciferase signal was mainly from the spleen. In contrast, the Kras; p53/p53; Cre
10 mouse had pancreatic tumors, and the signal was mainly from the pancreatic tumor.

Another study was performed in a Cre-mediated genetic manipulation heterozygous model (FIG. 2A). Mice designated "p53/+; Cre" have one wild type p53 allele and one p53 allele flanked by LoxP sites (LoxP-p53-LoxP). Breeding between the Kras; p53/p53 and p53/+; Cre strains gives rise to offspring in which the Pdx1 promoter-driven Cre recombinase mediates the deletion of a
15 single allele of the tumor suppressor p53 and activation of the mutant $Kras^{G12D}$ in pancreatic cells. These heterozygous mice, designated "Kras; p53/+; Cre," develop tumors later in life (at 4-9 months of age) due to the fact that they have one wild type allele of p53. This wild type allele must be lost or mutated in order for pancreatic tumors to develop. AdSyn-CO176 was injected intravenously into 4-month old p53/+; Cre and Kras; p53/+; Cre mice. Seventy-two hours after the
20 injection of virus, tissues were collected, incubated with luciferin for 5 minutes, and then scanned for 1 minute using the IVIS™ imaging system. The p53/+; Cre mouse had a normal pancreas, and the signal was mainly from spleen. In contrast, the Kras; p53/+; Cre mouse at 4 months of age had a pancreatic tumor, and the signal was mainly from the tumor and liver.

The heterozygous Kras; p53/+; Cre mice develop pancreatic tumors in 4-9 months. To test
25 whether AdSyn-CO176 can infect pancreatic tumors at a very early stage of tumor development (before tumors are visible), AdSyn-CO176 was injected intravenously into Kras; p53/+; Cre mice at 2 months of age. Seventy-two hours after the injection of virus, tissues were collected, incubated with luciferin for 5 minutes, and scanned for 4 minutes using the IVIS™ imaging system. The pancreas of Kras; p53/+; Cre mouse at 2 months of age looked normal, but luciferase signal was
30 found in this tissue (FIG. 3A). H&E staining was performed to evaluate histology of the pancreas following infection with AdSyn-CO176. For comparison, FIG. 3B shows the typical histology of normal pancreas tissue and pancreatic tumor tissue. H&E staining of the pancreas from a Kras; p53/+; Cre mouse at 2 months of age showed that a small part of the pancreas was developing the

tumor (FIG. 3C, indicated by the polygon), while most of the pancreas tissue appeared normal. This result indicated that AdSyn-CO176 can infect pancreatic tumors at a very early stage.

In pancreatic tumors, only 10% of the cells are cancer cells; the remaining 90% are stromal cells. To determine which cell type was targeted by AdSyn-CO176, IHC and IF staining were performed. CK19 is a marker of tumor cells, while smooth muscle actin (SMA) is a marker of stromal cells. IHC staining of a pancreatic tumor infected with AdSyn-CO176 showed that GFP, which was expressed from AdSyn-CO176, overlapped with SMA staining, indicating that AdSyn-CO176 targeted stromal cells (FIG. 4A). IF staining of a pancreatic tumor infected by AdSyn-CO176 also demonstrated that GFP overlapped with SMA staining (FIG. 4B), confirming that AdSyn-CO176 infects stromal cells.

Glioblastoma model

Shown in FIG. 5A is a schematic of a Cre-mediated genetic manipulation glioblastoma model. Lentiviruses were injected directly into the brain of GFAP-Cre mice. Glial fibrillary acidic protein (GFAP) promoter-driven Cre recombinase cleaves out RFP from the lentivirus-encoded gene and induces the expression of $HRas^{V12}$ and GFP primarily in astrocytes. Expression of lentivirus-encoded U6-p53 shRNA knocks down the expression of p53 in the brain cells that take up the virus. The expression of $HRas^{V12}$ and the knock down of p53 induces tumorigenesis in the brain from 1 week after the injection. GFP signal is used to indicate the formation of glioblastoma. Saline, AdSyn-CO171, or AdSyn-CO176 were injected via intravenous (IV) administration into GFAP-Cre mice that had received the tumor-inducing lentiviruses 4 weeks earlier. Forty-eight hours after the injection of virus, mice were scanned for 1 minute using the IVIS™ imaging system 5 minutes after the intraperitoneal injection of luciferin (FIG. 5B). A luciferase signal was detected in mice injected with AdSyn-CO176, but not in mice injected with saline or AdSyn-CO171.

Wild type mice (normal brain) and GFAP-Cre mice that had received injection of tumor-inducing lentiviruses were injected with AdSyn-CO171 or AdSyn-CO176. Brain tissues were collected 72 hours after the injection of synthetic adenoviruses, incubated with luciferin for 5 minutes, and scanned for 5 minutes using the IVIS™ imaging system (FIG. 5C). Only the GFAP-Cre mouse injected with the tumor-inducing lentiviruses showed a luciferase signal from AdSyn-CO176. This demonstrates that AdSyn-CO176 traffics to the brain tissue only when a tumor is present. Brain tissues were also scanned for the GFP signal (FIG. 5D). The GFP signal is used to identify the glioblastoma. Both of the GFAP-Cre mice that received the tumor-inducing lentiviruses had the GFP signal in the brain, while no GFP was detected in wild type mice. The

GFP signal overlapped with the luciferase signal perfectly in the GFAP-Cre mouse that received the tumor-inducing lentiviruses and AdSyn-CO176.

The injection of the tumor-inducing lentiviruses causes temporary injury to the brain at the injection site. Although the synthetic adenoviruses (AdSyn-CO171 and AdSyn-CO176) were injected 4 weeks after the initial injection of lentiviruses, it was still unclear whether trafficking of AdSyn-CO176 to the glioblastoma was driven by the tumor or by the injection site injury. To answer this question, GFAP-Cre mice were injected with synthetic adenovirus 4 weeks after either no injection, sham-injection or injection with tumor-inducing lentivirus. GFAP-Cre mice were injected with either Hanks' balanced salt solution (HBSS) or tumor-inducing lentiviruses. After 4 weeks, AdSyn-CO171 was injected intravenously. As shown in FIG. 6A, there was no luciferase signal from AdSyn-CO171 in the brain in either group of mice. GFAP-Cre mice were injected with HBSS or tumor-inducing lentiviruses, or received no injection. After 4 weeks, mice were injected intravenously with AdSyn-CO176. As shown in FIG. 6B, the luciferase signal was detected only in the brain of the mouse that received the tumor-inducing lentiviruses, while the mouse that received HBSS or no injection produced no signal. These results demonstrate that the specificity of AdSyn-CO176 is driven by the tumor and not injection site injury.

Example 3: AdSyn-CO176 traffics to human glioblastoma tumors in a xenograft model

This example describes the finding that a synthetic adenovirus expressing a chimeric fiber protein with an Ad34 knob domain is capable of targeting human glioblastoma tumors.

The U87-tdTomato cell line is a human glioblastoma cell line that expresses the tdTomato fluorescent protein as a reporter to enable monitoring of tumor growth. When U87-tdTomato cells are injected intracranially into NSG mice to generate glioblastoma tumors, it typically takes 4-8 weeks for tumors to develop (FIG. 7A). This glioblastoma xenograft model was used to determine whether AdSyn-CO176 could traffic to a human glioblastoma tumor. AdSyn-CO171 (SEQ ID NO: 1) and AdSyn-CO176 (SEQ ID NO: 2) were injected intravenously into NSG mice by tail vein injection four weeks after the mice had received an intracranial injection of U87-tdTomato cells. Forty-eight hours after the injection of viruses, liver, spleen and brain tissue were collected, incubated with luciferin for five minutes and then scanned for 1 minute using the IVIS™ imaging system. As shown in FIG. 7B, only the AdSyn-CO176 injected mice showed a luciferase signal in the brain, and this signal completely overlapped with tdTomato expression. Thus, these results demonstrate that AdSyn-CO176 can traffic to human glioblastoma tumors, while AdSyn-CO171 cannot.

Example 4: A synthetic adenovirus targeting tumor stroma and expressing a therapeutic transgene reduces tumor size in an animal model

This example demonstrates that a synthetic adenovirus expressing a chimeric fiber protein with an Ad34 knob domain and a therapeutic payload is capable of tracking to tumor stroma and reducing tumor size.

Studies were performed to determine whether a therapeutic transgene could be incorporated into AdSyn-CO176 (SEQ ID NO: 2) to enable treatment of tumors. To conduct this study, the KPCL ($Kras^{G12D}$; p53 knockout; Pdx1-Cre; firefly Luciferase) mouse model was used (FIG. 8A). KPCL mice are similar to homozygous "Kras; p53/p53; Cre" mice, which specifically express $Kras^{G12D}$ in the pancreas and have the p53 gene knocked out only in the pancreas. However, KPCL mice also specifically express firefly luciferase in the pancreas. The development of tumors in KPCL mice is also similar to the "Kras; p53/p53; Cre" mice.

Two additional synthetic adenoviruses were generated – AdSyn-CO987 (SEQ ID NO: 5) and AdSyn-CO989 (SEQ ID NO: 6). AdSyn-CO987 is a synthetic adenovirus based upon AdSyn-CO176. The herpes simplex virus-1 thymidine kinase (TK)/ganciclovir (GCV) suicide gene was cloned into AdSyn-CO176 to replace the firefly luciferase/GFP gene. A Renilla luciferase was also inserted just after TK in the genome of AdSyn-CO176. Control virus AdSyn-CO989 was generated by cloning TK-P2A-renilla luciferase into AdSyn-CO171 to replace the original firefly luciferase/GFP gene.

KPCL mice were injected intravenously via the tail vein with 1 x 10^6 PFU of AdSyn-CO987 or AdSyn-CO989 at 5-6 weeks of age. Two days later, the mice were i.p. or i.v. injected with GCV. Three control groups were used: AdSyn-CO989+GCV; AdSyn-CO987 followed with saline injection (AdSyn-CO987+saline); and GCV injection only (i.p. or i.v.). FIG. 8B provides a table showing the average survival of KPCL mice for each treatment group. Treatment with AdSyn-CO987+GCV extended mouse survival compared with controls.

The firefly luciferase signal expressed by tumors was analyzed during treatment to monitor tumor growth. The results are shown in FIG. 8C. The treatment for mouse Z619R was AdSyn-CO987+saline, which served as the control. Mice Z601R and Z607R were treated with AdSyn-CO987+GCV (i.p.). While the strength of the firefly luciferase signal increased in the control mouse Z619R (indicating an increase in tumor size), the signal decreased in mice Z601R and Z607R (indicating a reduction in tumor size).

Histology of the pancreatic tumors was also evaluated by H&E staining (FIG. 9A). Mice Z655, 1806, Z619 and Z621 were all control mice. Mouse Z655 was treated with i.p. injected GCV only; mouse 1806 was treated with i.v. injected GCV only; mouse Z619 was treated with AdSyn-CO987+saline; and mouse Z621 received no treatment. Mouse Z656 received treatment with
5 AdSyn-CO987+GCV i.v. Compared to the controls, the tumor from Z656 had more regions of necrosis (FIG. 9B).

In view of the many possible embodiments to which the principles of the disclosure may be applied, it should be recognized that the illustrated embodiments are only examples of the invention
10 and should not be taken as limiting the scope of the invention. Rather, the scope of the invention is defined by the following claims. We therefore claim as our invention all that comes within the scope and spirit of these claims.

CLAIMS

1. A method of expressing a transgene in tumor cells of a subject, comprising administering to the subject a synthetic adenovirus comprising:

5 the transgene;

a native or modified capsid that detargets the synthetic adenovirus from the liver; and

an adenovirus type 34 (Ad34) fiber protein or a chimeric fiber protein comprising an adenovirus type 5 (Ad5) shaft domain and an Ad34 knob domain.

10 2. The method of claim 1, wherein the transgene is a diagnostic transgene.

3. The method of claim 2, wherein the diagnostic transgene encodes a fluorescent protein.

15 4. The method of claim 3, wherein the fluorescent protein comprises a green fluorescent protein (GFP), a yellow fluorescent protein (YFP), a cyan fluorescent protein (CFP), a red fluorescent protein (RFP), a blue fluorescent protein (BFP), or an orange fluorescent protein.

5. The method of claim 2, wherein the diagnostic transgene encodes an enzyme.

20

6. The method of claim 5, wherein the enzyme is a luciferase.

7. The method of claim 2, wherein the diagnostic transgene comprises a positron emission tomography (PET) reporter gene.

25

8. The method of claim 1, wherein the transgene is a therapeutic transgene.

9. The method of claim 8, wherein the therapeutic transgene encodes an anti-cancer agent.

30

10. The method of claim 8, wherein the therapeutic transgene encodes an agent that disrupts or kills tumor stromal cells.

11. A method of diagnosing a subject as having a tumor, comprising administering to the subject a synthetic adenovirus comprising:

a diagnostic transgene;

a native or modified capsid that detargets the synthetic adenovirus from the liver; and

5 an adenovirus type 34 (Ad34) fiber protein or a chimeric fiber protein comprising an adenovirus type 5 (Ad5) shaft domain and an Ad34 knob domain.

12. The method of claim 11, wherein the diagnostic transgene comprises a positron emission tomography (PET) reporter gene.

10

13. The method of claim 11, wherein the diagnostic transgene encodes a fluorescent protein.

14. The method of claim 13, wherein the fluorescent protein comprises a green 15 fluorescent protein (GFP), a yellow fluorescent protein (YFP), a cyan fluorescent protein (CFP), a red fluorescent protein (RFP), a blue fluorescent protein (BFP), or an orange fluorescent protein.

15. The method of claim 11, wherein the diagnostic transgene encodes an enzyme.

20 16. The method of claim 15, wherein the enzyme is a luciferase.

17. A method of treating a tumor in a subject, comprising administering to the subject a synthetic adenovirus comprising:

a therapeutic transgene;

25 a native or modified capsid that detargets the synthetic adenovirus from the liver; and

an adenovirus type 34 (Ad34) fiber protein or a chimeric fiber protein comprising an adenovirus type 5 (Ad5) shaft domain and an Ad34 knob domain.

18. The method of claim 17, wherein the therapeutic transgene encodes an anti-cancer 30 agent.

19. The method of claim 17, wherein the therapeutic transgene encodes an agent that disrupts or kills tumor stromal cells.

20. The method of any one of claims 1-19, wherein the synthetic adenovirus comprises a modified capsid that detargets the synthetic adenovirus from the liver.

5 21. The method of claim 20, wherein the synthetic adenovirus comprises a modified hexon protein.

22. The method of claim 21, wherein the modified hexon protein comprises an E451Q mutation.

10

23. The method of any one of claims 1-22, wherein the synthetic adenovirus further comprises one or more binding sites for a liver-specific microRNA.

24. The method of claim 23, wherein the liver-specific microRNA is miR-122.

15

25. The method of claim 23 or claim 24, wherein the one or more binding sites are in the 3'UTR of the transgene.

26. The method of any one of claims 1-25, wherein the synthetic adenovirus further

20 comprises one or more binding sites for a spleen-specific microRNA.

27. The method of claim 26, wherein the spleen-specific microRNA is miR142-3p.

28. The method of claim 26 or claim 27, wherein the one or more binding sites are in the

25 3'UTR of the transgene.

29. The method of any one of claims 1-28, wherein expression of the transgene is regulated by a tissue-specific promoter.

30 30. The method of any one of claims 1-29, wherein the synthetic adenovirus is generated from an Ad5 vector genome.

31. The method of claim 30, wherein the synthetic adenovirus comprises Ad5 capsid proteins and a chimeric fiber protein comprising an Ad5 shaft domain and an Ad34 knob domain.

32. The method of any one of claims 1-31, wherein the tumor is a pancreatic tumor.

5

33. The method of any one of claims 1-31, wherein the tumor is a glioblastoma.

34. The method of any one of claims 1-33, wherein the genome of the synthetic adenovirus comprises a nucleotide sequence at least 95% identical to SEQ ID NO: 2 or SEQ ID

10 NO: 5.

35. The method of claim 34, wherein the genome of the synthetic adenovirus comprises the nucleotide sequence of SEQ ID NO: 2 or SEQ ID NO: 5.

15 36. A synthetic adenovirus genome, comprising a nucleotide sequence at least 95% identical to SEQ ID NO: 2 or SEQ ID NO: 5.

37. The synthetic adenovirus genome of claim 36, comprising SEQ ID NO: 2 or SEQ ID NO: 5.

20

FIG. 1A

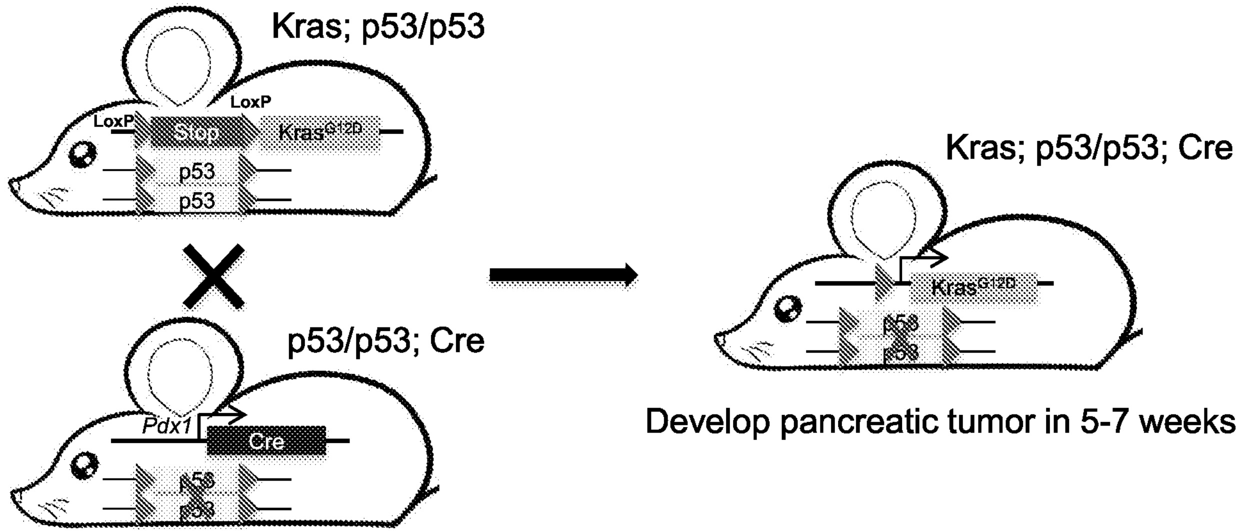


FIG. 1B

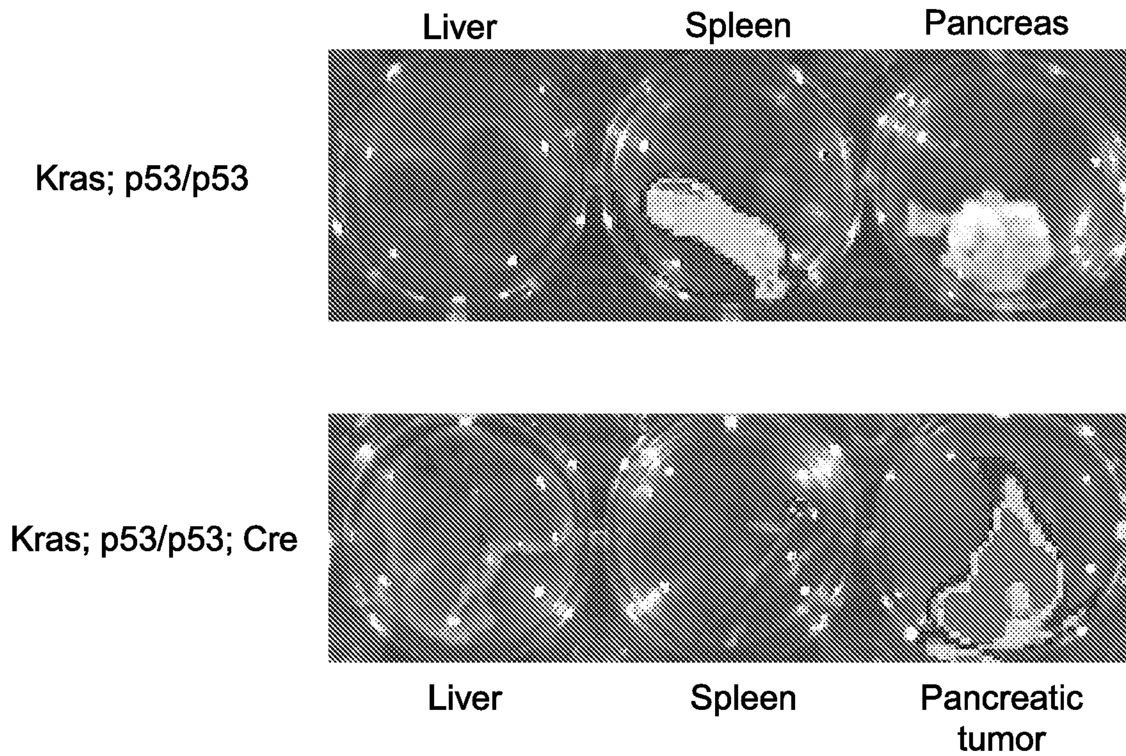


FIG. 2A

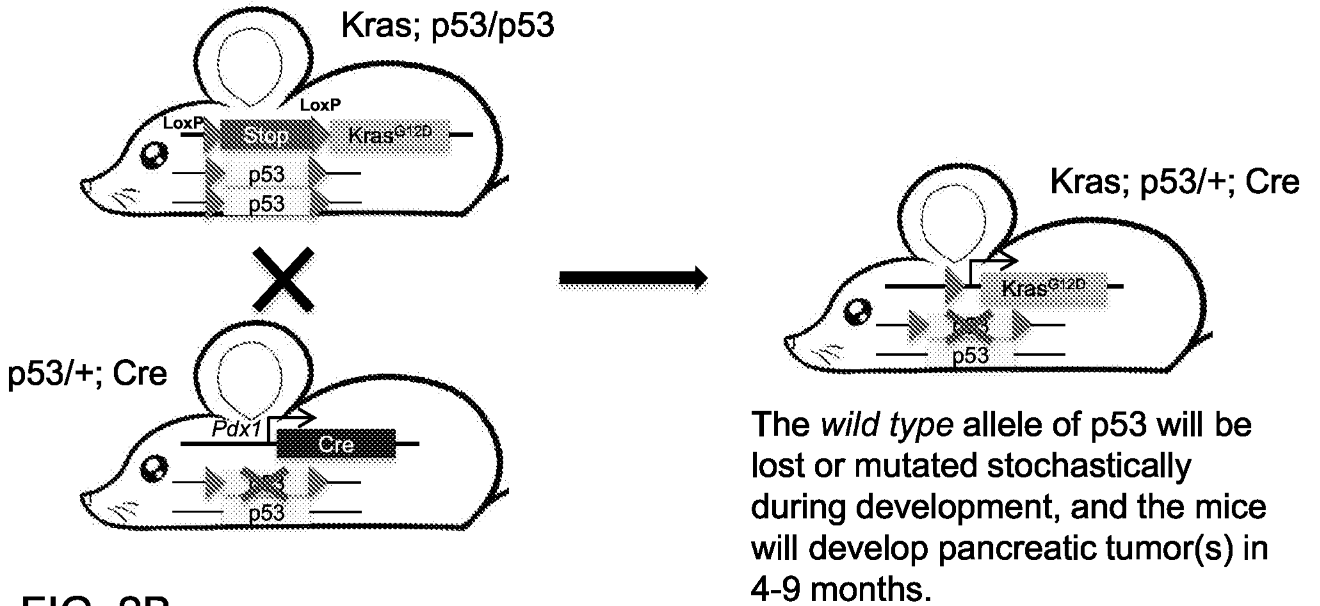


FIG. 2B

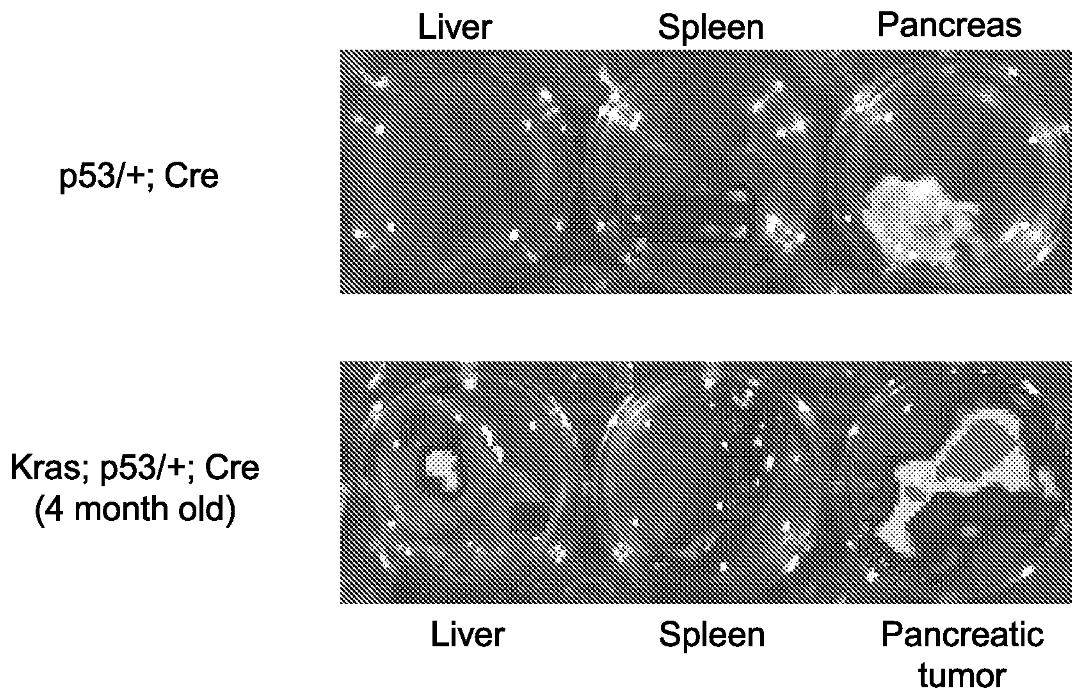


FIG. 3A

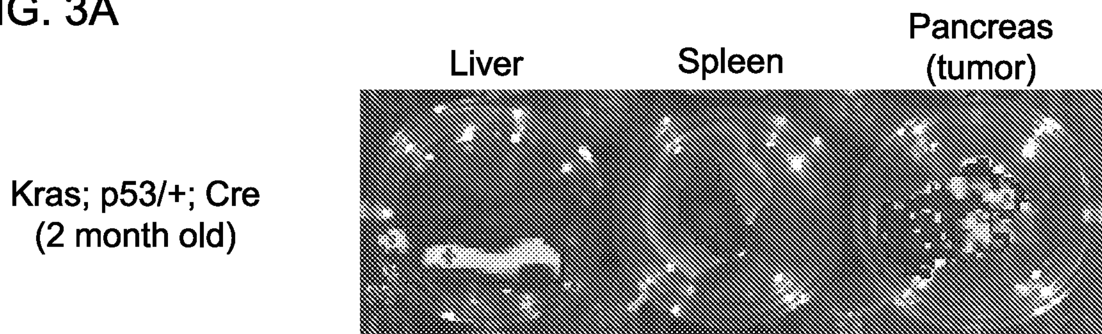


FIG. 3B

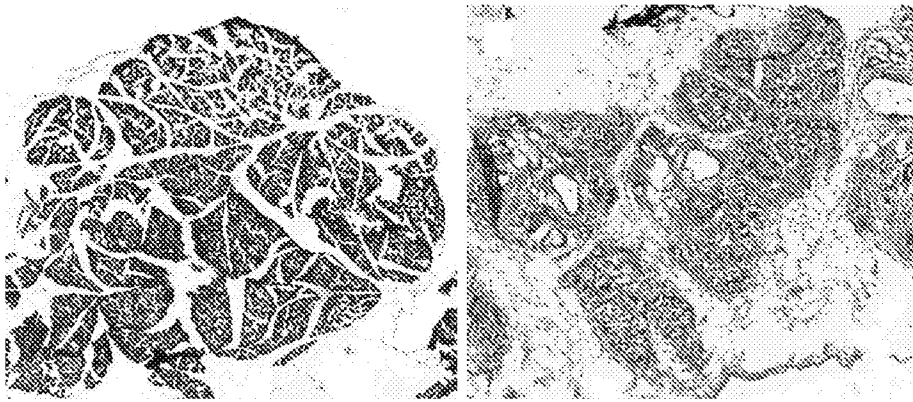


FIG. 3C

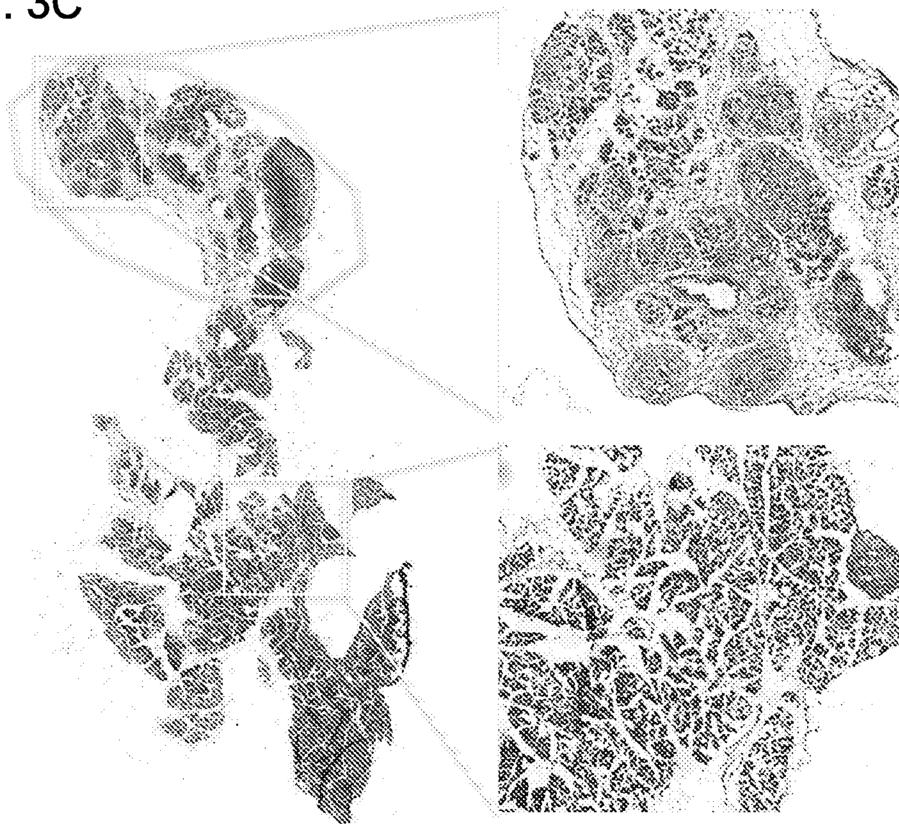


FIG. 4A

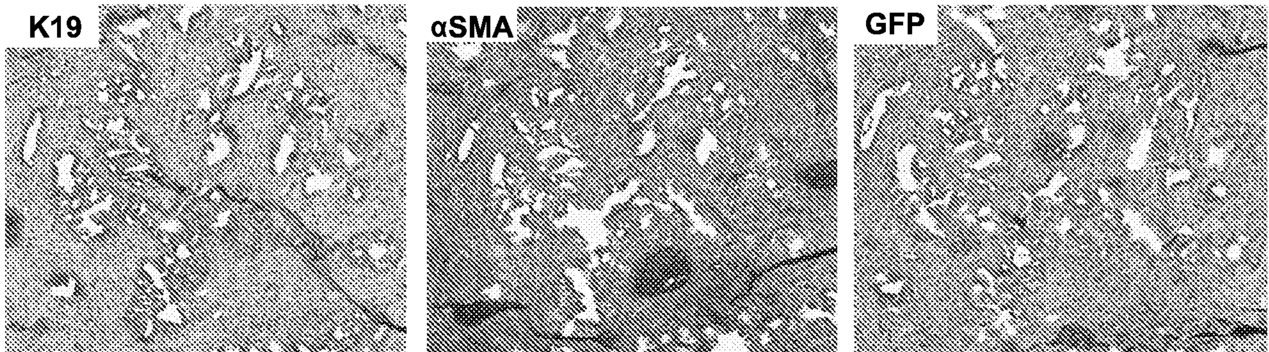


FIG. 4B

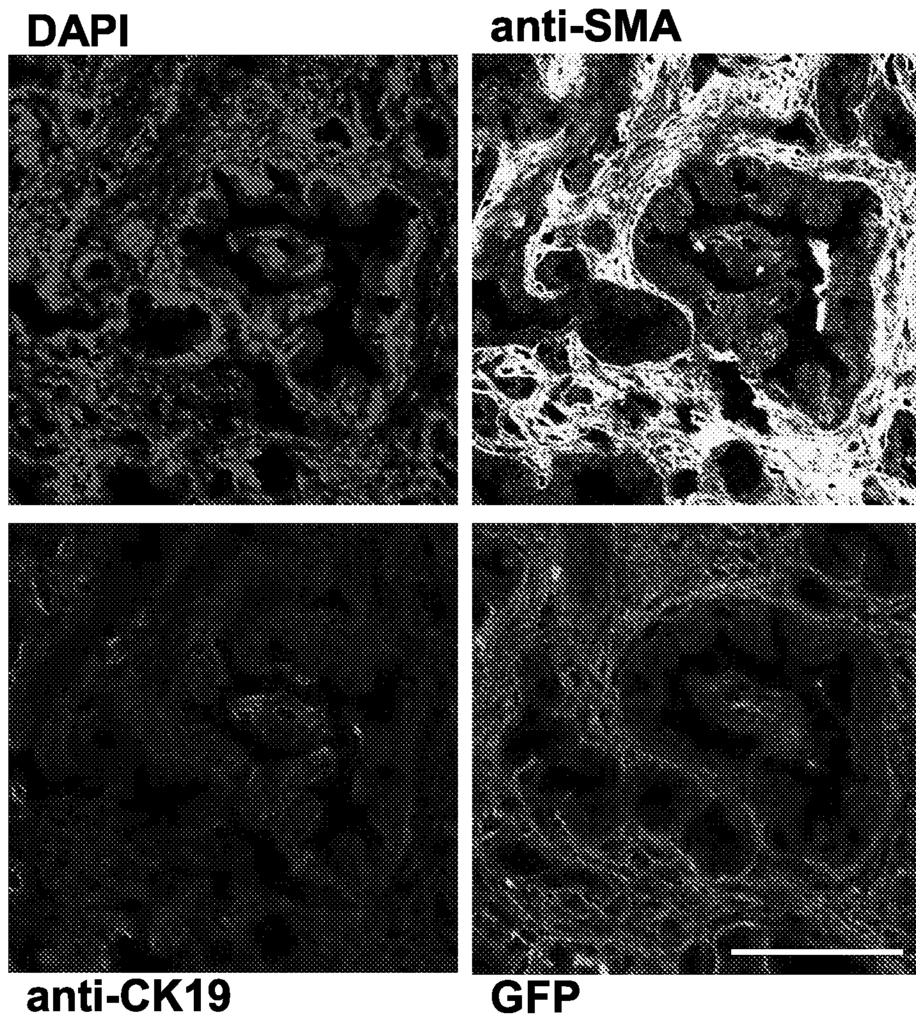
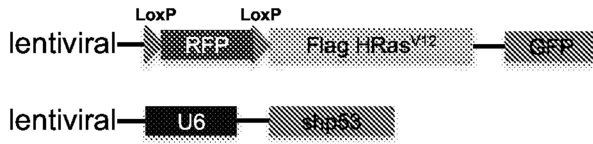


FIG. 5A



Develop glioblastoma from 1 week after the injection.

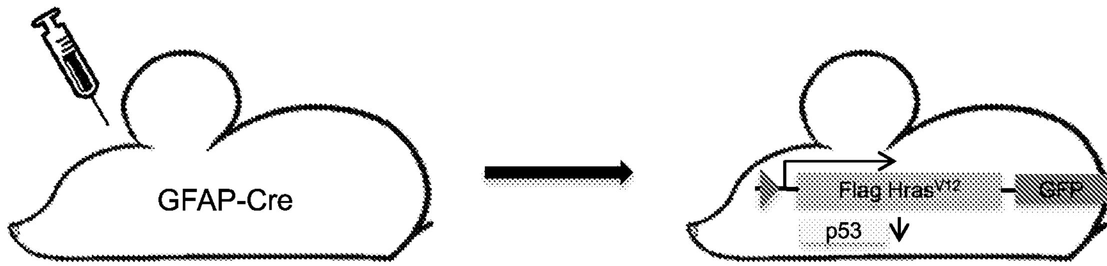


FIG. 5B

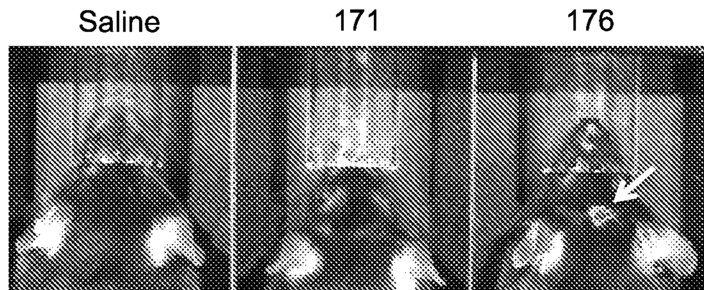


FIG. 5C

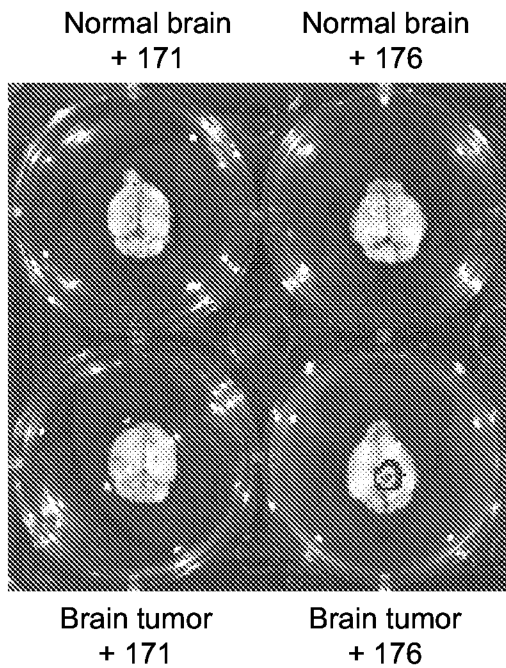


FIG. 5D

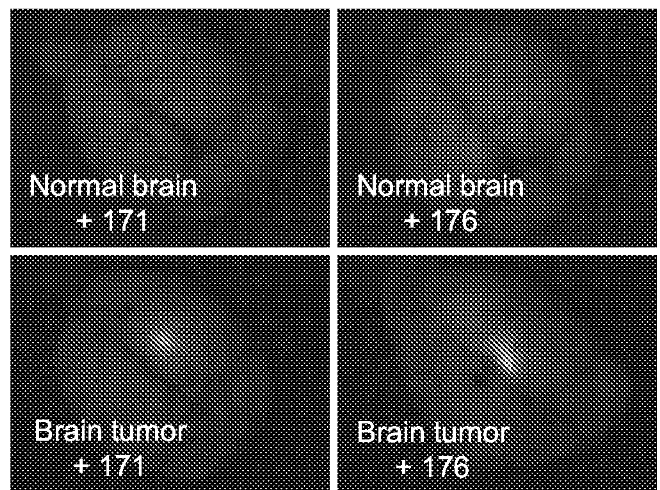


FIG. 6A

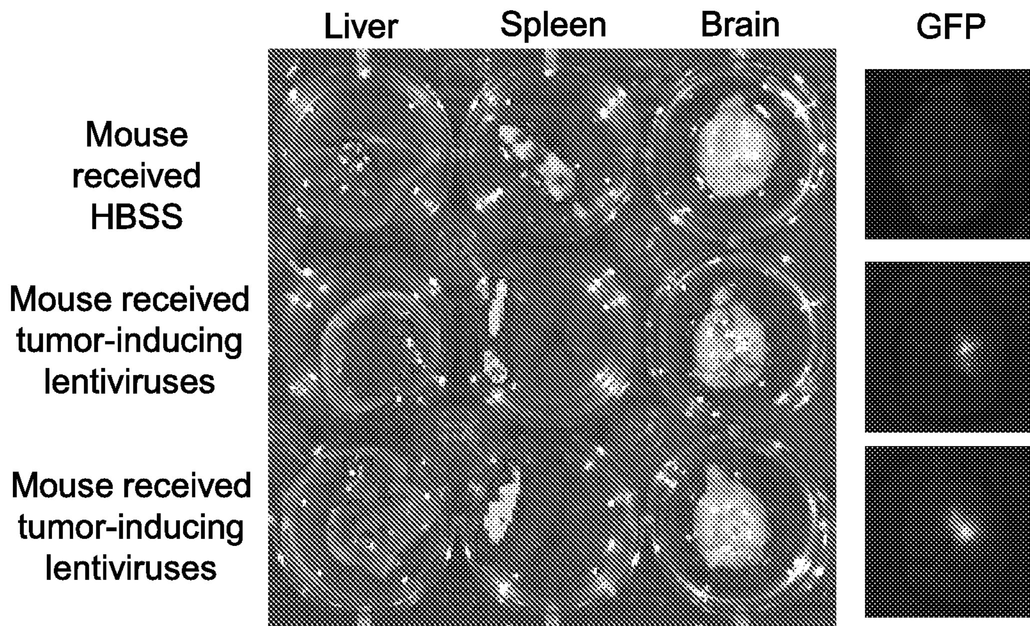


FIG. 6B

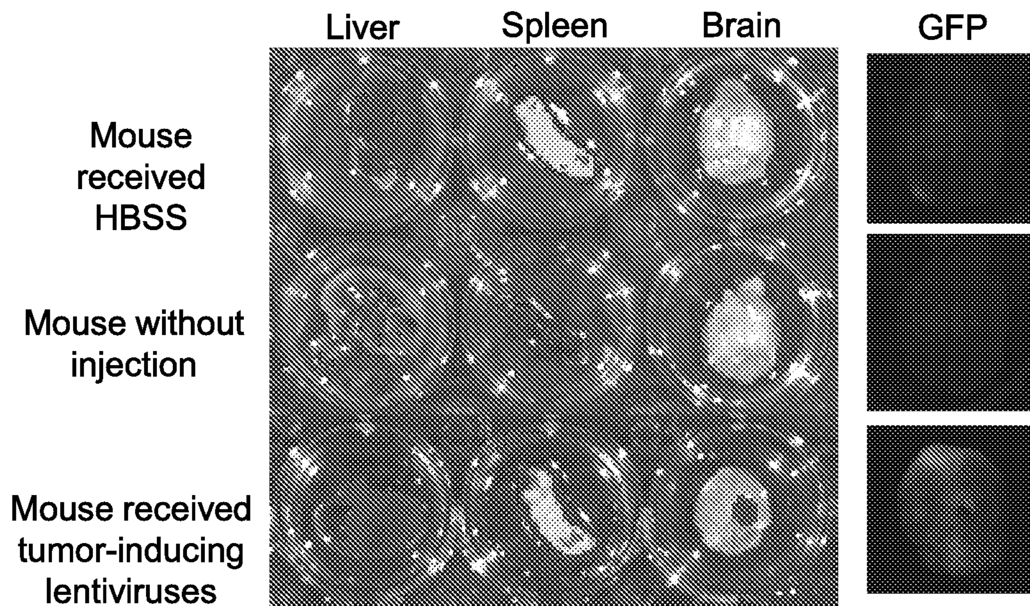


FIG. 7A

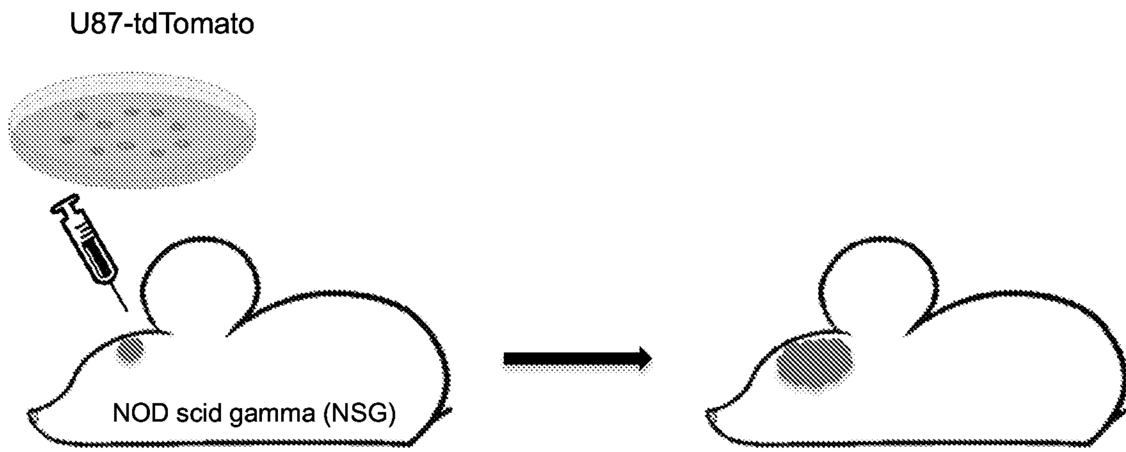


FIG. 7B

Liver Spleen Brain Brain

AdSyn-CO171 injected

AdSyn-CO176 injected

AdSyn-CO176 injected

AdSyn-CO176 injected

Luciferase signal

tdTomato fluorescence

FIG. 8A

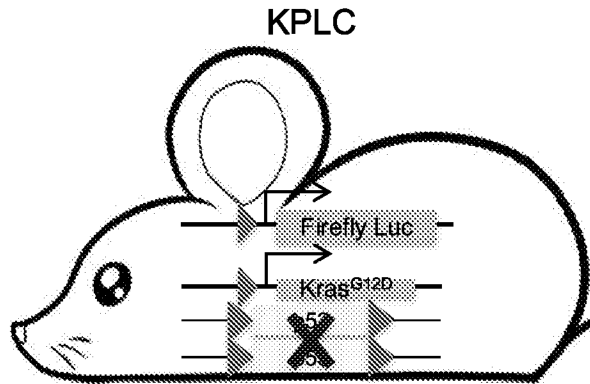


FIG. 8B

Treatment	Ave. Survival (days)
No treatment	37.5
AdSyn-CO989 i.v. + GCV i.p.	42
AdSyn-CO987 i.v. + saline i.p.	42
Only GCV i.p.	46.5
Only GCV i.v.	48
AdSyn-CO987 i.v. + GCV i.p.	55
AdSyn-CO987 i.v. + GCV i.v.	54

FIG. 8C

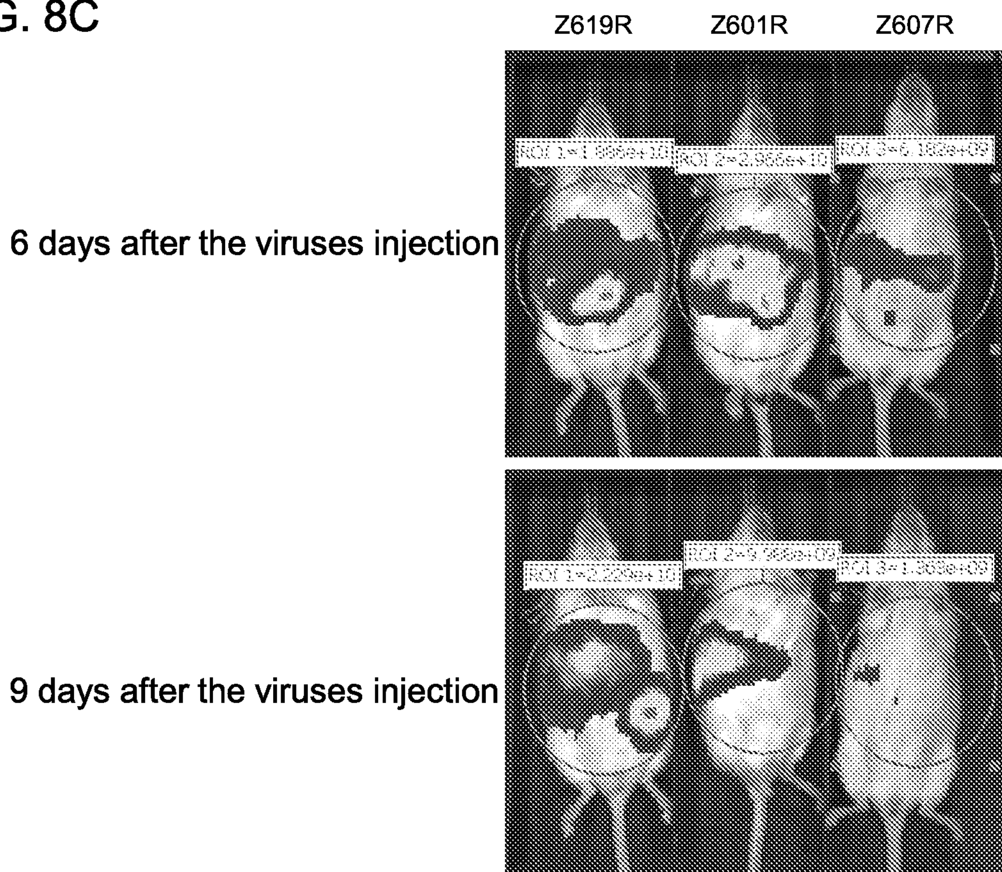


FIG. 9A

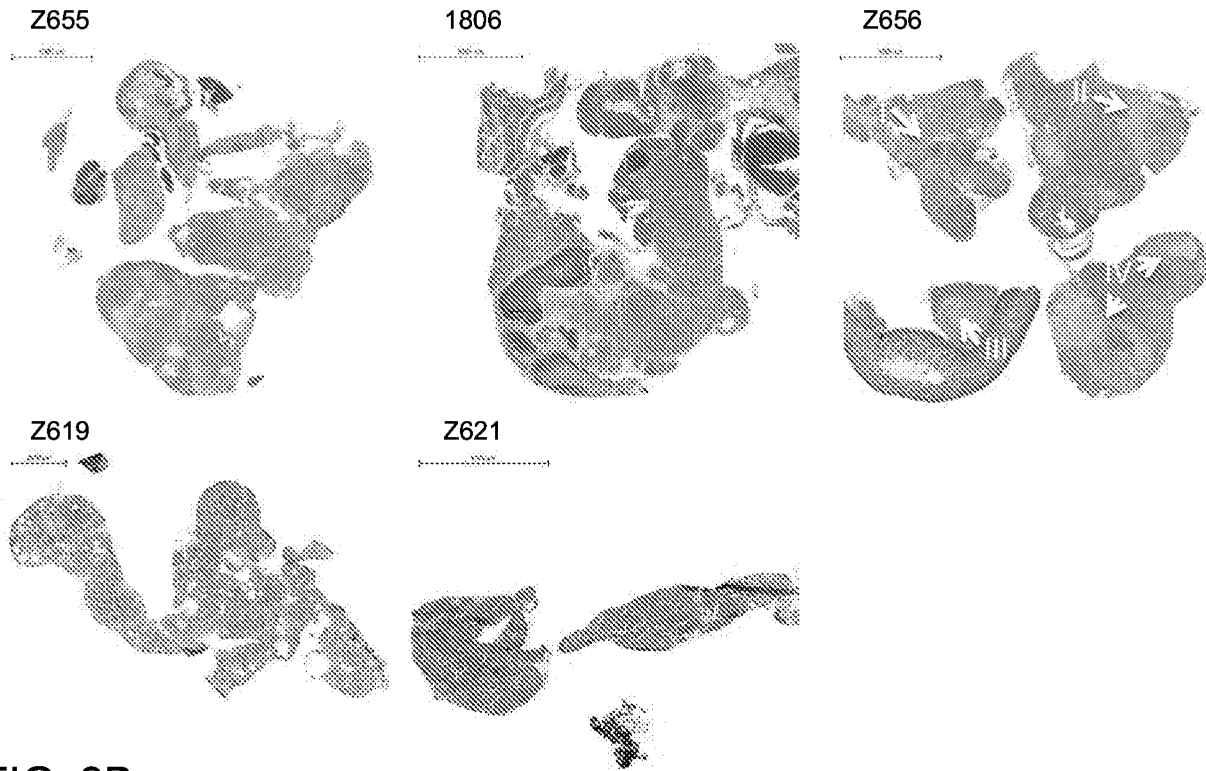
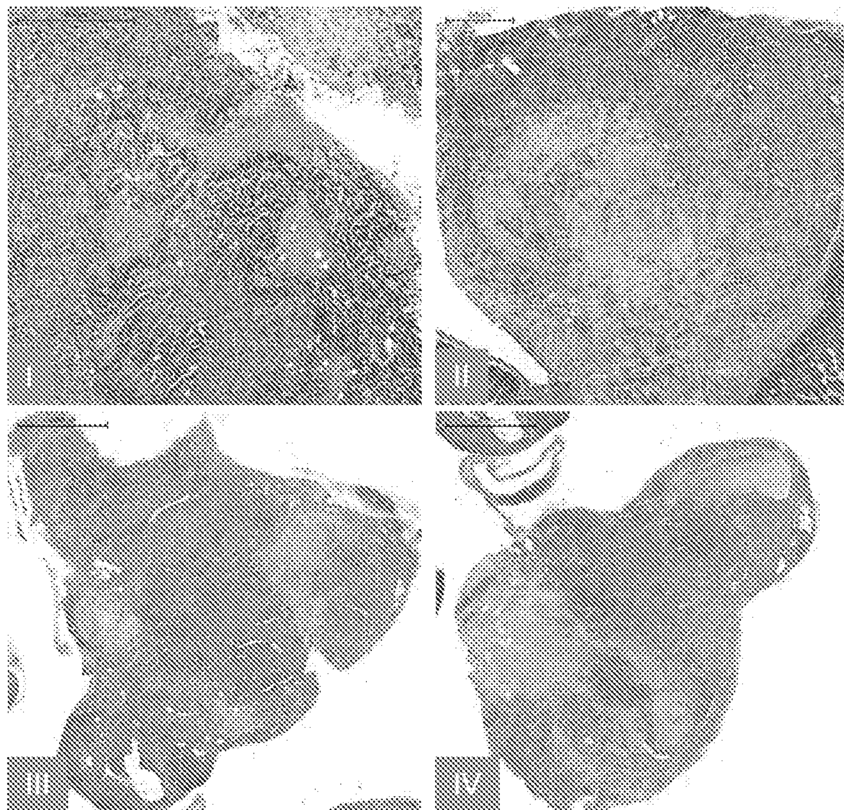


FIG. 9B



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2017/065604

A. CLASSIFICATION OF SUBJECT MATTER

A61K 35/761 (2015.01) C12N 7/01 (2006.01) C12N 15/33 (2006.01) C07K 14/075 (2006.01) A61P 35/00 (2006.01)

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)

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)

WPIAP, EPODOC, MEDLINE, NPL, PATENW (Full text databases), BIOSIS, CAPLUS, EMBASE: adenovirus, Ad5, Ad34, fiber, knob, shaft, chimera, cancer, detarget, miRNA and like terms.

Inventor/Applicant name search: Internal databases provided by IP Australia and external databases (ESPACENET, PATENTSCOPE, AUSPAT)

GENOMEQUEST sequence search: SEQ ID NOs 2, 5

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	Documents are listed in the continuation of Box C	

[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
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- "O" document referring to an oral disclosure, use, exhibition or other means
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- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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Date of the actual completion of the international search	Date of mailing of the international search report
23 January 2018	23 January 2018
Name and mailing address of the ISA/AU	**Authorised officer**
AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA Email address: pct@ipaustralia.gov.au	Suzanne Malik AUSTRALIAN PATENT OFFICE (ISO 9001 Quality Certified Service) Telephone No. +61262832058

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2016/049201 A1 (SALK INSTITUTE FOR BIOLOGICAL STUDIES) 31 March 2016 abstract, pages 3, 32, 35, 46-47, Table 3, Example 3, Figure 23D, SEQ ID NO: 10, 73	1-37
X	US 2014/0199688 A1 (MIZUGUCHI, H. et al.) 17 July 2014 paragraphs 0050, 0064, 0088-0089, 0096-0098, 0119, 0127, 0147, 0185, Example 1	1-6, 8-11, 13-20, 26-32
X	WO 2012/024351 A2 (SALK INSTITUTE FOR BIOLOGICAL STUDIES) 23 February 2012 paragraphs 0004, 0097;Table 3, page 64; Fig 29A	1-22, 29-33
A	US 2003/0215948 A1 (KALEKO, M. et al.) 20 November 2003 abstract, paragraphs 0022, 0204; SEQ ID NO: 9, 69, 70	1-37
A	WO 2013/036791 A2 (BETH ISRAEL DEACONESS MEDICAL CENTER, INC.) 14 March 2013 page 2	1-37
P,X	WO 2017/062511 A1 (SALK INSTITUTE FOR BIOLOGICAL STUDIES) 13 April 2017 SEQ ID NOs: 1-7, 10	36-37

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2017/065604

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c 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 sequence listing filed or furnished:

   a. (means)

   - [ ] on paper
   - [X] in electronic form

   b. (time)

   - [ ] in the international application as filed
   - [X] together with the international application in electronic form
   - [ ] 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 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:

INTERNATIONAL SEARCH REPORT Information on patent family members	International application No. **PCT/US2017/065604**

This Annex lists known patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document/s Cited in Search Report		**Patent Family Member/s**	
Publication Number	**Publication Date**	**Publication Number**	**Publication Date**
WO 2016/049201 A1	31 March 2016	WO 2016049201 A1	31 Mar 2016
		AU 2015320665 A1	06 Apr 2017
		CA 2961748 A1	31 Mar 2016
		EP 3198009 A1	02 Aug 2017
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		AU 2011292120 B2	30 Jul 2015
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		AU 2017239633 A1	02 Nov 2017
		CA 2808389 A1	23 Feb 2012
		CN 103237889 A	07 Aug 2013
		CN 103237889 B	05 Apr 2017
		CN 107090440 A	25 Aug 2017
		EP 2606127 A2	26 Jun 2013
		JP 2013539363 A	24 Oct 2013

Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.

INTERNATIONAL SEARCH REPORT Information on patent family members	International application No. **PCT/US2017/065604**

This Annex lists known patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document/s Cited in Search Report		**Patent Family Member/s**	
Publication Number	**Publication Date**	**Publication Number**	**Publication Date**
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		JP 2016198111 A	01 Dec 2016
		KR 20130126589 A	20 Nov 2013
		KR 101721725 B1	31 Mar 2017
		KR 20170036118 A	31 Mar 2017
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		US 2014348791 A1	27 Nov 2014
WO 2017/062511 A1	13 April 2017	WO 2017062511 A1	13 Apr 2017

End of Annex

Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.