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 oncolytic 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, wherein the first therapeutic transgene and the second therapeutic transgenes are selected from the group consisting of CD80, CD137L, IL-23A/p19, endostatin, angiostatin, ICAM-1, and a TGF-β trap.

[0006b] In another aspect, the present disclosure refers to a recombinant oncolytic 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), wherein the first therapeutic transgene and the second therapeutic transgenes are selected from the group consisting of CD80, CD137L, IL-23A/p19, endostatin, angiostatin, ICAM-1, and a TGF-β trap.

[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 recombinant adenovirus disclosed herein for use in to expressing two or three therapeutic transgenes in a target cell.

[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

WHAT IS CLAIMED IS:

1. A recombinant oncolytic 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, wherein the first therapeutic transgene and the second therapeutic transgenes are selected from the group consisting of CD80, CD137L, IL-23A/p19, endostatin, angiostatin, ICAM-1, and a TGF-β trap.

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 oncolytic 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), wherein the first therapeutic transgene and the second therapeutic transgene are selected from the group consisting of CD80, CD137L, IL-23A/p19, endostatin, angiostatin, ICAM-1, and a TGF-β trap.

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 TAAAAAAAAT (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 and second therapeutic transgene encode a first and second subunit, respectively, of a heterodimeric cytokine.

36. The recombinant adenovirus of any one of claim 1-34, wherein the first, second and/or third therapeutic transgenes are selected from the group consisting of CD80, CD137L, and ICAM-1.

37. The recombinant adenovirus of claim 36, wherein the recombinant adenovirus comprises a nucleotide sequence encoding the amino acid sequence that is encoded by SEQ ID NO: 5, a nucleotide sequence encoding the amino acid sequence that is encoded by SEQ ID NO: 7, and/or a nucleotide sequence encoding the amino acid sequence that is encoded by SEQ ID NO: 32.

38. The recombinant adenovirus of claim 36 or 37, wherein the recombinant adenovirus comprises the nucleotide sequence of SEQ ID NO: 6 and/or the nucleotide sequence of SEQ ID NO: 8.

39. The recombinant adenovirus of any one of claims 36-38, wherein the recombinant adenovirus comprises the nucleotide sequence of SEQ ID NO: 27, SEQ ID NO: 31, or SEQ ID NO: 9.

40. The recombinant adenovirus of any one of claims 36-39, wherein the recombinant adenovirus comprises the nucleotide sequence of SEQ ID NO: 22.

41. The recombinant adenovirus of any one of claims 1-34, wherein the first and/or second therapeutic transgenes are selected from the group consisting of endostatin and angiostatin.

42. The recombinant adenovirus of any one of claims 1-41, wherein the recombinant adenovirus further comprises a deletion of a Pea3 binding site, or a functional fragment thereof.

43. 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.

44. The recombinant adenovirus of any one of claims 1-43, 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.

45. A pharmaceutical composition comprising the recombinant adenovirus of any one of claims 1-44 and at least one pharmaceutically acceptable carrier or diluent.

46. A recombinant adenovirus of any one of claims 1-44 for use in expressing two or three therapeutic transgenes in a target cell.

47. Use of a recombinant adenovirus of any one of claims 1-44 in the manufacture of a medicament for treating cancer.

48. A recombinant adenovirus of any one of claims 1-44 for use in treating cancer.