CLAIMS

What is Claimed is:

1. A miRNA expression construct comprising a promoter element, a spacer at least 50 nucleotides in length, and a miRNA hairpin.

2. The miRNA expression construct of claim 1, wherein the spacer is between 50 and 1,000 nucleotides in length.

3. The miRNA expression construct of claim 1, wherein the spacer is between 50 and 900; 50 and 800; 100 and 800; or 50 and 800 nucleotides in length.

4. The miRNA expression construct of claim 1, wherein the spacer is at least 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180 or 200 nucleotides in length.

5. The miRNA expression construct of claim 1, wherein the spacer is heterologous with respect to the promoter element.

6. The miRNA expression construct of claim 1, wherein the spacer comprises an encoded open reading frame.

7. The miRNA expression construct of claim 1, wherein the spacer is at least 375 nucleotides in length.

8. The miRNA expression construct of claim 1, wherein the promoter is a eukaryotic promoter.

9. The miRNA expression construct of claim 8, wherein the eukaryotic promoter is a Pol II or Pol III promoter.

10. The miRNA expression construct of claim 9, wherein the eukaryotic promoter is a Pol II promoter.

11. The miRNA expression construct of claim 1, wherein the promoter is an inducible, tissue-specific-or cell lineage-specific promoter.

12. The miRNA expression construct of any of claims 1-11, wherein the promoter element is selected from the promoter elements of Table 1.

13. The miRNA expression construct of claim 12, wherein the promoter element is at least 80% identical the EF1α promoter.

14. The miRNA expression construct of claim 13, wherein the EF1α promoter is a splice variant of the EF1α promoter.

15. The miRNA expression construct of claim 14, wherein the splice variant of the EF1α promoter is EF1s.

16. The miRNA expression construct of claim 15, wherein the EF1s promoter has a sequence at least 80%, 85%, 90% or 95% identical to SEQ ID NO: 45.

17. The miRNA expression construct of any of claims 1-16, wherein the spacer is selected from the spacers in Table 9.

18. The miRNA expression construct of claim 17, wherein the spacer is at least 80% identical to SEQ ID NO: 46.

19. The miRNA expression construct of claim 18, wherein the sequence of the spacer is SEQ ID NO: 46.

20. The miRNA expression construct of claim 17, wherein the spacer is at least 80% identical to SEQ ID NO: 47.

21. The miRNA expression construct of claim 20, wherein the sequence of the spacer is SEQ ID NO: 47.

22. The miRNA expression construct of claim 17, wherein the spacer is at least 80% identical to SEQ ID NO: 48.

23. The miRNA expression construct of claim 22, wherein the sequence of the spacer is SEQ ID NO: 48.

24. The miRNA expression construct of claim 17, wherein the spacer is at least 80% identical to SEQ ID NO: 49.

25. The miRNA expression construct of claim 24, wherein the sequence of the spacer is SEQ ID NO: 49.

26. The miRNA expression construct of any of claims 1-25, wherein the miRNA hairpin comprises from 5' to 3' and in the order from (a)-(g):

(a) a mir-16 flanking sequence comprising the sequence of SEQ ID NO: 25;

(b) a first lower stem sequence comprising the mir-16 sequence of SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29 or SEQ ID NO: 30;

(c) an anti-sense target sequence 22 nucleotides in length;

(d) a mir-30 loop sequence comprising the sequence of SEQ ID NO: 31;

(e) a sense sequence wherein the sequence is complementary to the sequence of (c) except that the sequence comprises one or two mismatches relative to the sequence of (c), wherein the one or two mismatches comprise:

i) a mismatch located at the position 8 to 14 of the sense sequence; or

ii) a mismatch at the final 3' position (position 22) of the sense sequence;

(f) a second lower stem sequence wherein the sequence is complementary to the sequence of (b); and

(g) a second flanking sequence.

27. The miRNA expression construct of claim 26, wherein the sense sequence (e) of the miRNA hairpin comprises one mismatch relative to sequence (c) located at nucleotide position 11 of the sense sequence (e).

28. The miRNA expression construct of claim 26, wherein the sense sequence (e) of the miRNA hairpin comprises two mismatches relative to sequence (c) located (i) at position 11 of the sense sequence (e) and (ii) at the last 3' nucleotide (position 22) of the sense sequence (e).

29. The miRNA expression construct of any of claims 26-28, wherein flanking sequence (g) is not complementary to the mir-16 flanking sequence (a).

30. The miRNA expression construct of any of claims 1-29, wherein the miRNA hairpin sequence is selected from the sequences listed in Table 6.

31. The miRNA expression construct of any one of claims 1-30, wherein the nucleic acid molecule is RNA.

32. The miRNA expression construct of any one of claims 1-30, wherein the nucleic acid molecule is DNA.

33. The miRNA expression construct of any one of claims 1-32, wherein the nucleic acid molecule comprises at least 2 repeats of the miRNA hairpin.

34. The miRNA expression construct of claim 33, wherein the at least 2 repeats are separated by an intervening sequence.

35. The miRNA expression construct of any one of claims 26-34, wherein the anti-sense target sequence is complementary to a CCR5 mRNA sequence.

36. An expression vector comprising the miRNA expression construct of any of claims 1-35.

37. The expression vector of claim 36, comprising 2 or more copies the nucleic acid sequence of claim 1.

38. The expression vector of claim 37, wherein the 2 or more copies of the nucleic acid sequence of claim 1 form a polycistronic transcript coding sequence.

39. The expression vector of claim 36, wherein the expression vector is an adenovirus, adeno-associated virus, retrovirus or lentivirus vector.

40. The expression vector of claim 36, further comprising at least one drug resistance marker.

41. A host cell comprising the nucleic acid molecule of any one of claims 1-35 or the expression vector of any one of claims 36-40.

42. The host cell of claim 41, wherein the host cell in an immune effector cell.

43. The host cell of claim 41, wherein the host cell in a T-cell or a CAR T-cell.

44. A method for reducing expression of a gene in a cell comprising expressing a nucleic acid molecule of any one of claims 26-35 in the cell wherein the anti-sense target sequence (c) is complementary to the sense strand of the gene.

45. The method of claim 44, wherein expressing the nucleic acid molecule in the cell comprises transfecting the cell with a nucleic acid.

46. The method of claim 44, wherein expressing the nucleic acid molecule in the cell comprises inserting the nucleic acid molecule into the genome of the cell by a TALE nuclease, Zinc Finger Nuclease, or CRISPR/Cas9.

47. The method of claim 44, wherein expressing the nucleic acid molecule in the cell comprises expressing the nucleic acid molecule from an expression vector.

48. The method of claim 47, wherein the expression vector is an adenovirus, adeno-associated virus, retrovirus or lentivirus vector.

49. The method of claim 44, wherein the gene is CCR5.

50. The method of claim 44, wherein the cell is a human cell.

51. The method of claim 44, further defined as an *in vivo* method.

52. The method of claim 44, further defined as an *in vitro* or *ex vivo* method.

53. The method of claim 52, further comprising transplanting the cell expressing the nucleic acid molecule into an organism.

54. The method of claim 44, wherein the cell is comprised in an organism.

55. A recombinant nucleic acid molecule comprising a promoter element, a spacer at least 50 nucleotides in length, and at least one miRNA hairpin, the miRNA hairpin from 5' to 3' comprising in the order from (a)-(g):

(a) a mir-16 flanking sequence;
(b) a first lower stem sequence comprising a mir-16 sequence;
(c) an anti-sense target sequence 22 nucleotides in length;
(d) a mir-30 loop sequence;
(e) a sense sequence wherein the sequence is complementary to the sequence of (c) except that the sequence comprises one or two mismatches relative to the sequence of (c), wherein the one or two mismatches comprise:
    i) a mismatch located at the position 8 to 14 of the sense sequence; or
    ii) a mismatch at the final 3' position (position 22) of the sense sequence;
(f) a second lower stem sequence wherein the sequence is complementary to the sequence of (b), wherein the lower stem is at least 11 nucleotides in length; and

(g) a second flanking sequence.

56. The nucleic acid molecule of claim 55, wherein the lower stem is 11, 12, 13, 14, 15, 16 or 17 nucleotides in length.

57. The nucleic acid molecule of claim 55, wherein the first lower stem (b) comprises the mir-16 sequence of SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29 or SEQ ID NO: 30.

58. The nucleic acid molecule of claim 55, wherein the mir-16 flanking sequence (a) comprises the sequence of SEQ ID NO: 25.

59. The nucleic acid molecule of claim 55, wherein the mir-30 loop sequence comprises the sequence of SEQ ID NO: 31.

60. The nucleic acid molecule of claim 55, wherein the sense sequence (e) comprises one mismatch relative to sequence (c) located at nucleotide position 11 of the sense sequence (e).

61. The nucleic acid molecule of claim 55, wherein the sense sequence (e) comprises two mismatches relative to sequence (c) located (i) at position 11 of the sense sequence (e) and (ii) at the last 3' nucleotide (position 22) of the sense sequence (e).

62. The nucleic acid molecule of any one of claims 55-61, comprising a flanking sequence (g), wherein the flanking sequence is not complementary to the mir-16 flanking sequence (a).

63. The nucleic acid molecule of any one of claims 55-62, wherein the miRNA hairpin sequence is selected from the sequences listed in Table 6 or Table 10.

64. The nucleic acid molecule of any one of claims 55-63, wherein the nucleic acid molecule is RNA.

65. The nucleic acid molecule of any one of claims 55-63, wherein the nucleic acid molecule is DNA.

66. The nucleic acid molecule of any one of claims 55-65, wherein the nucleic acid molecule comprises at least 2 repeats of the sequences (a)-(g).

67. The nucleic acid molecule of claim 66, wherein the at least 2 repeats are separated by an intervening sequence.

68. The nucleic acid molecule of any one of claims 55-67, wherein the anti-sense target sequence is complementary to a CCR5 mRNA sequence.

69. An expression vector comprising a nucleic acid of any one of claim 55-68.

70. The expression vector of claim 69, wherein the promoter is a eukaryotic promoter.

71. The expression vector of claim 70, wherein the eukaryotic promoter is a Pol II or Pol III promoter.

72. The expression vector of claim 71, wherein the eukaryotic promoter is a Pol II promoter.

73. The expression vector of claim 69, wherein the promoter is an inducible, tissue-specific-or cell lineage-specific promoter.

74. The expression vector of claim 69, comprising 2 or more copies the nucleic acid sequence of claim 55.

75. The expression vector of claim 74, wherein the 2 or more copies of the nucleic acid sequence of claim 55 form a polycistronic transcript coding sequence.

76. The expression vector of claim 69, wherein the expression vector is an adenovirus, adeno-associated virus, retrovirus or lentivirus vector.

77. The expression vector of claim 69, further comprising at least one drug resistance marker.

78. A host cell comprising the nucleic acid molecule of any one of claims 55-68 or the expression vector of any one of claims 69-77.

79. A method for reducing expression of a gene in a cell comprising expressing a nucleic acid molecule of any one of claims 55-68 in the cell wherein the anti-sense target sequence (c) is complementary to the sense strand of the gene.

80. The method of claim 79, wherein expressing the nucleic acid molecule in the cell comprises incorporating the nucleic acid molecule into the genome of the cell using a TALE nuclease, zinc finger nuclease, or CRISPR/Cas9.

81. The method of claim 79, wherein expressing the nucleic acid molecule in the cell comprises transfecting the cell with a nucleic acid.

82. The method of claim 79, wherein expressing the nucleic acid molecule in the cell comprises expressing the nucleic acid molecule from an expression vector.

83. The method of claim 82, wherein the expression vector is an adenovirus, adeno-associated virus, retrovirus or lentivirus vector.

84. The method of claim 79, wherein the gene is CCR5.

85. The method of claim 79, wherein the cell is a human cell.

86. The method of claim 79, further defined as an *in vivo* method.

87. The method of claim 79, further defined as an *in vitro* or *ex vivo* method.

88. The method of claim 87, further comprising transplanting the cell expressing the nucleic acid molecule into an organism.

89. The method of claim 79, wherein the cell is comprised in an organism.

90. A miRNA expression construct comprising a promoter sequence and at least two miRNA hairpins wherein said at least two miRNA hairpins are targeted to transcripts of immune checkpoint genes.

91. The miRNA expression construct of claim 90, wherein the at least two miRNA hairpins are identical.

92. The miRNA expression construct of claim 90, wherein the at least two miRNA hairpins target different sequences.

93. The miRNA expression construct of claim 92, wherein the at least two miRNA hairpins target different sequences of transcripts of the same gene.

94. The miRNA expression construct of claim 92, wherein the miRNA hairpins are targeted to different transcripts.

95. The miRNA expression construct of claim 94, wherein the miRNA hairpins are targeted to transcripts of different genes.

96. The miRNA expression construct of claim 90, comprising at least three miRNA hairpins.

97. The miRNA expression construct of claim 96, wherein the at least three miRNA hairpins are each different.

98. The miRNA expression construct of claim 96, wherein at least two of the at least three miRNA hairpins are different.

99. The miRNA expression construct of claim 97, wherein said at least three different miRNA hairpins are targeted to transcripts of two or three different immune checkpoint genes.

100. The miRNA expression construct of claim 90, comprising at least 4, 5, 6, 7, 8, 9 or 10 miRNA hairpins.

101. The miRNA expression construct of claim 100, wherein at least two of the at least 4, 5, 6, 7, 8, 9 or 10 miRNA hairpins are different.

102. The miRNA expression construct of claim 100, wherein the at least 4, 5, 6, 7, 8, 9 or 10 miRNA hairpins are each different.

103. The miRNA expression construct of claim 102, wherein said at least 4, 5, 6, 7, 8, 9 or 10 different miRNA hairpins are targeted to transcripts of 2, 3, 4, or 5 different immune checkpoint genes.

104. The miRNA expression construct of claim 90, further comprising a spacer sequence position between the promoter and the at least two miRNA hairpins.

105. The miRNA expression construct of claim 90, wherein the spacer is between 50 and 1,000 nucleotides in length.

106. The miRNA expression construct of claim 90, wherein the at least two miRNA hairpins are targeted to transcripts of at least one immune checkpoint gene selected from the group consisting of: PD1, CTLA4, LAG3, TIM3, TIGIT, CD96, BTLA, KIRs, adenosine A2a receptor, ARG2 (arginase 2), Vista, IDO, FAS, SIRP alpha, CISH, SHP-1, FOXP3, LAIR1, PVRIG, PPP2CA, PPP2CB, PTPN6, PTPN22, CD160, CRTAM, SIGLEC7, SIGLEC9, CD244, TNFRSF10B, TNFRSF10A, CASP8, CASP10, CASP3, CASP6, CASP7, FADD, TGFBRII, TGFRBRI, SMAD2, SMAD3, SMAD4, SMAD10, SKI, SKIL, TGIF1, IL10RA, IL10RB, HMOX2, IL6R, IL6ST, EIF2AK4, CSK, PAG1, SIT1, PRDM1, BATF, GUCY1A2, GUCY1A3, GUCY1B2, and GUCY1B3.

107. The miRNA expression construct of any of claims 90-106, wherein the promoter sequence is at least 80% identical to the EF1s promoter sequence.

108. The miRNA expression construct of any of claims 90-106, wherein the promoter sequence is at least 80% identical to the UBI promoter sequence.

109. The miRNA expression construct of any of claims 90-108, wherein the miRNA expression construct further comprises a chimeric antigen receptor sequence.

110. The miRNA expression construct of any of claims 90-108, wherein the miRNA expression construct further comprises a T cell receptor sequence.

111. The miRNA expression construct of any of claims 90-110, wherein the miRNA expression construct further comprises a selection gene.

112. The miRNA expression construct of claim 111, wherein the selection gene is LNGFR or a derivative thereof.

113. The miRNA expression construct of any of claims 90-110, wherein the miRNA expression construct further comprises a suicide gene.

114. The miRNA expression construct of claim 113, wherein the suicide gene is selected from the group consisting of herpes simplex virus thymidine kinase (HSV-tk), inducible caspase 9 (iCasp9), truncated endothelial growth factor receptor (tEGFR), RQR8, dihydrofolate reductase (DHFR), and thymidylate synthase (TYMS).

115. The miRNA expression construct of any of claims 90-113, wherein the miRNA expression construct further comprises a peptide cleavage site.

116. The miRNA expression construct of claim 115, wherein the peptide cleavage site is a 2A peptide.

117. The miRNA expression construct of claim 116, wherein the 2A peptide is selected from the group comprising: 2A, P2A, T2A, E2A, F2A, BmCPV 2A, and BmIFV 2A.

118. The miRNA expression construct of claim 117, wherein the 2A peptide is T2A.

119. A vector comprising the miRNA expression construct of any of claims 90-118.

120. The vector of claim 119, wherein the vector is a viral vector.

121. The vector of claim 120, wherein the viral vector is an adenovirus, adeno-associated virus, retrovirus or lentiviral vector.

122. A mammalian cell comprising the miRNA expression construct of any of claims 90-118 or the vector of any of claims 119-121.

123. The mammalian cell of claim 122, wherein the mammalian cell is an immune effector cell.

124. The mammalian cell of claim 122, wherein the cell is engineered for reduced CCR5 expression.

125. The immune effector cell of claim 123, wherein the immune effector cell is selected from the group comprising: T cells, TILS, TCR-engineered T cells, CAR T cells, NK cells, NK/T cells, T regulatory cells, monocytes and macrophages.

126. A method for preparing engineered immune effector cells comprising transfecting or transducing the immune effector cells with the miRNA expression construct of any of claims 90-118 or transducing the immune effector cells with the vector of any of claims 118-121.

127. A method for preparing engineered immune effector cells comprising transfecting or transducing a chimeric antigen receptor sequence or T cell receptor sequence

into immune effector cells and then transfecting or transducing the miRNA expression construct of any of claims 90-118 or the vector of any of claims 119-121 into the cells.

128. A method for preparing engineered immune effector cells comprising incorporating the miRNA expression construct of any of claims 90-118 into the genome of the cell by a TALE nuclease, megaTAL, zinc finger nuclease, or CRISPR/Cas9.

129. A method for preparing engineered immune effector cells from a patient comprising:

(a) collecting immune effector cells from the patient; and

(b) transfecting the immune effector cells with the miRNA expression construct of any of claims 90-118 or transducing the immune effector cells with the vector of any of claims 119-121 to generate engineered immune effector cells.

130. A method for preparing engineered immune effector cells from a patient comprising:

(a) collecting immune effector cells from the patient;

(b) transducing or transfecting the immune effector cells with a chimeric antigen receptor or T cell receptor to generate a modified immune effector cells; and

(c) transducing or transfecting the modified immune effector cells with the miRNA expression construct of any of claims 90-118 or the vector of any of claims 119-121 to generate engineered immune effector cells.

131. The method of claim 130, wherein the chimeric antigen receptor or T cell receptor is encoded by the miRNA expression construct or vector.

132. The method of any of claims 129-131, wherein transducing or transfecting the immune effector cells with the miRNA expression construct comprises incorporating the miRNA expression construct into the genome by a TALE nuclease, megaTAL, zinc finger nuclease, or CRISPR/Cas9.

133. A method of treating a patient in need thereof, comprising introducing the cells of any of claims 122-132 into the patient.

134. An immune effector cell comprising one or more miRNA expression constructs that target a CCR5 and an immune checkpoint inhibitor gene.

135. The cell of claim 134, wherein the checkpoint inhibitor gene is PD1, CTLA4, LAG3, TIM3, TIGIT, CD96, BTLA, KIRs, adenosine A2a receptor, ARG2 (arginase 2), Vista, IDO, FAS, SIRP alpha, CISH, SHP-1, FOXP3, LAIR1, PVRIG, PPP2CA, PPP2CB, PTPN6, PTPN22, CD160, CRTAM, SIGLEC7, SIGLEC9, CD244, TNFRSF10B, TNFRSF10A, CASP8, CASP10, CASP3, CASP6, CASP7, FADD, TGFBRII, TGFRBRI, SMAD2, SMAD3, SMAD4, SMAD10, SKI, SKIL, TGIF1, IL10RA, IL10RB, HMOX2, IL6R, IL6ST, EIF2AK4, CSK, PAG1, SIT1, PRDM1, BATF, GUCY1A2, GUCY1A3, GUCY1B2, or GUCY1B3.

136. The cell of claim 134, wherein the checkpoint inhibitor gene is PD1.

137. The cell of claim 134, wherein the PD1 target sequence is SEQ ID NO: 59.

138. The cell of claim 134, wherein the immune effector cell is selected from the group consisting of: T cells, TILS, TCR-engineered T cells, CAR T cells, NK cells, NK/T cells, T regulatory cells, monocytes and macrophages.

139. The cell of claim 134, wherein the immune effector cell is a T-cell.

140. The cell of claim 138, wherein the immune effector cell is a CAR T-cell.

141. The cell of claim 140, wherein the CAR targets HIV infected cells.

142. The cell of claim 134, comprising one or more miRNA expression constructs that target a CCR5 and at least two immune checkpoint inhibitor genes.

143. The cell of claim 134, comprising a miRNA expression construct of any of claims 90-118 or the vector of any of claims 119-121 into the cells.

144. The cell of claim 134, wherein the miRNA expression construct is in accordance with anyone of claims 1-40.

145. A expression construct comprising a miRNA sequence that target a CCR5 and an immune checkpoint inhibitor gene.

146. The construct of claim 145, wherein the miRNA sequence is in accordance with anyone of claims 1-40.

147. The construct of claim 145, further comprising a CAR expression sequence.

148. The construct of claim 147, wherein the CAR targets HIV infected cells.