and E2F, proteins that regulate adenovirus expression of E1a, the earliest gene to be transcribed after virus entry into the host cell, through binding to specific DNA sequences.

[0005] Despite the efforts to date, there is a need for improved oncolytic viruses for treating cancers and hyperproliferative disorders in human patients.

SUMMARY

[0006] The present disclosure is based, in part, upon the discovery that adenoviruses such as oncolytic viruses, unexpectedly can efficiently express, when inserted into particular insertion sites, multiple (two or more) therapeutic transgenes without the use of an exogenous promoter and that the viruses can replicate and efficiently express the two or more therapeutic transgenes despite the size of the transgenes incorporated into the viral genome.

[0006a] In one aspect, the present disclosure refers to a recombinant adenovirus comprising:

(a) a first nucleotide sequence encoding a first therapeutic transgene inserted into an E1b-19K insertion site; wherein the E1b-19K insertion site is located between the start site of E1b-19K and the start site of E1b-55K; and

(b) a second nucleotide sequence encoding a second therapeutic transgene inserted into an E3 insertion site, wherein the E3 insertion site is located between the stop site of pVIII and the start site of Fiber.

[0006b] In another aspect, the present disclosure refers to a recombinant adenovirus comprising:

(a) a first nucleotide sequence encoding a first therapeutic transgene inserted into an E1b-19k insertion site; and

(b) a second nucleotide sequence encoding a second therapeutic transgene inserted into the E1b-19k insertion site,

wherein the E1b-19k insertion site is located between the start site of E1b-19k and the start site of E1b-55k, and wherein the first nucleotide sequence and the second nucleotide sequence are separated by a first internal ribosome entry site (IRES).

[0006c] In a further aspect, the present disclosure refers to a pharmaceutical composition comprising the recombinant adenovirus disclosed herein and at least one pharmaceutically acceptable carrier or diluent.

[0006d] In another aspect, the present disclosure refers to a method of expressing two or three therapeutic transgenes in a target cell comprising exposing the cell to an effective amount of the recombinant adenovirus disclosed herein to express the two therapeutic transgenes.

[0006e] In a further aspect, the present disclosure refers to a use of a recombinant adenovirus disclosed herein in the manufacture of a medicament for treating cancer.

[0006f] In another aspect, the present disclosure refers to a recombinant disclosed herein for use in treating cancer.

[0007] In one example the present disclosure provides a recombinant adenovirus comprising: (a) a first nucleotide sequence encoding a first therapeutic transgene inserted into an E1b-19K insertion site; wherein the E1b-19K insertion site is located between the start site of E1b-19K and the start site of E1b-55K; and (b) a second nucleotide sequence encoding a second therapeutic transgene inserted into an E3 insertion site, wherein the E3 insertion site is located between the stop site of pVIII and the start site of Fiber.

[0008] In certain examples, the recombinant adenovirus is a type 5 adenovirus (Ad5).

[0009] In certain examples, the E1b-19K insertion site is located between the start site of E1b-19K and the stop site of E1b-19K. In certain examples, the E1b-19K insertion site comprises a deletion of from about 100 to about 305, about 100 to about 300, about 100 to about 250, about 100 to about 200, about 100 to about 150, about 150 to about 305, about 150 to about 300, about 150 to about 250, or about 150 to about 200 nucleotides adjacent the start site of E1b-19K. In certain examples, the E1b-19K insertion site comprises a deletion of about 200 nucleotides, *e.g.,* 202 or 203 nucleotides adjacent the start site of E1b-19K. In certain examples, the E1b-19K insertion site comprises a deletion corresponding to nucleotides 1714-1917 or 1714-1916 of the Ad5 genome (SEQ ID NO: 23). In certain examples, the first therapeutic transgene is inserted between nucleotides corresponding to 1714 and 1917 or between nucleotides corresponding to 1714 and 1916 of the Ad5 genome (SEQ ID NO: 23). In certain

examples, the first therapeutic transgene is inserted between CTGACCTC (SEQ ID NO: 1) and TCACCAGG (SEQ ID NO: 2), *e.g.,* the recombinant adenovirus comprises, in a 5' to 3' orientation, CTGACCTC (SEQ ID NO: 1), the first therapeutic transgene, and TCACCAGG (SEQ ID NO: 2).

[0010] In certain examples, the E3 insertion site comprises a deletion of from about 500 to about 3185, from about 500 to about 3000, from about 500 to about 2500, from about 500 to about 2000, from about 500 to about 1500, from about 500 to about 1000, from about 1000 to about 3185, from about 1000 to about 3000, from about 1000 to about 2500, from about 1000 to about 2000, from about 1000 to about 1500, from about 1500 to about 3185, from about 1500 to about 3000, from about 1500 to about 2000, from about 2000 to about 3185, from about 2000 to about 3000, from about 2000 to about 2500, from about 2500 to about 3185, from about 2500 to about 3000, or from about 3000 to about 3185 nucleotides. In certain examples, the E3 insertion site is located between the stop site of E3-10.5K and the stop site of E3-14.7K. In certain examples, the E3 insertion site comprises a deletion of from about 500 to about 1551, from about 500 to about 1500, from about 500 to about 1000, from about 1000 to about 1551, from about 1000 to about 1500, or from about 1500 to about 1551 nucleotides adjacent the stop site of E3-10.5K. In certain examples, the E3 insertion site comprises a deletion of about 1050 nucleotides adjacent the stop site of E3-10.5K, *e.g.,* the E3 insertion site comprises a deletion of 1063 or 1064 nucleotides adjacent the stop site of E3-10.5K. In certain examples, the E3 insertion site comprises a deletion corresponding to the Ad5 dl309 E3 deletion. In certain examples, the E3 insertion site comprises a deletion corresponding to nucleotides 29773-30836 of the Ad5 genome (SEQ ID NO: 23). In certain examples, the second therapeutic transgene is inserted between nucleotides corresponding to 29773 and 30836 of the Ad5 genome (SEQ ID NO: 23). In certain examples, the second therapeutic transgene is inserted between CAGTATGA (SEQ ID NO: 3) and TAATAAAAAA (SEQ ID NO: 4), *e.g.,* the recombinant adenovirus comprises, in a 5' to 3' orientation, CAGTATGA (SEQ ID NO: 3), the second therapeutic transgene, and TAATAAAAAA (SEQ ID NO: 4). In certain examples, the E3 insertion site is located between stop site of E3-gp19K and the stop site of E3-14.7K. In certain examples, the E3 insertion site comprises a deletion of from about 500 to about 1824, from about 500 to about

1500, from about 500 to about 1000, from about 1000 to about 1824, from about 1000 to about 1500, or from about 1500 to about 1824 nucleotides adjacent the stop site of E3-gp19K. In certain examples, the E3 insertion site comprises a deletion of about 1600 nucleotides adjacent the stop site of E3-gp19K. *e.g.,* the E3 insertion site comprises a deletion of 1622 nucleotides adjacent the stop site of E3-gp19K. In certain examples, the E3 insertion site comprises a deletion corresponding to nucleotides 29218-30839 of the Ad5 genome (SEQ ID NO: 23). In certain examples, the second therapeutic transgene is inserted between nucleotides corresponding to 29218 and 30839 of the Ad5 genome (SEQ ID NO: 23). In certain examples, the second therapeutic transgene is inserted between TGCCTTAA (SEQ ID NO: 29) and TAAAAAAAAAT (SEQ ID NO: 30), *e.g.,* the recombinant adenovirus comprises, in a 5' to 3' orientation, TGCCTTAA (SEQ ID NO: 29), the second therapeutic transgene, and TAAAAAAAAAT (SEQ ID NO: 30).

[0011] In another example, the present disclosure provides a recombinant adenovirus comprising: (a) a first nucleotide sequence encoding a first therapeutic transgene inserted into an E1b-19k insertion site; and (b) a second nucleotide sequence encoding a second therapeutic transgene inserted into the E1b-19k insertion site, wherein the E1b-19k insertion site is located between the start site of E1b-19k and the start site of E1b-55k, and wherein the first nucleotide sequence and the second nucleotide sequence are separated by a first internal ribosome entry site (IRES).

[0012] In certain examples, the recombinant adenovirus is a type 5 adenovirus (Ad5).

[0013] In certain examples, the E1b-19K insertion site is located between the start site of E1b-19K and the stop site of E1b-19K. In certain examples, the E1b-19K insertion site comprises a deletion of from about 100 to about 305, about 100 to about 300, about 100 to about 250, about 100 to about 200, about 100 to about 150, about 150 to about 305, about 150 to about 300, about 150 to about 250, or about 150 to about 200 nucleotides adjacent the start site of E1b-19K. In certain examples, the E1b-19K insertion site comprises a deletion of about 200 nucleotides, *e.g.,* 202 or 203 nucleotides adjacent the start site of E1b-19K. In certain examples, the E1b-19K insertion site comprises a deletion corresponding to nucleotides 1714-1917 or 1714-1916 of the Ad5 genome (SEQ ID NO: 23). In certain examples, the first and second therapeutic transgenes are inserted between nucleotides corresponding to 1714 and 1917 or

between nucleotides corresponding to 1714 and 1916 of the Ad5 genome (SEQ ID NO: 23). In certain examples, the first and second therapeutic transgenes are inserted between CTGACCTC (SEQ ID NO: 1) and TCACCAGG (SEQ ID NO: 2), *e.g.,* the recombinant adenovirus comprises, in a 5' to 3' orientation, CTGACCTC (SEQ ID NO: 1), the first therapeutic transgene, the first IRES, the second therapeutic transgene, and TCACCAGG (SEQ ID NO: 2).

[0014] In certain examples the recombinant adenovirus comprises an E3 deletion. In certain examples, the E3 deletion comprises a deletion of from about 500 to about 3185, from about 500 to about 3000, from about 500 to about 2500, from about 500 to about 2000, from about 500 to about 1500, from about 500 to about 1000, from about 1000 to about 3185, from about 1000 to about 3000, from about 1000 to about 2500, from about 1000 to about 2000, from about 1000 to about 1500, from about 1500 to about 3185, from about 1500 to about 3000, from about 1500 to about 2000, from about 2000 to about 3185, from about 2000 to about 3000, from about 2000 to about 2500, from about 2500 to about 3185, from about 2500 to about 3000, or from about 3000 to about 3185 nucleotides. In certain examples, the E3 deletion site is located between the stop site of pVIII and the start site of Fiber. In certain examples, the E3 deletion site is located between the stop site of E3-10.5K and the stop site of E3-14.7K. In certain examples, the E3 deletion comprises a deletion of from about 500 to about 1551, from about 500 to about 1500, from about 500 to about 1000, from about 1000 to about 1551, from about 1000 to about 1500, or from about 1500 to about 1551 nucleotides adjacent the stop site of E3-10.5K. In certain examples, the E3 deletion comprises a deletion of about 1050 nucleotides adjacent the stop site of E3-10.5K, *e.g.,* the E3 deletion comprises a deletion of 1063 or 1064 nucleotides adjacent the stop site of E3-10.5K. In certain examples, the E3 deletion comprises a deletion corresponding to the Ad5 dl309 E3 deletion. In certain examples, the E3 deletion comprises a deletion corresponding to nucleotides 29773-30836 of the Ad5 genome (SEQ ID NO: 23). In certain examples, the E3 deletion is located between stop site of E3-gp19K and the stop site of E3-14.7K. In certain examples, the E3 deletion comprises a deletion of from about 500 to about 1824, from about 500 to about 1500, from about 500 to about 1000, from about 1000 to about 1824, from about 1000 to about 1500, or from about 1500 to about 1824 nucleotides adjacent the stop site of E3-gp19K. In certain examples, the E3 deletion comprises a deletion of about 1600

nucleotides adjacent the stop site of E3-gp19K. *e.g.,* the E3 insertion site comprises a deletion of 1622 nucleotides adjacent the stop site of E3-gp19K. In certain examples, the E3 deletion comprises a deletion corresponding to nucleotides 29218-30839 of the Ad5 genome (SEQ ID NO: 23).

[0015] In certain examples, the recombinant adenovirus comprises a third nucleotide sequence encoding a third therapeutic transgene. The third therapeutic transgene may be inserted into the E1b-19k insertion site wherein, *e.g.*, the second nucleotide sequence and the third nucleotide sequence are separated by a second internal ribosome entry site (IRES). In certain examples, the first, second, and third therapeutic transgenes are inserted between CTGACCTC (SEQ ID NO: 1) and TCACCAGG (SEQ ID NO: 2), *e.g.,* the recombinant adenovirus comprises, in a 5' to 3' orientation, CTGACCTC (SEQ ID NO: 1), the first therapeutic transgene, the first IRES, the second therapeutic transgene, the second IRES, the third therapeutic transgene, and TCACCAGG (SEQ ID NO: 2). The third therapeutic transgene may also be inserted into the E3 deletion site, *i.e.,* in certain examples the recombinant adenovirus comprises a third nucleotide sequence encoding a third therapeutic transgene inserted into an E3 insertion site. In certain examples, the third therapeutic transgene is inserted between nucleotides corresponding to 29773 and 30836 of the Ad5 genome. In certain examples, the third therapeutic transgene is inserted between CAGTATGA (SEQ ID NO: 3) and TAATAAAAAA (SEQ ID NO: 4), *e.g.,* the recombinant adenovirus comprises, in a 5' to 3' orientation, CAGTATGA (SEQ ID NO: 3), the third therapeutic transgene, and TAATAAAAAA (SEQ ID NO: 4). In certain examples, the third therapeutic transgene is inserted between nucleotides corresponding to 29218 and 30839 of the Ad5 genome (SEQ ID NO: 23). In certain examples, the third therapeutic transgene is inserted between TGCCTTAA (SEQ ID NO: 29) and TAAAAAAAAAT (SEQ ID NO: 30), *e.g.,* the recombinant adenovirus comprises, in a 5' to 3' orientation, TGCCTTAA (SEQ ID NO: 29), the third therapeutic transgene, and TAAAAAAAAAT (SEQ ID NO: 30).

[0016] The IRES may, *e.g.,* be selected from the group consisting of the encephalomyocarditis virus (EMCV) IRES, the foot-and-mouth disease virus (FMDV) IRES, and the poliovirus IRES.

[0017] In certain examples, in any of the foregoing viruses, the recombinant adenovirus further comprises an E4 deletion. In certain examples, the E4 deletion is located between the

start site of E4-ORF6/7 and the right inverted terminal repeat (ITR). In certain examples, the E4 deletion is located between the start site of E4-ORF6/7 and the start site of E4-ORF1. In certain examples, the E4 deletion comprises a deletion of from about 500 to about 2500, from about 500 to about 2000, from about 500 to about 1500, from about 500 to about 1000, from about 1000 to about 2500, from about 1000 to about 2000, from about 1000 to about 1500, from about 1500 to about 2500, from about 1500 to about 2000, or from about 2000 to about 2500 nucleotides. In certain examples, the E4 deletion comprises a deletion of from about 250 to about 1500, from about 250 to about 1250, from about 250 to about 1000, from about 250 to about 750, from about 250 to about 500, from 500 to about 1500, from about 500 to about 1250, from about 500 to about 1000, from about 500 to about 750, from 750 to about 1500, from about 750 to about 1250, from about 750 to about 1000, from about 1000 to about 1500, or from about 1000 to about 1250 nucleotides adjacent the start site of E4-ORF6/7. In certain examples, the E4 deletion comprises a deletion of about 1450 nucleotides adjacent the start site of E4-ORF6/7, *e.g.,* the E4 deletion comprises a deletion of about 1449 nucleotides adjacent the start site of E4-ORF6/7. In certain examples, the E4 deletion comprises a deletion corresponding to nucleotides 34078-35526 of the Ad5 genome (SEQ ID NO: 23).

[0018] In certain examples, in any of the foregoing viruses, the first and/or second therapeutic transgenes, the first, second, and/or third therapeutic transgenes, or all of the therapeutic transgenes, are not operably linked to an exogenous promoter sequence.

[0019] In certain examples, the size of the first and second therapeutic transgenes, the size of the first, second, and third therapeutic transgenes, or the size of all of the therapeutic transgenes, when combined, comprise from about 500 to about 5000, from about 500 to about 4000, from about 500 to about 3000, from about 500 to about 2000, from about 500 to about 1000, from about 1000 to about 5000, from about 1000 to about 4000, from about 1000 to about 3000, from about 1000 to about 2000, from about 2000 to about 5000, from about 2000 to about 4000, from about 2000 to about 3000, from about 3000 to about 5000, from about 3000 to about 4000, or from about 4000 to about 5000 nucleotides. In certain examples, the size of the first and second therapeutic transgenes, the size of the first, second, and third therapeutic transgenes, or the size of all of the therapeutic transgenes, when combined, comprise from about 500 to about 7000, from

about 500 to about 6000, from about 500 to about 5000, from about 500 to about 4000, from about 500 to about 3000, from about 500 to about 2000, from about 500 to about 1000, from about 1000 to about 7000, from about 1000 to about 6000, from about 1000 to about 5000, from about 1000 to about 4000, from about 1000 to about 3000, from about 1000 to about 2000, from about 2000 to about 7000, from about 2000 to about 6000, from about 2000 to about 5000, from about 2000 to about 4000, from about 2000 to about 3000, from about 3000 to about 7000, from about 3000 to about 6000, from about 3000 to about 5000, from about 3000 to about 4000, from about 4000 to about 7000, from about 4000 to about 6000, from about 4000 to about 5000 nucleotides, from about 5000 to about 7000, from about 5000 to about 6000, or from about 6000 to about 7000 nucleotides.

[0020] In certain examples, the size of the first and second therapeutic transgenes, the size of the first, second, and third therapeutic transgenes, or the size of all of the therapeutic transgenes, when combined, comprise at least about 500, about 1000, about 2000, about 3000, about 4000, or about 5000 nucleotides. In certain examples, the size of the first and second therapeutic transgenes, the size of the first, second, and third therapeutic transgenes, or the size of all of the therapeutic transgenes, when combined, comprise about 1650 nucleotides. In certain examples, the size of the first and second therapeutic transgenes, the size of the first, second, and third therapeutic transgenes, or the size of all of the therapeutic transgenes, when combined, comprise at least about 500, about 1000, about 2000, about 3000, about 4000, about 5000, about 6000, or about 7000 nucleotides. In certain examples, the size of the first and second therapeutic transgenes, the size of the first, second, and third therapeutic transgenes, or the size of all of the therapeutic transgenes, when combined, comprise about 3100 nucleotides.

[0021] In certain examples, in any of the foregoing viruses, the first and/or second therapeutic transgene, the first, second, and/or third therapeutic transgenes, or any of the therapeutic transgenes encode a therapeutic polypeptide selected from the group consisting of CD80, CD137L, IL-23A/p19, p40, endostatin, angiostatin, ICAM-1, and a TGF-β trap.

[0022] In certain examples, in any of the foregoing viruses, the first and/or second therapeutic transgene, the first, second, and/or third therapeutic transgenes, or any of the therapeutic transgenes encode a therapeutic polypeptide selected from the group consisting of

CD80, CD137L, IL-23, IL-23A/p19, p40, IL-27, IL-27A/p28, IL-27B/EBI3, endostatin, angiostatin, ICAM-1, a TGF-β trap, TGF-β, CD19, CD20, IL-1, IL-3, IL-4, IL-5, IL-6, IL-8, IL-9, CD154, CD86, BORIS/CTCFL, FGF, IL-24, MAGE, NY-ESO-1, acetylcholine, interferon-gamma, DKK1/Wnt, p53, thymidine kinase, an anti-PD-1 antibody heavy chain or light chain, and an anti-PD-L1 antibody heavy chain or light chain.

[0023] In certain examples, the first and second therapeutic transgene encode a first and second subunit, respectively, of a heterodimeric cytokine.

[0024] In certain examples, in any of the foregoing viruses, the first and/or second therapeutic transgenes are selected from the group consisting of CD80 and CD137L, *e.g.,* the first therapeutic transgene encodes CD80 and the second therapeutic transgene encodes CD137L. In certain examples, the recombinant adenovirus comprises a nucleotide sequence encoding an amino acid sequence that is encoded by SEQ ID NO: 5, and/or SEQ ID NO: 7, or comprises the nucleotide sequence of SEQ ID NO: 6, and/or SEQ ID NO: 8. In certain examples, the recombinant adenovirus comprises the nucleotide sequence of SEQ ID NO: 27.

[0025] In certain examples, in any of the foregoing viruses, the first, second, and/or third therapeutic transgenes are selected from the group consisting of CD80, CD137L, and ICAM-1., *e.g.,* the first therapeutic transgene encodes CD80, the second therapeutic transgene encodes CD137L, and the third therapeutic transgene encodes ICAM-1. In certain examples, the recombinant adenovirus comprises a nucleotide sequence encoding an amino acid sequence that is encoded by SEQ ID NO: 5, SEQ ID NO: 7, and/or SEQ ID NO: 32. In certain examples, the recombinant adenovirus comprises the nucleotide sequence of SEQ ID NO: 31, SEQ ID NO: 9, or SEQ ID NO: 22.

[0026] In certain examples, in any of the foregoing viruses, the first and/or second therapeutic transgenes are selected from the group consisting of IL-23A/p19 and p40, which make up the heterodimeric cytokine IL-23. For example, in certain examples, the first therapeutic transgene encodes IL-23A/p19 and the second therapeutic transgene encodes p40. In certain examples, the recombinant adenovirus comprises a nucleotide sequence encoding an amino acid sequence that is encoded by SEQ ID NO: 12 and/or SEQ ID NO: 10, or comprises the nucleotide sequence of SEQ ID NO: 13.

[0027] In certain examples, in any of the foregoing viruses, the first and/or second therapeutic transgenes are selected from the group consisting of IL-27A/p28 and IL-27B/EBI3, , which make up the heterodimeric cytokine IL-27. For example, in certain examples, the first therapeutic transgene encodes IL-27A/p28 and the second therapeutic transgene encodes IL-27B/EBI3.

[0028] In certain examples, in any of the foregoing viruses, the first and/or second therapeutic transgenes are selected from the group consisting of endostatin and angiostatin *e.g.,* the first therapeutic transgene encodes endostatin and the second therapeutic transgene encodes angiostatin. In certain examples, the recombinant adenovirus comprises a nucleotide sequence encoding the amino acid sequence of SEQ ID NO: 37 or SEQ ID NO: 38. In certain examples, the recombinant adenovirus comprises a nucleotide sequence encoding the amino acid sequence of SEQ ID NO: 39, SEQ ID NO: 40, SEQ ID NO: 41, SEQ ID NO: 42, SEQ ID NO: 43 or SEQ ID NO: 44. In certain examples, the recombinant adenovirus comprises the nucleotide sequence of SEQ ID NO: 11.

[0029] In certain examples, any of the foregoing recombinant adenoviruses may comprise a deletion of at least one Pea3 binding site, or a functional portion thereof, *e.g.,* the virus may comprise a deletion of nucleotides corresponding to about -300 to about -250 upstream of the initiation site of E1a or a deletion of nucleotides corresponding to -305 to -255 or -304 to -255 upstream of the initiation site of E1a.

[0030] In certain examples, in any of the foregoing compositions, the recombinant oncolytic adenovirus may comprise a deletion of at least one E2F binding site, or a functional portion thereof. In certain examples, the recombinant oncolytic adenovirus may comprise a deletion of at least one E2F binding site, or a functional portion thereof, and not comprise a deletion of a Pea3 binding site.

[0031] In another example, the present disclosure provides a recombinant adenovirus comprising SEQ ID NO: 14, or a sequence having 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity to SEQ ID NO: 14.

[0032] In certain examples, each of the foregoing recombinant adenoviruses may selectively replicate in a hyperproliferative cell. In certain examples, any of the foregoing recombinant adenoviruses may selectively express two or more therapeutic transgenes in a hyperproliferative cell. The hyperproliferative cell may be a cancer cell, *e.g.*, a lung cancer cell, a colon cancer cell, and a pancreatic cancer cell. In certain examples, each of the foregoing recombinant adenoviruses may be an oncolytic adenovirus.

[0033] In another example, the present disclosure provides a pharmaceutical composition comprising each of the foregoing recombinant adenoviruses and at least one pharmaceutically acceptable carrier or diluent.

[0034] In another example, the present disclosure provides a method of treating cancer in a subject. The method comprises administering to the subject an effective amount of a recombinant adenovirus described herein to treat the cancer disease in the subject. In certain examples, the cancer is selected from the group consisting of melanoma, squamous cell carcinoma of the skin, basal cell carcinoma, head and neck cancer, breast cancer, anal cancer, cervical cancer, non-small cell lung cancer, mesothelioma, small cell lung cancer, renal cell carcinoma, prostate cancer, gastroesophageal cancer, colorectal cancer, testicular cancer, bladder cancer, ovarian cancer, hepatocellular carcinoma, cholangiocarcinoma, brain cancer, endometrial cancer, neuroendocrine cancer, merkel cell carcinoma, gastrointestinal stromal tumors, a sarcoma, and pancreatic cancer.

[0035] In another example, the present disclosure provides a method of inhibiting proliferation of a tumor cell in a subject. The method comprises administering to the subject an effective amount of a recombinant adenovirus described herein to inhibit proliferation of the tumor cell.

[0036] In another example, the present disclosure provides a method of inhibiting tumor growth in a subject. The method comprises administering to the subject an effective amount of a recombinant adenovirus described herein to inhibit proliferation of the tumor cell.

[0037] In each of the foregoing methods, the recombinant adenovirus can, *e.g.*, be administered in combination with one or more therapies selected from the group consisting of surgery, radiation, chemotherapy, immunotherapy, hormone therapy, and virotherapy. In each of the foregoing methods, the effective amount of the recombinant adenovirus can be, *e.g.*, 10^2-10^{15}

plaque forming units (pfus). In each of the foregoing methods, the subject can, *e.g.,* be a human, *e.g.,* a pediatric human, or an animal.

[0038] In each of the foregoing methods, the effective amount of the recombinant virus may, *e.g.,* be identified by measuring an immune response to an antigen in the subject. In certain examples, the immune response to the antigen is measured by injecting the subject with the antigen at an injection site on the skin of the subject and measuring the size of an induration at the injection site.

[0039] In another example, the present disclosure provides a method of expressing two or more therapeutic transgenes in a target cell. The method comprises exposing the cell to an effective amount of the recombinant adenovirus described herein to express the target transgenes.

[0040] These and other aspects and advantages of the invention are illustrated by the following figures, detailed description and claims.

DESCRIPTION OF THE DRAWINGS

[0041] The invention can be more completely understood with reference to the following drawings.

[0042] **FIGURE 1** depicts staining of ADS-12 cells for mouse CD80 or mouse CD137L two days following infection with the indicated virus at a multiplicity of infection (MOI) of 5.

[0043] **FIGURE 2** depicts staining of ADS-12 cells for mouse CD80 or mouse CD137L two days following infection with the indicated virus at a multiplicity of infection (MOI) of 5.

[0044] **FIGURE 3** depicts staining of 4T1 cells for mouse CD80 or mouse CD137L three days following infection with the indicated virus.

[0045] **FIGURE 4** depicts staining of 4T1 cells for mouse CD80 or mouse CD137L three days following infection with the indicated virus.

[0046] **FIGURE 5** depicts staining of non-cancerous (WI-38 and MRC5) or cancerous (A549) cells for human CD80 or human CD137L two days following infection with the indicated virus at a MOI of 2.

WHAT IS CLAIMED IS:

1. A recombinant adenovirus comprising:

(a) a first nucleotide sequence encoding a first therapeutic transgene inserted into an E1b-19K insertion site; wherein the E1b-19K insertion site is located between the start site of E1b-19K and the start site of E1b-55K; and

(b) a second nucleotide sequence encoding a second therapeutic transgene inserted into an E3 insertion site, wherein the E3 insertion site is located between the stop site of pVIII and the start site of Fiber.

2. The recombinant adenovirus of claim 1, wherein the recombinant adenovirus is a type 5 adenovirus (Ad5).

3. The recombinant adenovirus of claim 1 or 2, wherein the E1b-19K insertion site comprises a deletion of about 200 nucleotides adjacent the start site of E1b-19K.

4. The recombinant adenovirus of any one of claims 1-3, wherein the E1b-19K insertion site comprises a deletion corresponding to nucleotides 1714-1916 of the Ad5 genome (SEQ ID NO: 23).

5. The recombinant adenovirus of any one of claims 1-4, wherein the first therapeutic transgene is inserted between nucleotides corresponding to 1714 and 1916 of the Ad5 genome (SEQ ID NO: 23), and/or the recombinant adenovirus comprises, in a 5' to 3' orientation, CTGACCTC (SEQ ID NO: 1), the first therapeutic transgene, and TCACCAGG (SEQ ID NO: 2).

6. The recombinant adenovirus of any one of claims 1-5, wherein the E3 insertion site comprises a deletion of about 1050 nucleotides adjacent the stop site of E3-10.5K.

7. The recombinant adenovirus of any one of claims 1-6, wherein the E3 insertion site comprises a deletion corresponding to nucleotides 29773-30836 of the Ad5 genome (SEQ ID NO: 23).

8. The recombinant adenovirus of any one of claims 1-7, wherein the second therapeutic transgene is inserted between nucleotides corresponding to 29773 and 30836 of the Ad5 genome (SEQ ID NO: 23), and/or the recombinant adenovirus comprises, in a 5' to 3' orientation, CAGTATGA (SEQ ID NO: 3), the second therapeutic transgene, and TAATAAAAAA (SEQ ID NO: 4).

9. The recombinant adenovirus of any one of claims 1-5, wherein the E3 insertion site comprises a deletion of about 1600 nucleotides adjacent the stop site of E3-gp19K.

10. The recombinant adenovirus of claim 9, wherein the E3 insertion site comprises a deletion corresponding to nucleotides 29218-30839 of the Ad5 genome (SEQ ID NO: 23).

11. The recombinant adenovirus of claim 9 or 10, wherein the second therapeutic transgene is inserted between nucleotides corresponding to 29218 and 30839 of the Ad5 genome (SEQ ID NO: 23), and/or the recombinant adenovirus comprises, in a 5' to 3' orientation, TGCCTTAA (SEQ ID NO: 29), the second therapeutic transgene, and TAAAAAAAAAT (SEQ ID NO: 30).

12. A recombinant adenovirus comprising:

(a) a first nucleotide sequence encoding a first therapeutic transgene inserted into an E1b-19k insertion site; and

(b) a second nucleotide sequence encoding a second therapeutic transgene inserted into the E1b-19k insertion site,

wherein the E1b-19k insertion site is located between the start site of E1b-19k and the start site of E1b-55k, and wherein the first nucleotide sequence and the second nucleotide sequence are separated by a first internal ribosome entry site (IRES).

13. The recombinant adenovirus of claim 12, wherein the adenovirus is a type 5 adenovirus (Ad5).

14. The recombinant adenovirus of claim 12 or 13, wherein the E1b-19K insertion site comprises a deletion of about 200 nucleotides adjacent the start site of E1b-19K.

15. The recombinant adenovirus of any one of claims 12-14, wherein the E1b-19K insertion site comprises a deletion corresponding to nucleotides 1714-1916 of the Ad5 genome (SEQ ID NO: 23).

16. The recombinant adenovirus of any one of claims 12-15, wherein the first and second therapeutic transgenes are inserted between nucleotides corresponding to 1714 and 1916 of the Ad5 genome (SEQ ID NO: 23), and/or the recombinant adenovirus comprises, in a 5' to 3' orientation, CTGACCTC (SEQ ID NO: 1), the first therapeutic transgene, the IRES, the second therapeutic transgene, and TCACCAGG (SEQ ID NO: 2).

17. The recombinant adenovirus of any one of claims 12-16, wherein the recombinant adenovirus comprises a third nucleotide sequence encoding a third therapeutic transgene inserted into the E1b-19k insertion site wherein the second nucleotide sequence and the third nucleotide sequence are separated by a second internal ribosome entry site (IRES).

18. The recombinant adenovirus of claim 17, wherein the recombinant adenovirus comprises, in a 5' to 3' orientation, CTGACCTC (SEQ ID NO: 1), the first therapeutic transgene, the first IRES, the second therapeutic transgene, the second IRES, the third therapeutic transgene, and TCACCAGG (SEQ ID NO: 2).

19. The recombinant adenovirus of any of claims 12-18, wherein the recombinant adenovirus further comprises an E3 deletion, wherein the E3 deletion is located between the stop site of pVIII and the start site of Fiber.

20. The recombinant adenovirus of claim 19, wherein the E3 deletion comprises a deletion of about 1050 nucleotides adjacent the stop site of E3-10.5K.

21. The recombinant adenovirus of claim 19 or 20, wherein the E3 deletion comprises a deletion corresponding to nucleotides 29773-30836 of the Ad5 genome (SEQ ID NO: 23).

22. The recombinant adenovirus of claim 19, wherein the E3 deletion comprises a deletion of about 1600 nucleotides adjacent the stop site of E3-gp19K.

23. The recombinant adenovirus of claim 19 or 22, wherein the E3 deletion comprises a deletion corresponding to nucleotides 29218-30839 of the Ad5 genome (SEQ ID NO: 23).

24. The recombinant adenovirus of any of claims 12-16, wherein the recombinant adenovirus comprises a third nucleotide sequence encoding a third therapeutic transgene inserted into an E3 insertion site, wherein the E3 insertion site is located between the stop site of pVIII and the start site of Fiber.

25. The recombinant adenovirus of claim 24, wherein the E3 insertion site comprises a deletion of about 1050 nucleotides adjacent the stop site of E3-10.5K.

26. The recombinant adenovirus of claim 24 or 25, wherein the E3 insertion site comprises a deletion corresponding to nucleotides 29773-30836 of the Ad5 genome (SEQ ID NO: 23).

27. The recombinant adenovirus of any one of claims 24-26, wherein the third therapeutic transgene is inserted between nucleotides corresponding to 29773 and 30836 of the Ad5 genome (SEQ ID NO: 23), and/or the recombinant adenovirus comprises, in a 5' to 3' orientation, CAGTATGA (SEQ ID NO: 3), the third therapeutic transgene, and TAATAAAAAA (SEQ ID NO: 4).

28. The recombinant adenovirus of claim 24, wherein the E3 insertion site comprises a deletion of about 1600 nucleotides adjacent the stop site of E3-gp19K.

29. The recombinant adenovirus of claim 24 or 28, wherein the E3 insertion site comprises a deletion corresponding to nucleotides 29218-30839 of the Ad5 genome (SEQ ID NO: 23).

30. The recombinant adenovirus of any one of claims 24, 28, or 29, wherein the third therapeutic transgene is inserted between nucleotides corresponding to 29218 and 30839 of the Ad5 genome (SEQ ID NO: 23), and/or the recombinant adenovirus comprises, in a 5' to 3' orientation, TGCCTTAA (SEQ ID NO: 29), the third therapeutic transgene, and TAAAAAAAAAT (SEQ ID NO: 30).

31. The recombinant adenovirus of any of claims 1-30, wherein the recombinant adenovirus further comprises an E4 deletion, wherein the E4 deletion is located between the start site of E4-ORF6/7 and right inverted terminal repeat (ITR).

32. The recombinant adenovirus of claim 31, wherein the E4 deletion comprises a deletion of about 1450 nucleotides adjacent the start site of E4-ORF6/7.

33. The recombinant adenovirus of claim 31 or 32, wherein the E4 deletion comprises a deletion corresponding to nucleotides 34078-35526 of the Ad5 genome (SEQ ID NO: 23).

34. The recombinant adenovirus of any one of claims 1-33, wherein the combined size of the first, second, and/or third therapeutic transgenes comprises at least about 500, about 1000, about 2000, about 3000, about 4000, about 5000, about 6000, or about 7000 nucleotides.

35. The recombinant adenovirus of any one of claims 1-34, wherein the first, second and/or third therapeutic transgene encodes a therapeutic polypeptide selected from the group consisting of CD80, CD137L, IL-23, IL-23A/p19, IL-27, IL-27A/p28, IL-27B/EBI3, endostatin, angiostatin, ICAM-1, a TGF-β trap, TGF-β, CD19, CD20, IL-1, IL-3, IL-4, IL-5, IL-6, IL-8, IL-9, CD154, CD86, BORIS/CTCFL, FGF, IL-24, MAGE, NY-ESO-1, acetylcholine, interferon-gamma, DKK1/Wnt, p53, thymidine kinase, an anti-PD-1 antibody heavy chain or light chain, and an anti-PD-L1 antibody heavy chain or light chain.

36. The recombinant adenovirus of any one of claims 1-35, wherein the first and second therapeutic transgene encode a first and second subunit, respectively, of a heterodimeric cytokine.

37. The recombinant adenovirus of any one of claim 1-35, wherein the first, second and/or third therapeutic transgenes are selected from the group consisting of CD80, CD137L, and ICAM-1.

38. The recombinant adenovirus of claim 37, wherein the recombinant adenovirus comprises a nucleotide sequence encoding an amino acid sequence that is encoded by SEQ ID NO: 5, a nucleotide sequence encoding an amino acid sequence that is encoded by SEQ ID NO: 7, and/or a nucleotide sequence encoding an amino acid sequence that is encoded by SEQ ID NO: 32.

39. The recombinant adenovirus of claim 37 or 38, wherein the recombinant adenovirus comprises the nucleotide sequence of SEQ ID NO: 6 and/or the nucleotide sequence of SEQ ID NO: 8.

40. The recombinant adenovirus of any one of claims 37-39, wherein the recombinant adenovirus comprises the nucleotide sequence of SEQ ID NO: 27, SEQ ID NO: 31, or SEQ ID NO: 9.

41. The recombinant adenovirus of any one of claims 37-40, wherein the recombinant adenovirus comprises the nucleotide sequence of SEQ ID NO: 22.

42. The recombinant adenovirus of any one of claims 1-35, wherein the first and/or second therapeutic transgenes are selected from the group consisting of IL-27A/p28 and IL-27B/EBI3.

43. The recombinant adenovirus of any one of claims 1-35, wherein the first and/or second therapeutic transgenes are selected from the group consisting of endostatin and angiostatin.

44. The recombinant adenovirus of any one of claims 1-43, wherein the recombinant adenovirus further comprises a deletion of a Pea3 binding site, or a functional fragment thereof.

45. The recombinant adenovirus of claim 33, wherein the recombinant adenovirus comprises SEQ ID NO: 14, or a sequence having 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity to SEQ ID NO: 14.

46. The recombinant adenovirus of any one of claims 1-45, wherein the recombinant adenovirus selectively replicates in a hyperproliferative cell or selectively expresses the first, second, and/or third therapeutic transgene in a hyperproliferative cell..

47. The recombinant adenovirus of any one of claims 1-46, wherein the recombinant adenovirus is an oncolytic virus.

48. A pharmaceutical composition comprising the recombinant adenovirus of any one of claims 1-47 and at least one pharmaceutically acceptable carrier or diluent.

49. A method of expressing two or three therapeutic transgenes in a target cell comprising exposing the cell to an effective amount of the recombinant adenovirus of any one of claims 1-47 to express the two therapeutic transgenes.

50. Use of a recombinant adenovirus of any one of claims 1-47 in the manufacture of a medicament for treating cancer.

51. A recombinant adenovirus of any one of claims 1-47 for use in treating cancer.