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(54) **THERAPEUTIC DELIVERY AND EXPRESSION SYSTEM, METHODS AND USES THEREOF**

(52) **U.S. Cl.**
CPC *A61K 35/74* (2013.01); *A61K 48/0025* (2013.01); *A61K 39/145* (2013.01)
USPC *424/200.1*; *435/479*; *424/93.2*; *435/252.3*; *435/320.1*

(71) Applicant: **The University of Hong Kong**, Hong Kong (CN)

(72) Inventors: **Jian-Dong Huang**, Hong Kong (CN); **Lei Shi**, Hong Kong (CN); **Bin Yu**, Hong Kong (CN)

(73) Assignee: **The University of Hong Kong**, Hong Kong (CN)

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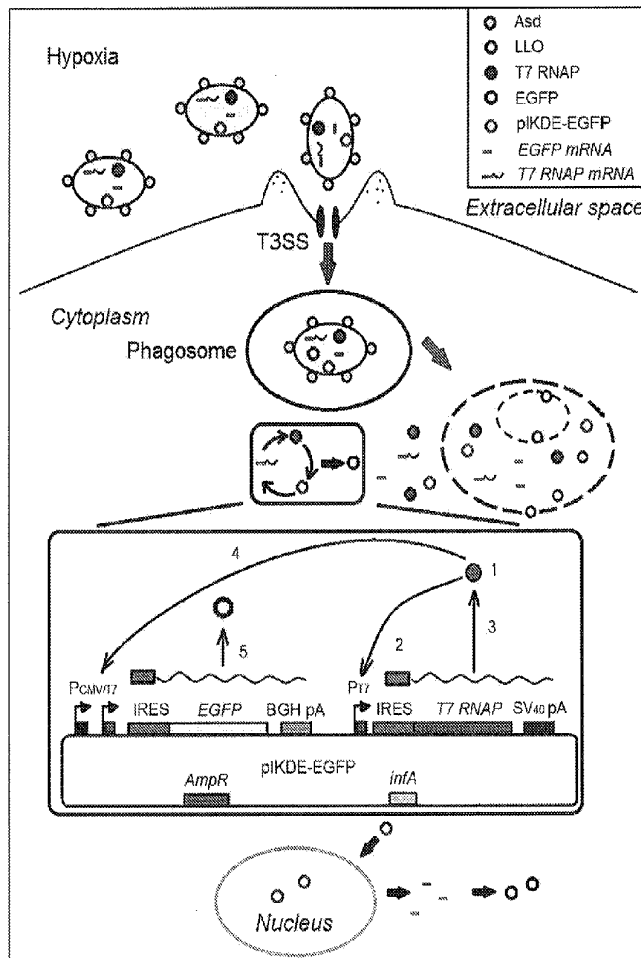
(22) Filed: **Sep. 5, 2013**

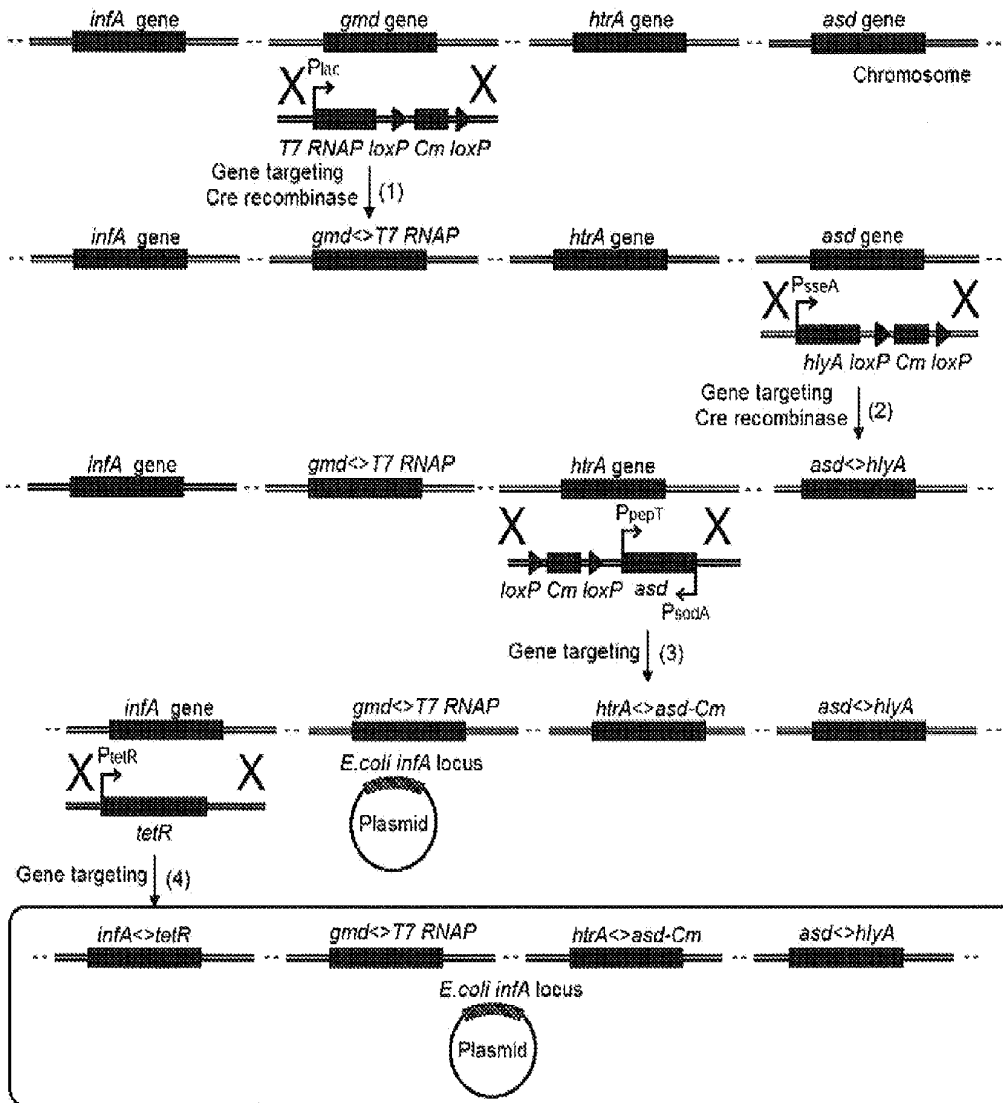
(57) **ABSTRACT**

Therapeutic methods for cancer treatments using a combined prokaryotic-eukaryotic delivery and expression system for the delivery of multiple therapeutic factors via a modified tumor-targeted bacteria. A targeted bacteria-vector system elicits an inter-kingdom dual expression (IKDE) of antitumor agents, in the nucleus or cytoplasm of eukaryotic cells, with priming and maintenance of the vector in the bacterium. The therapeutic factors include small interfering RNAs, tumoricidal proteins, DNA molecules, or a combination thereof. The system provides direct killing of tumor cells and alters the tumor microenvironment by expressing anti-angiogenic factors and cytokines in intracellular and/or extracellular environments. Also provided are methods of using natural exosomes comprising cargoes obtained from the bacterially infected cells. The bacteria-vector system is useful for many types of tumor and cancer as well as recombinant vaccines. The method causes significant regression of tumor and prolongs survival of tumor-bearing mice and subject without detectable systemic toxicity.

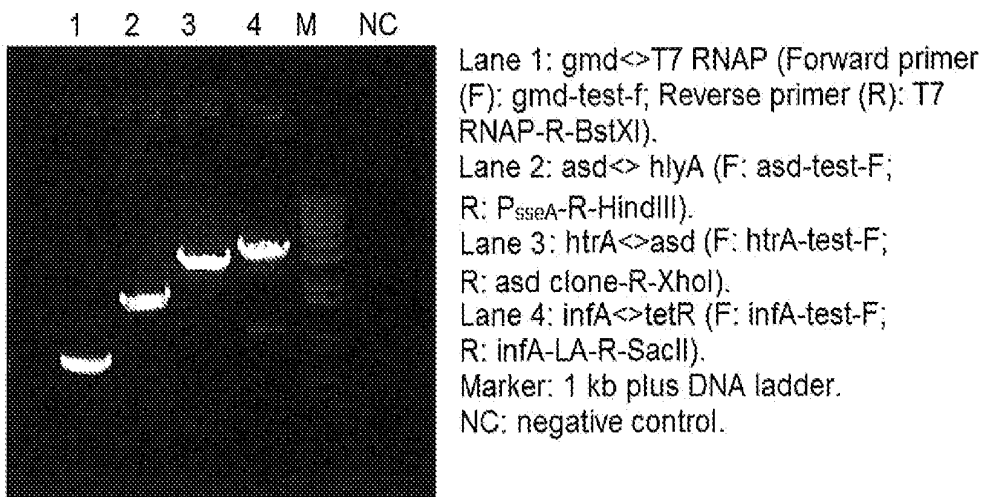
Publication Classification

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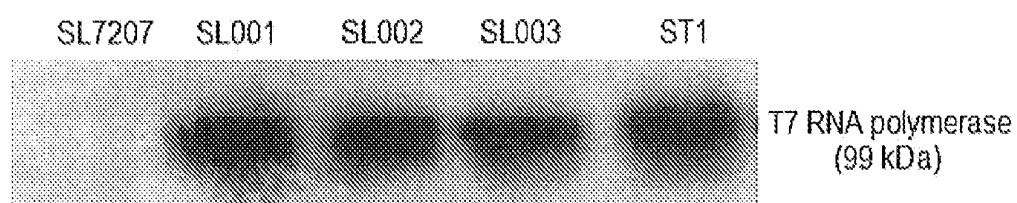




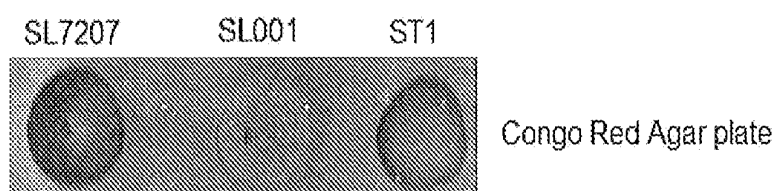
Figs. 1A



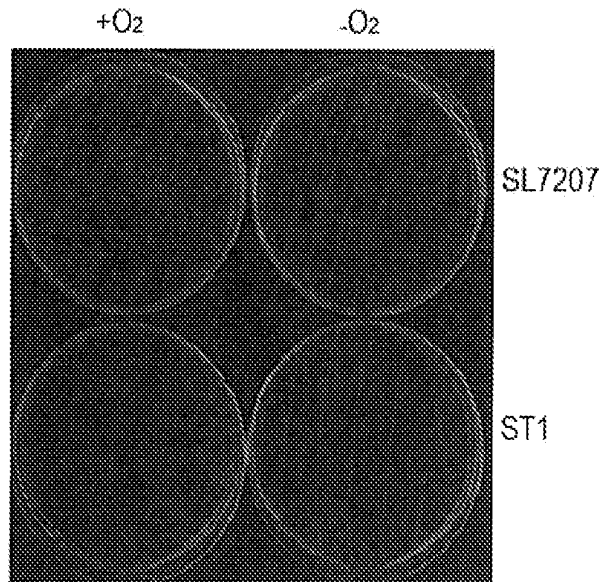
Figs. 1B



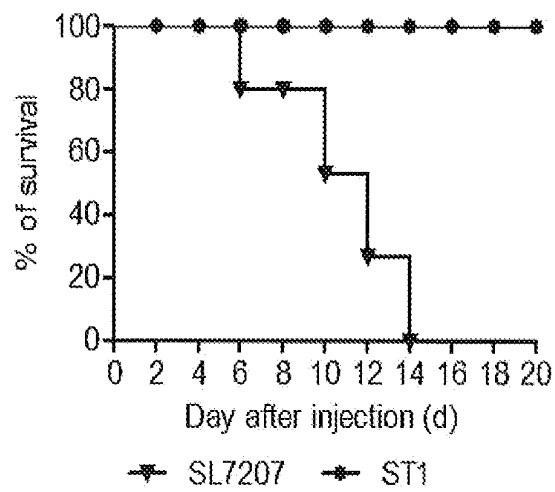
Figs. 2A



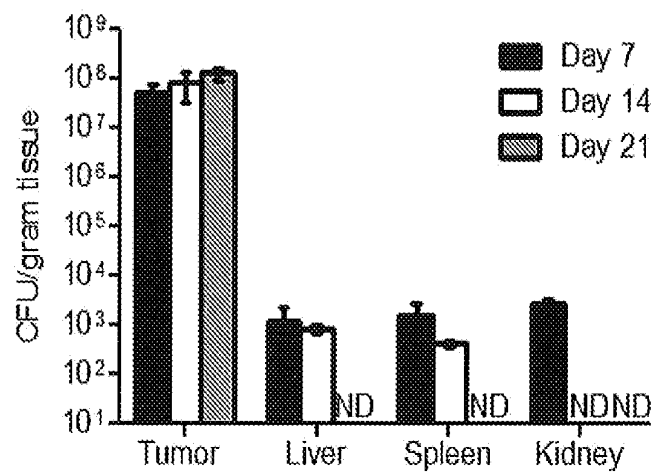
Figs. 2B



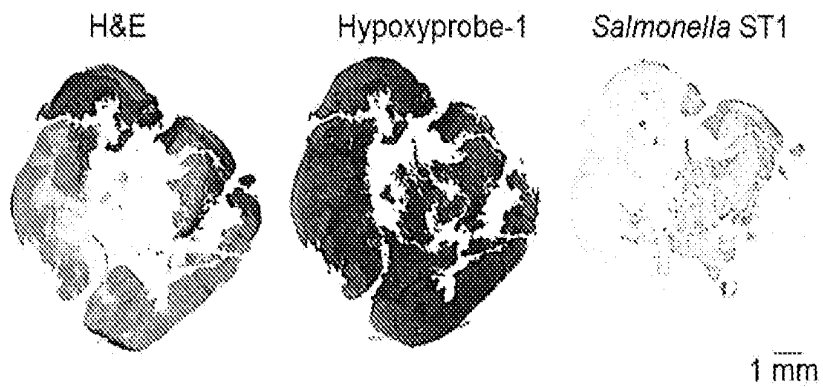
Figs. 3A



Figs. 3B



Figs. 4A



Figs. 4B

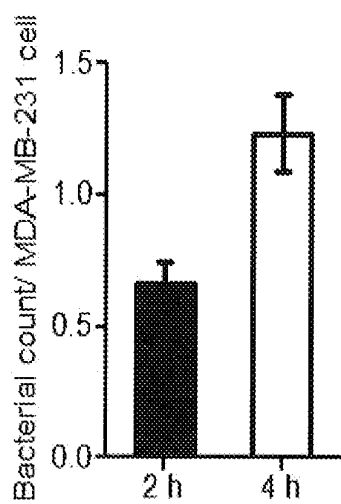


Fig. 5

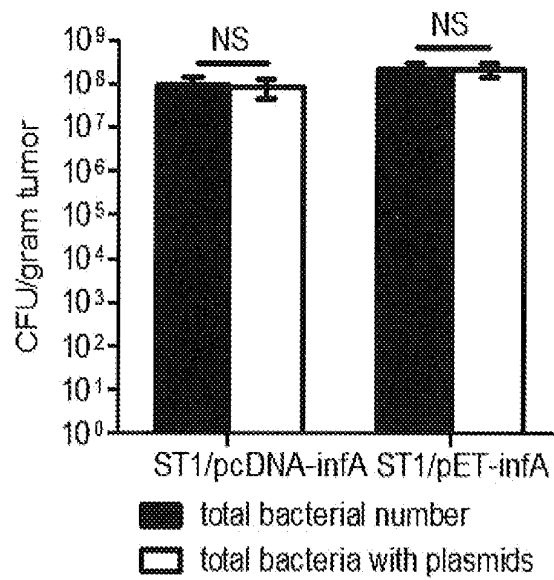


Fig. 7

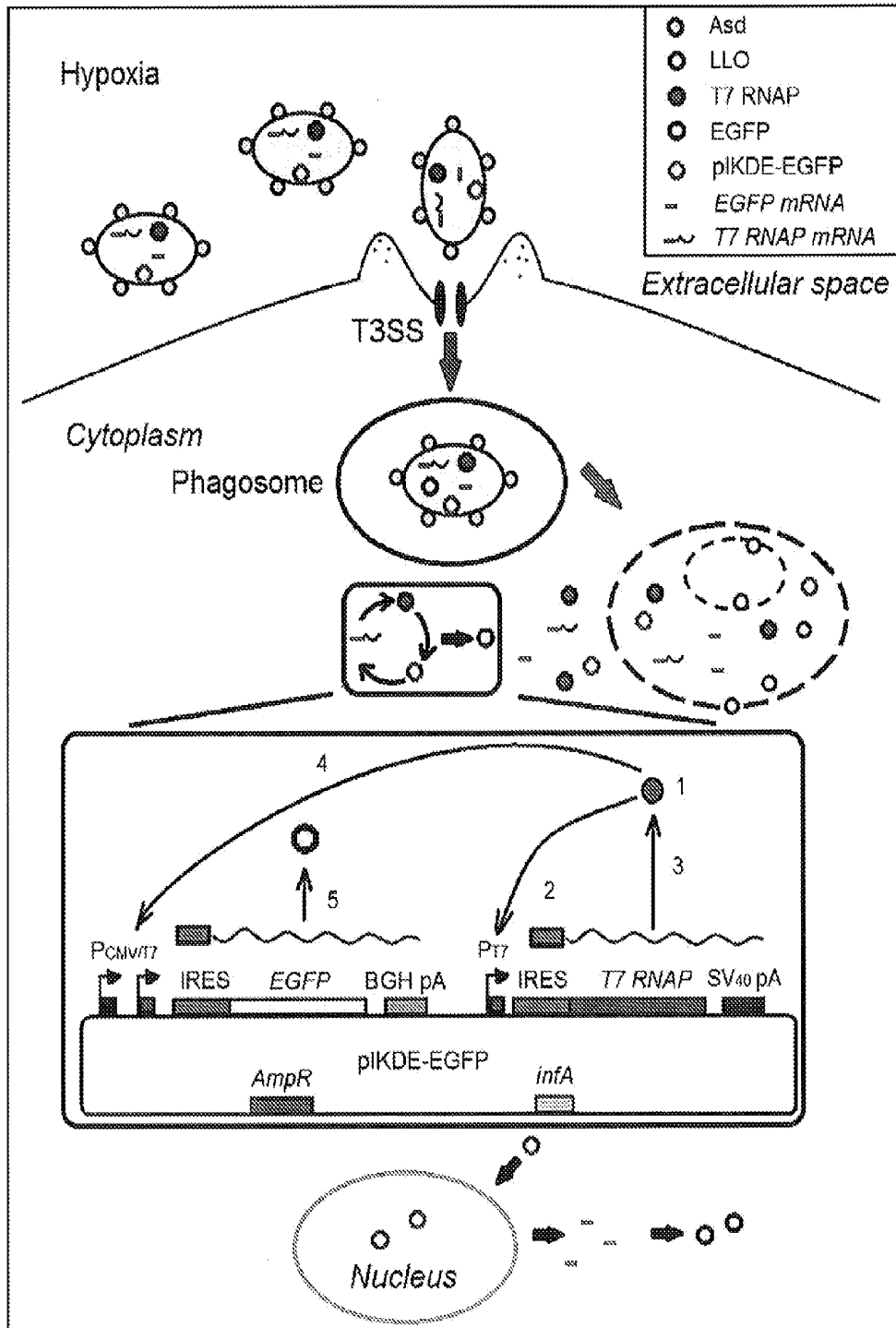


Fig. 8

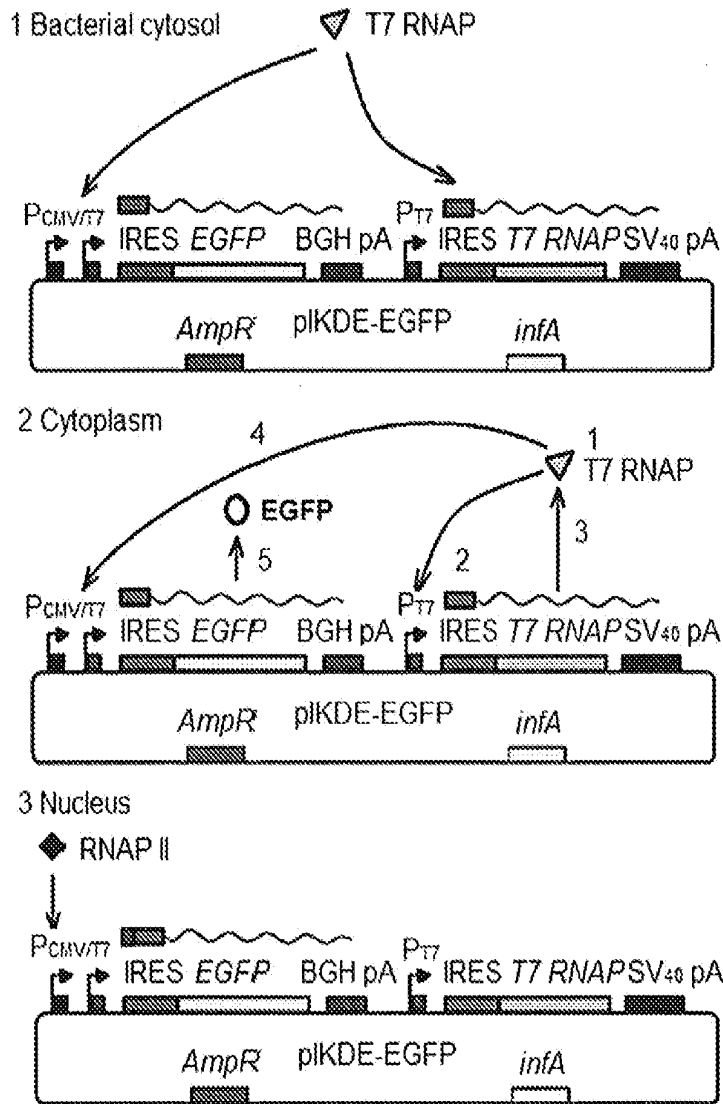


Fig. 9

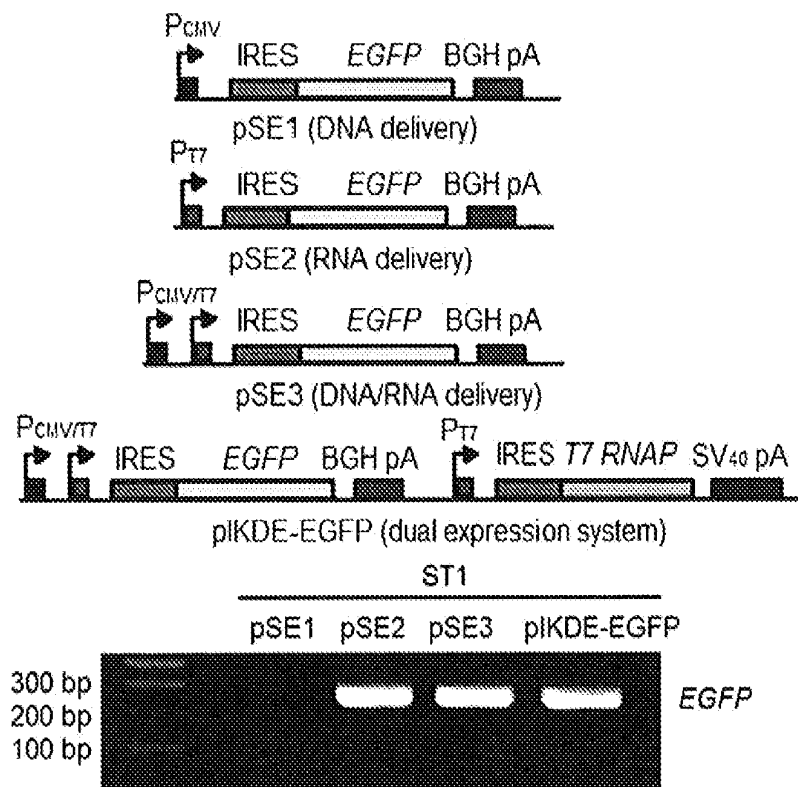


Fig. 10

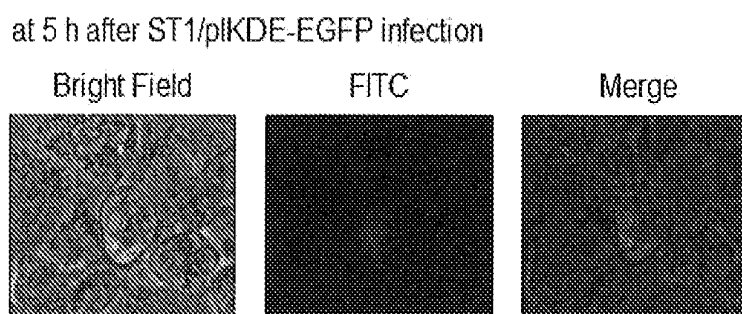


Fig. 11

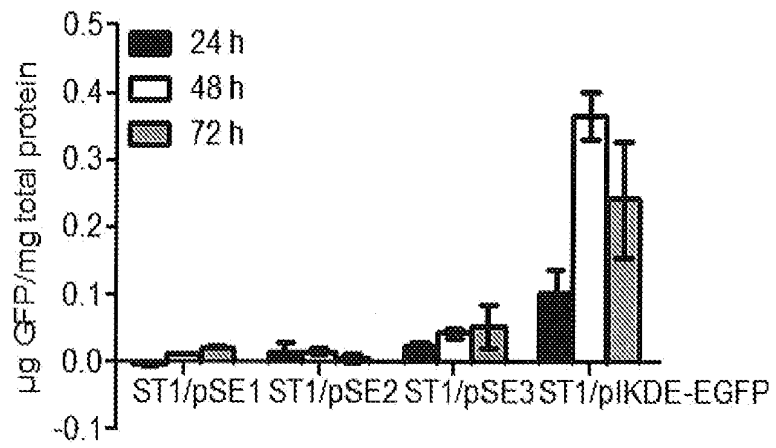


Fig. 12

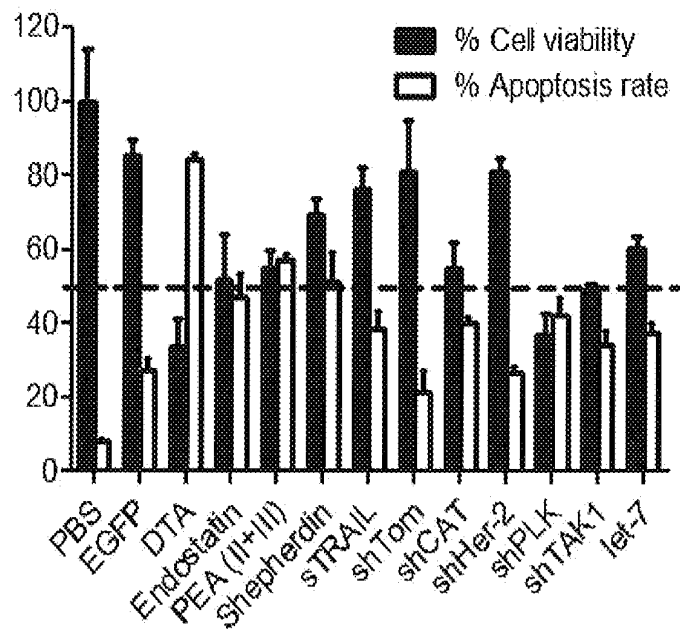


Fig. 13

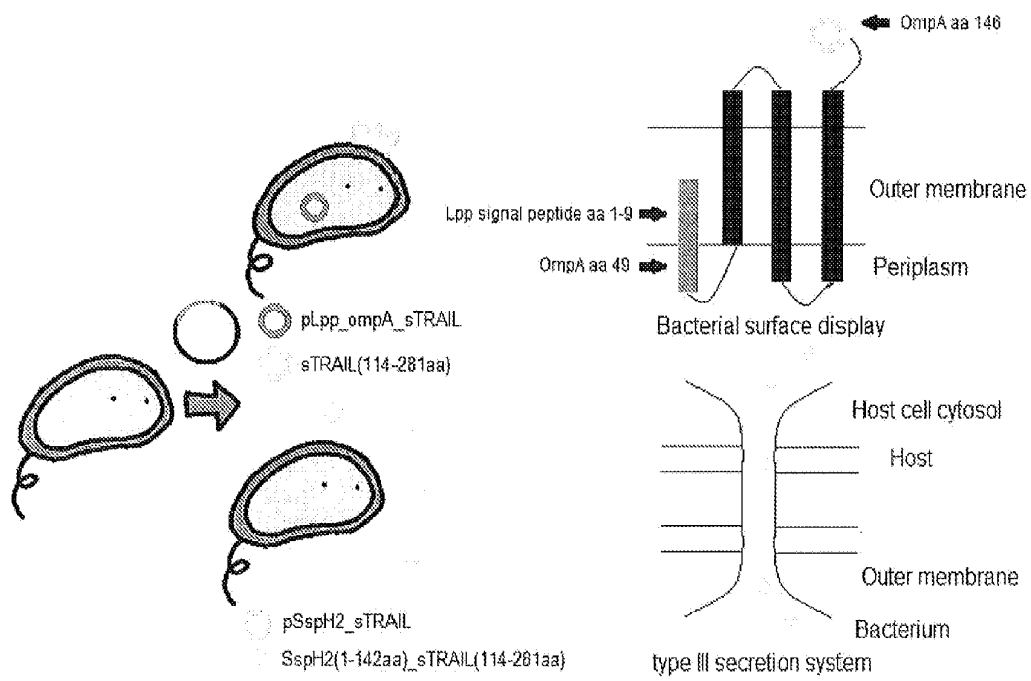


Fig. 14

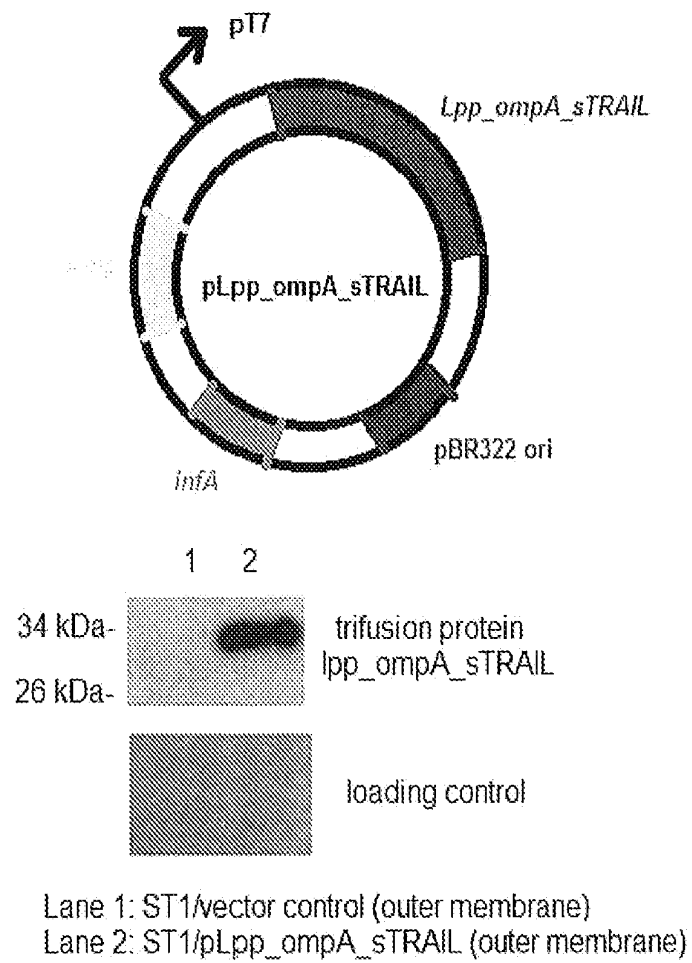


Fig. 15

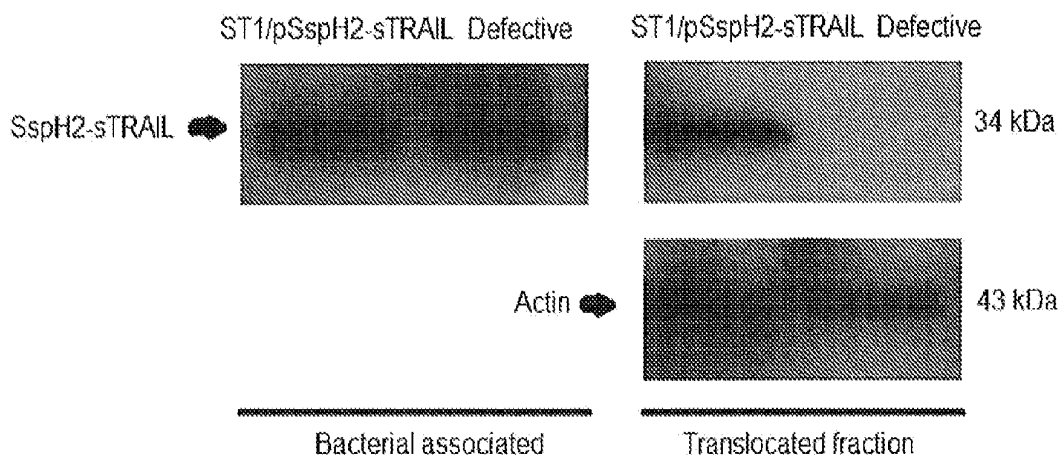


Fig. 16

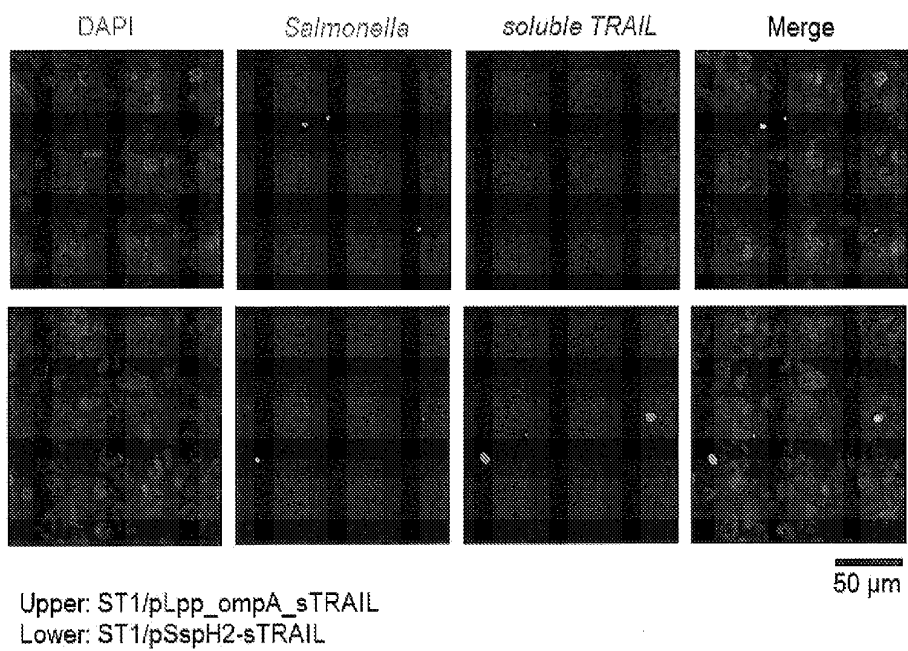
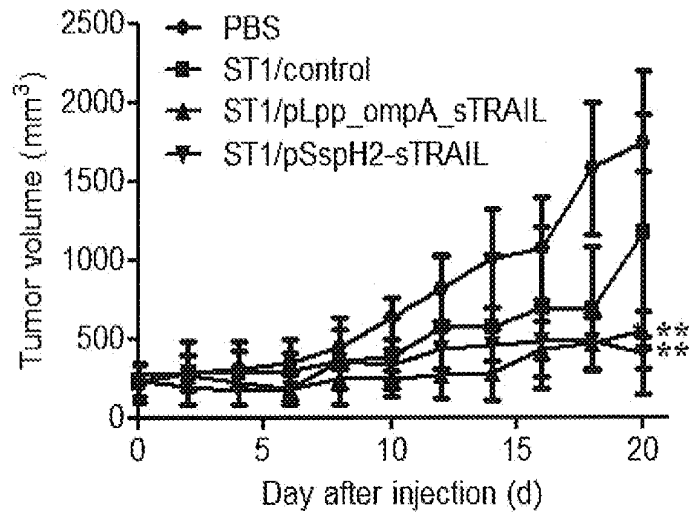
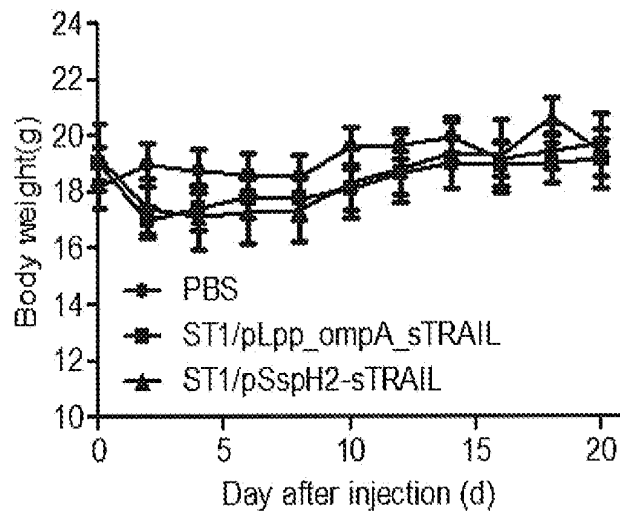


Fig. 17



Figs. 18A



Figs. 18B

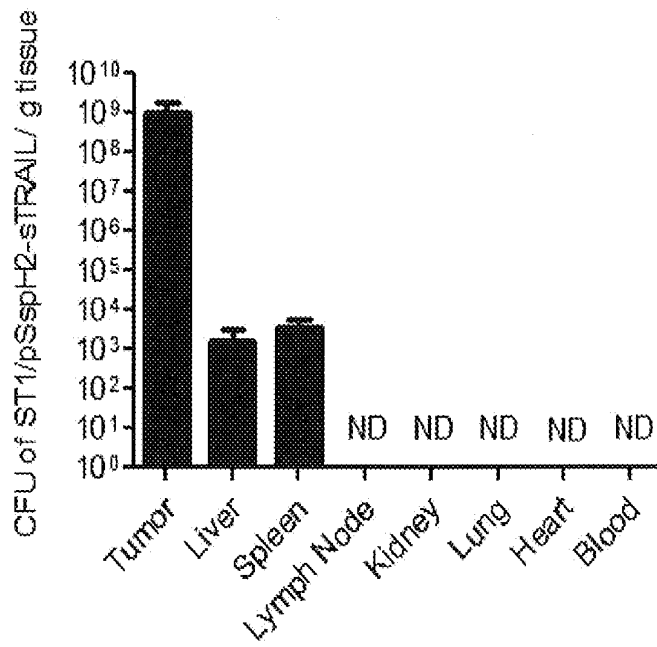


Fig. 19

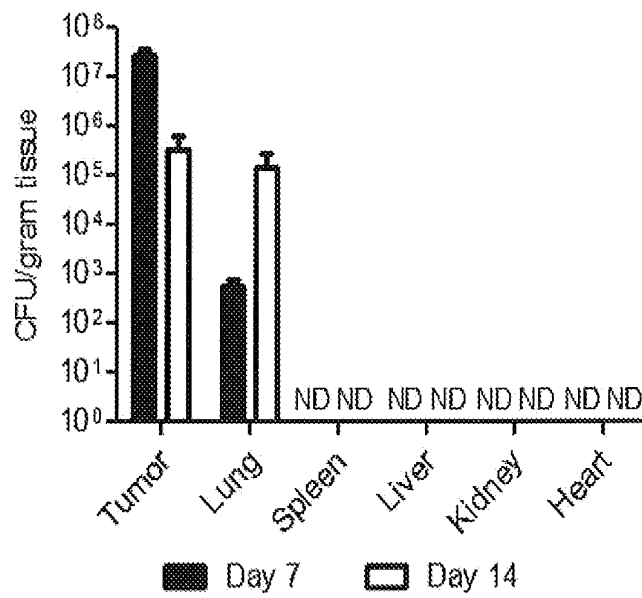
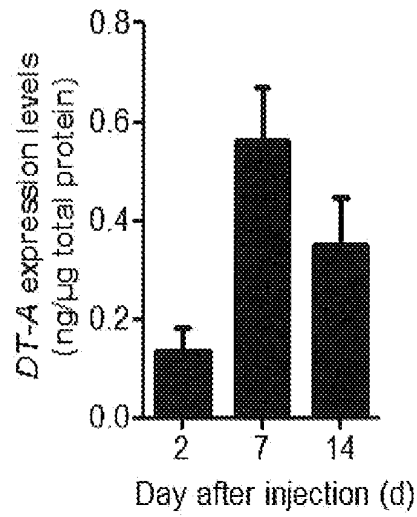
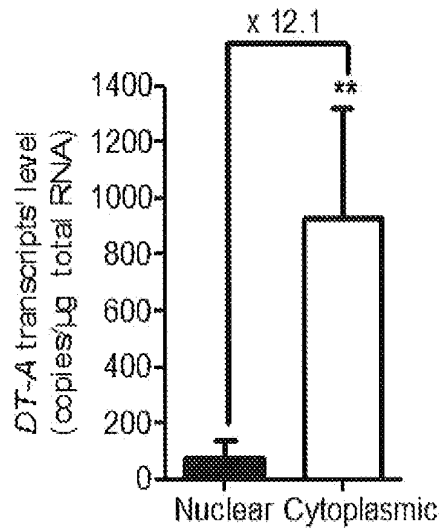


Fig. 20



Figs. 21A



Figs. 21B

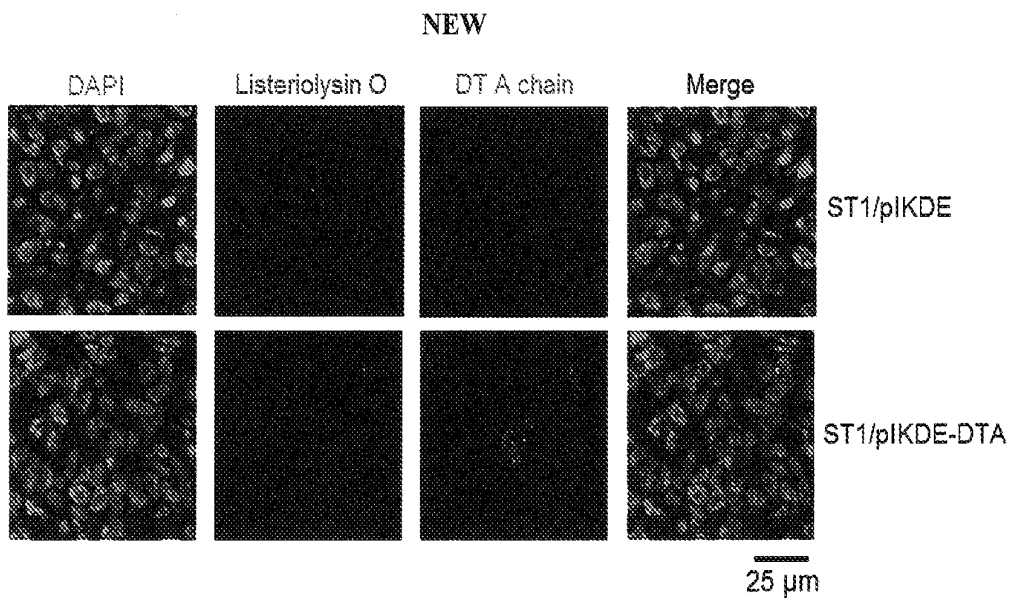
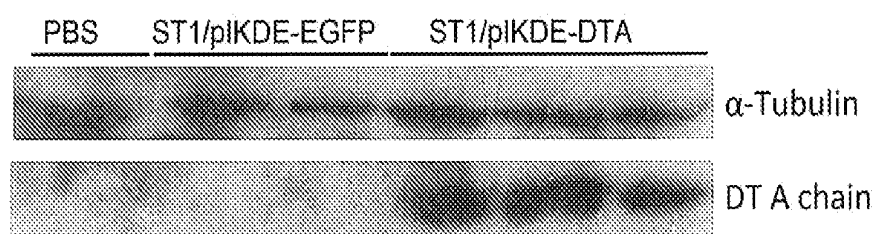
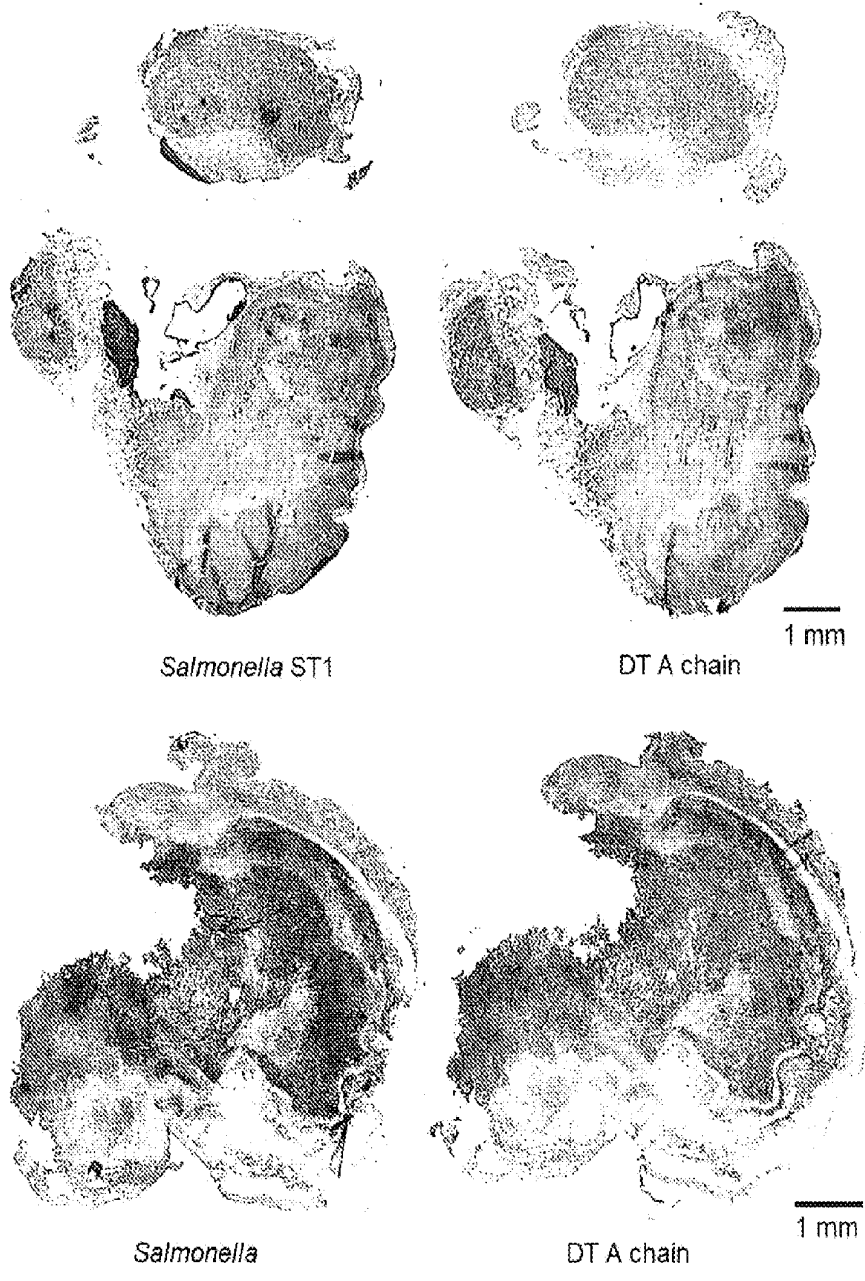
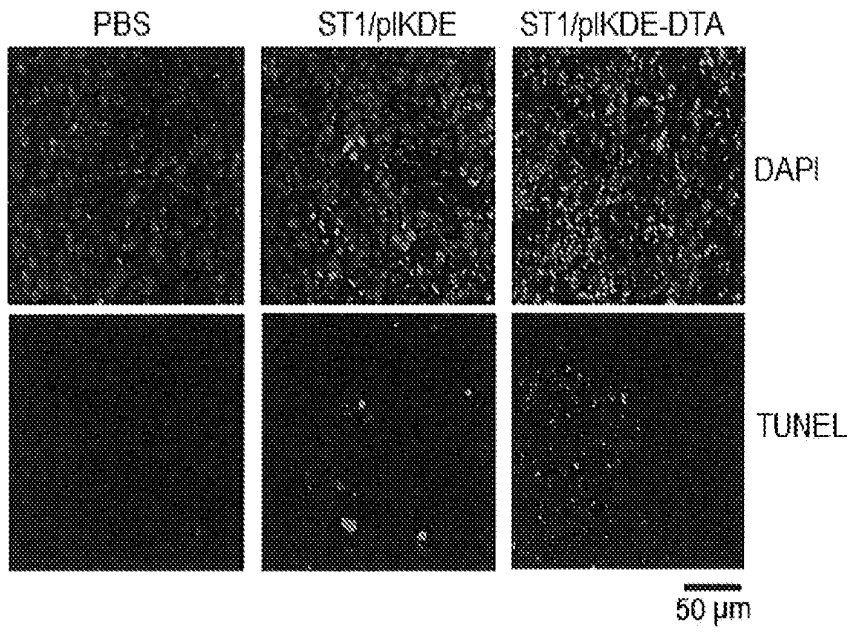


Fig. 22

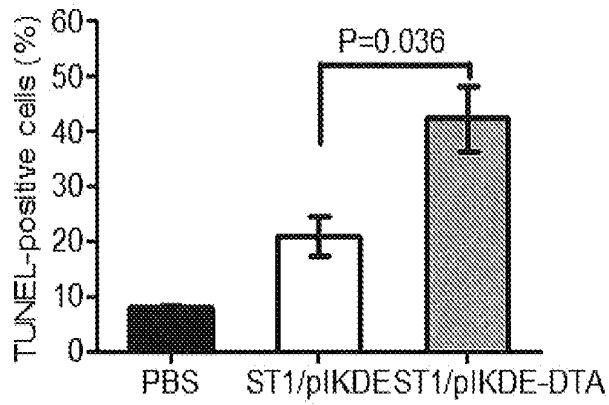
**Figs. 23A**



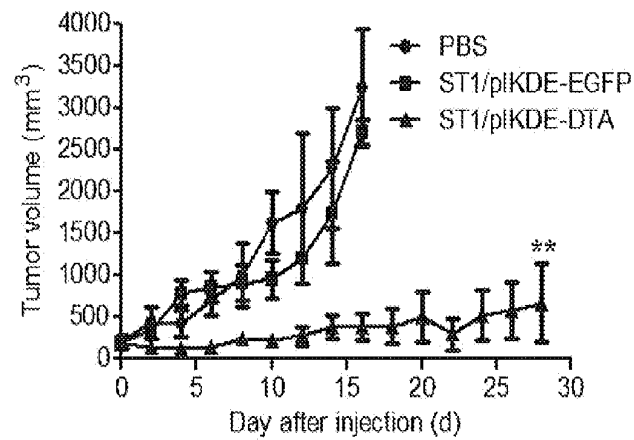
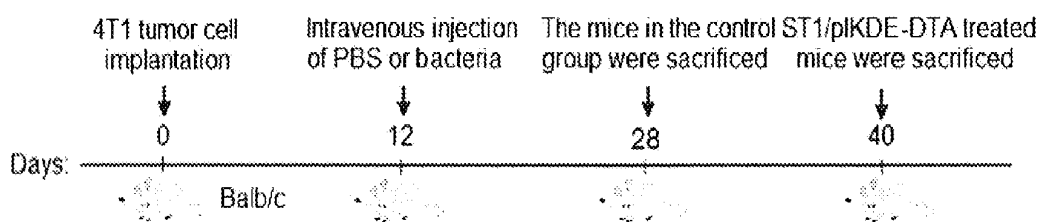
Figs. 23B



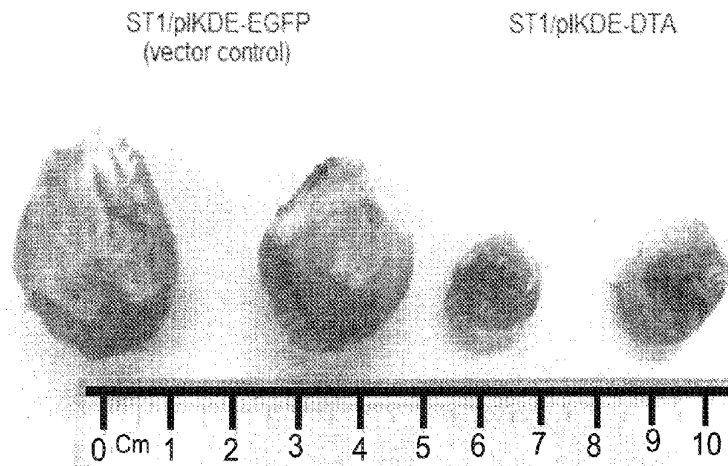
Figs. 24A



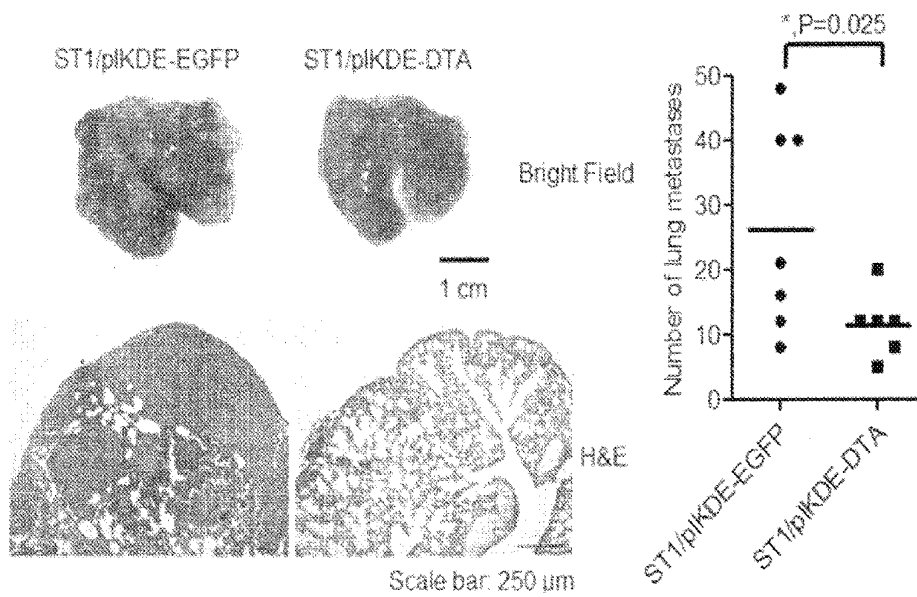
Figs. 24B



Figs. 25A



Figs. 25B



Figs. 25C

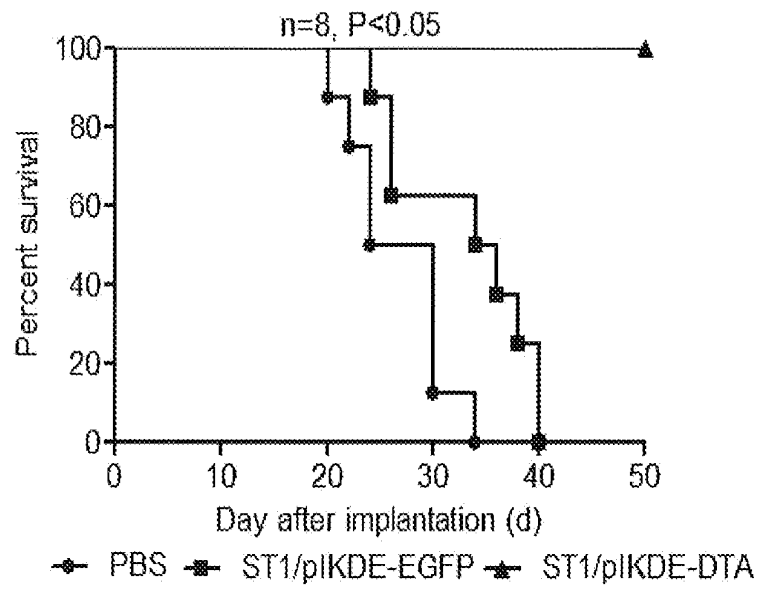
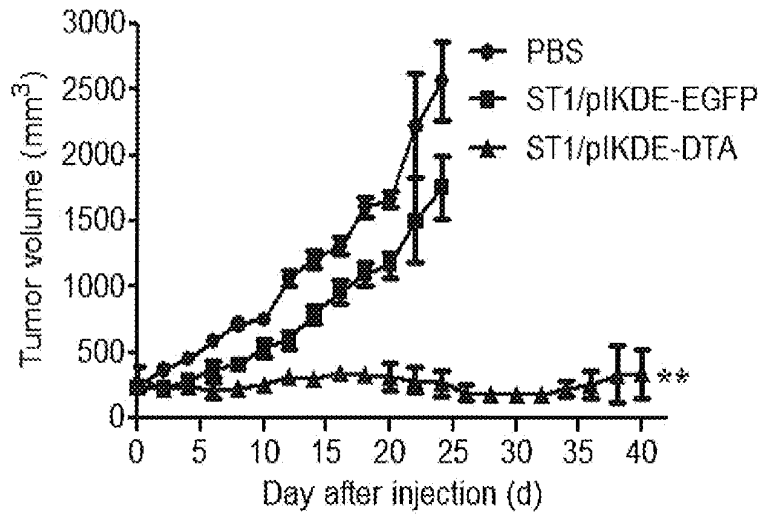
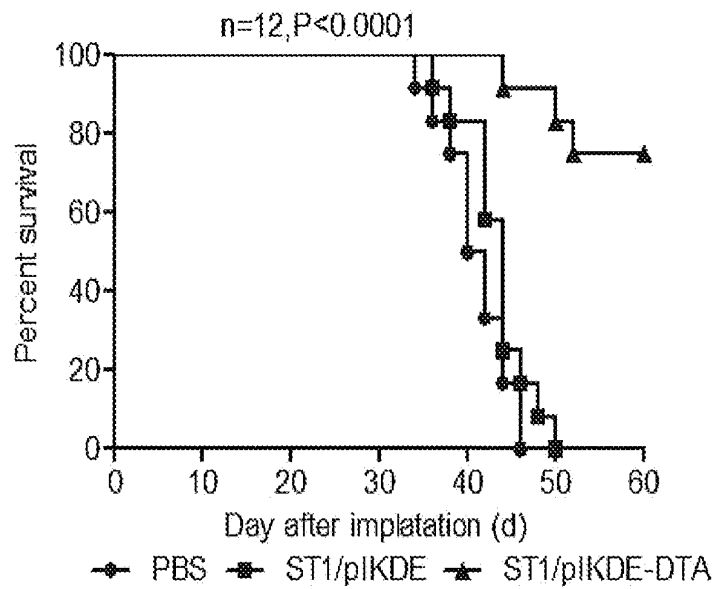


Fig. 26

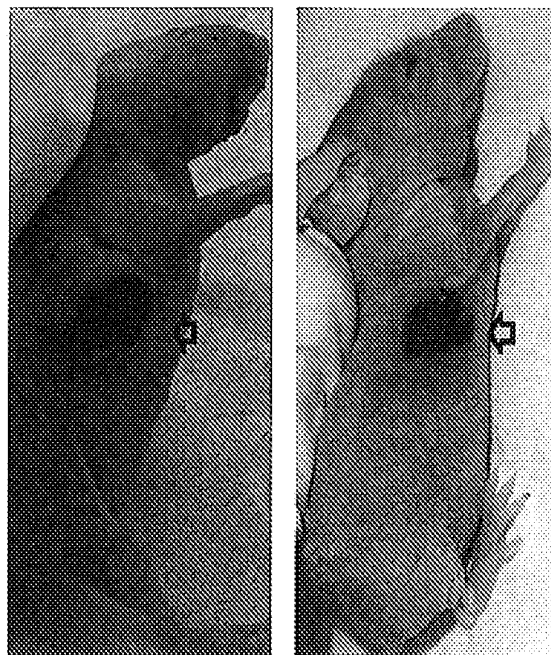


Figs. 27A



Figs. 27B

ST1/pIKDE-DTA treated



Figs. 27C

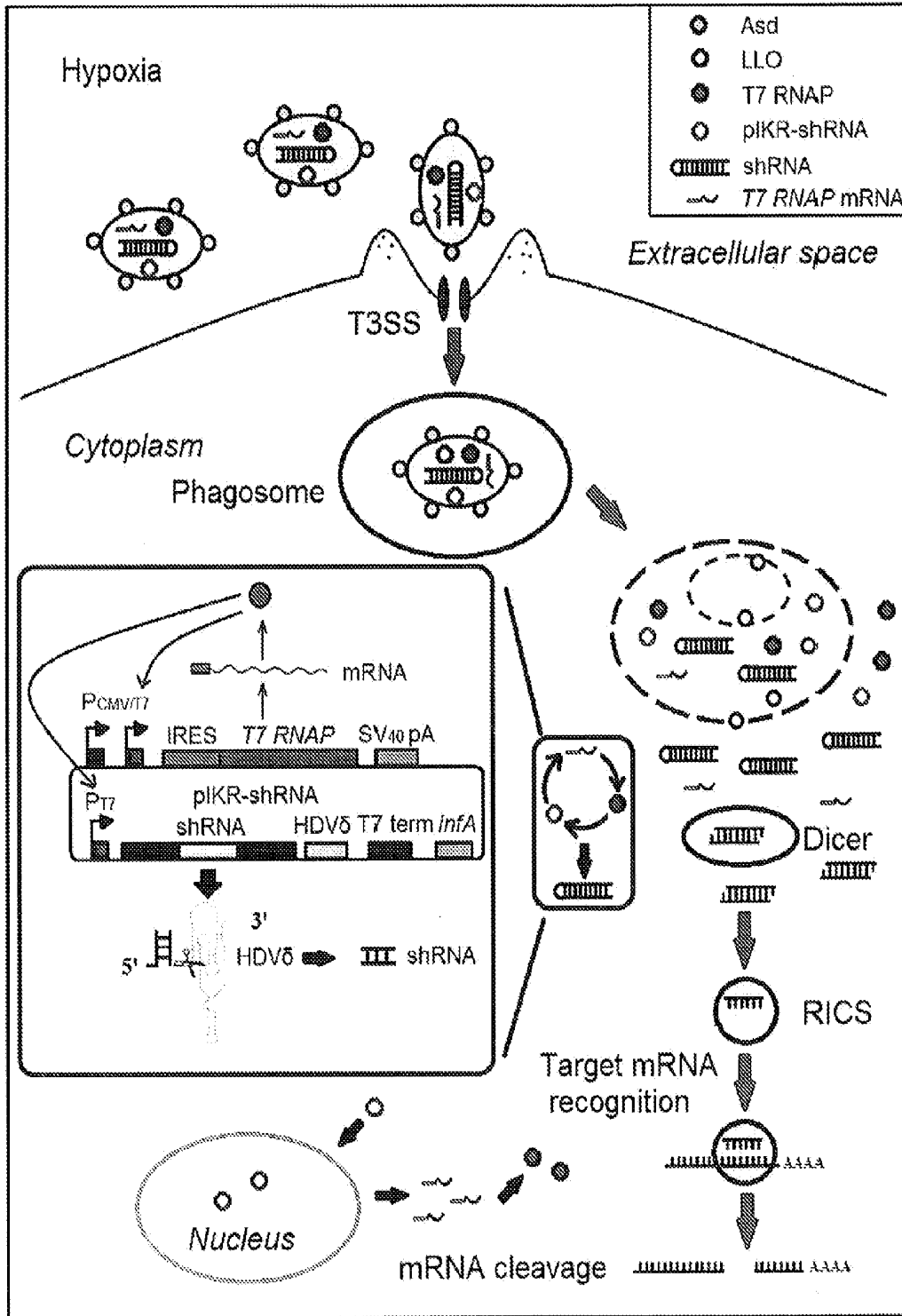
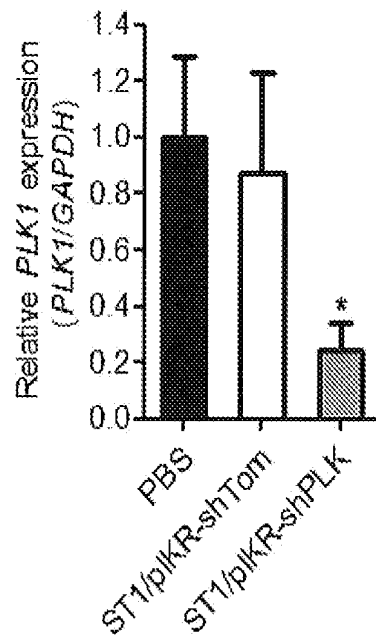


Fig. 28



Figs. 29A



Figs. 29B

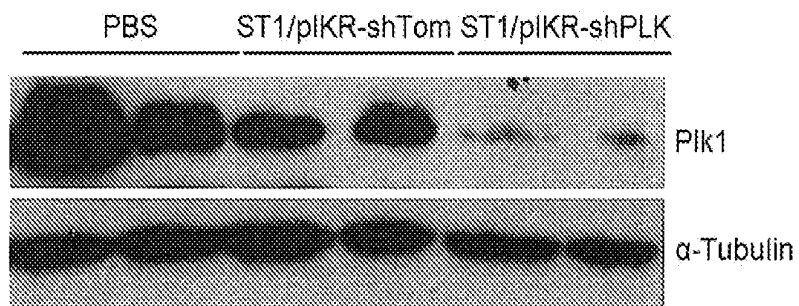


Fig. 30A

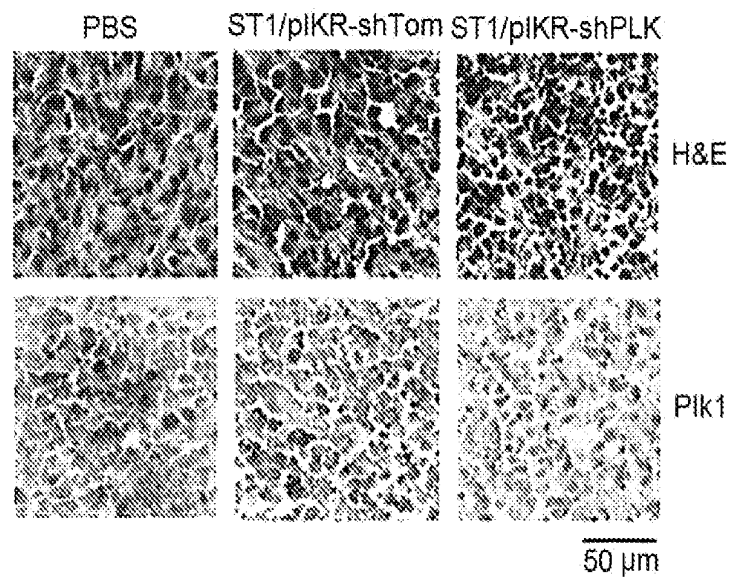


Fig. 30B

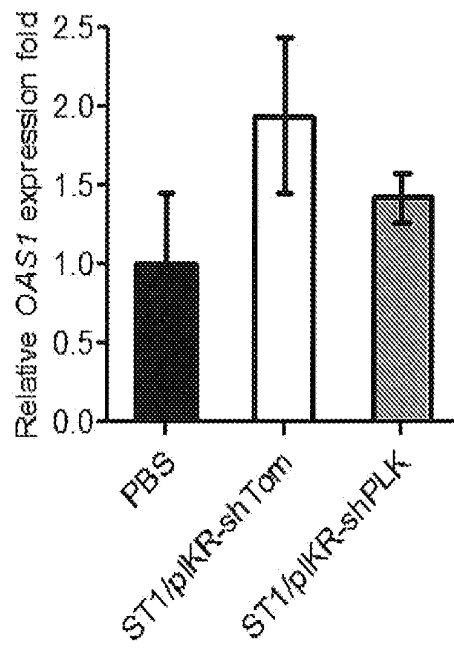
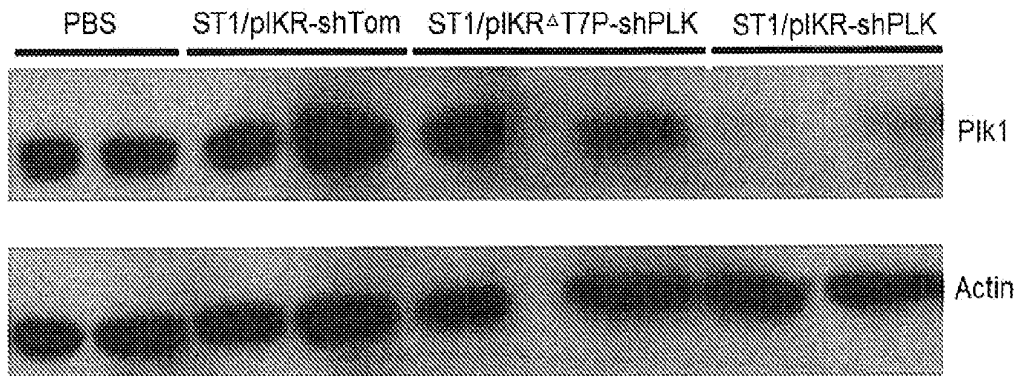
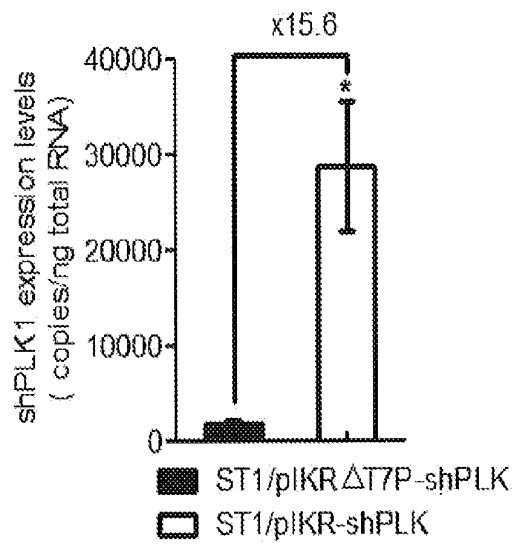


Fig. 31



Figs. 32A



Figs. 32B

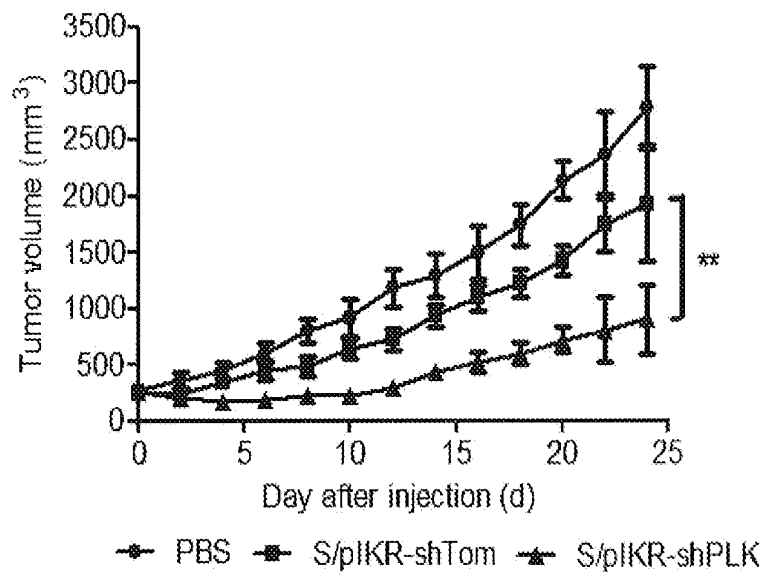


Fig. 33

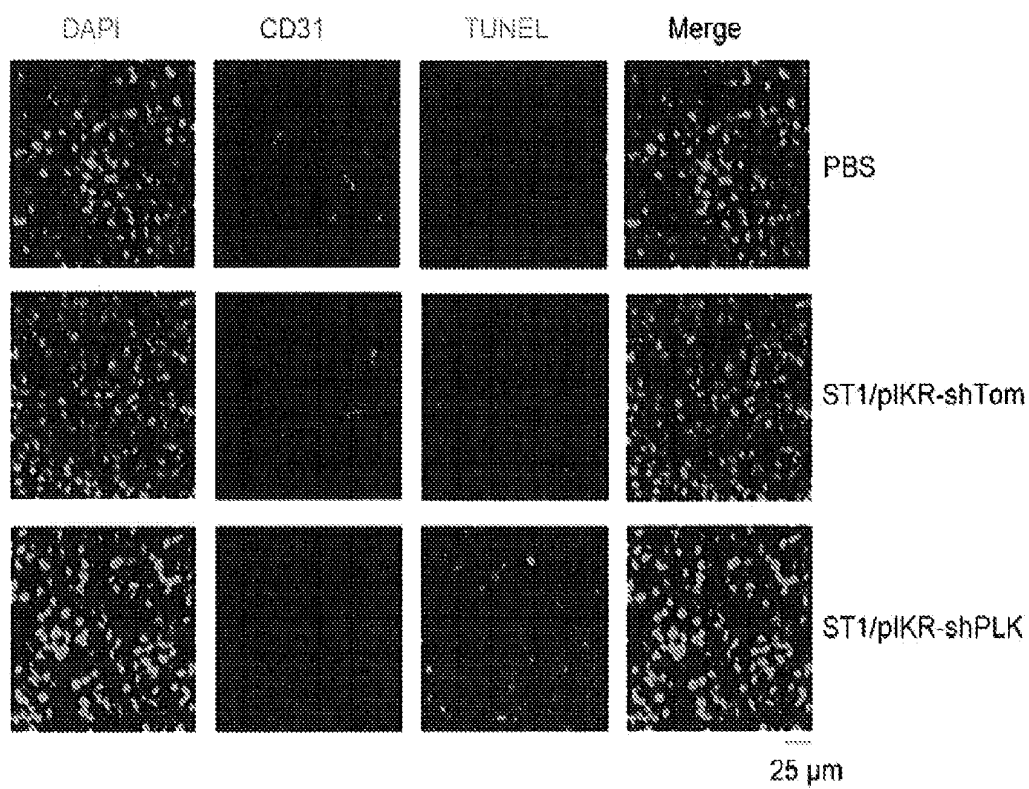
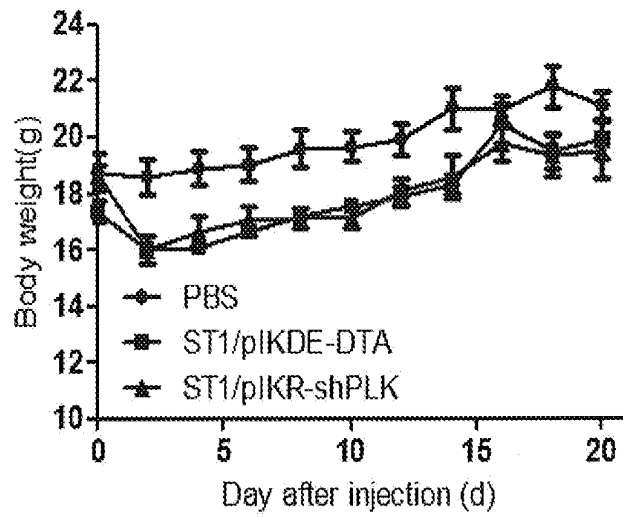
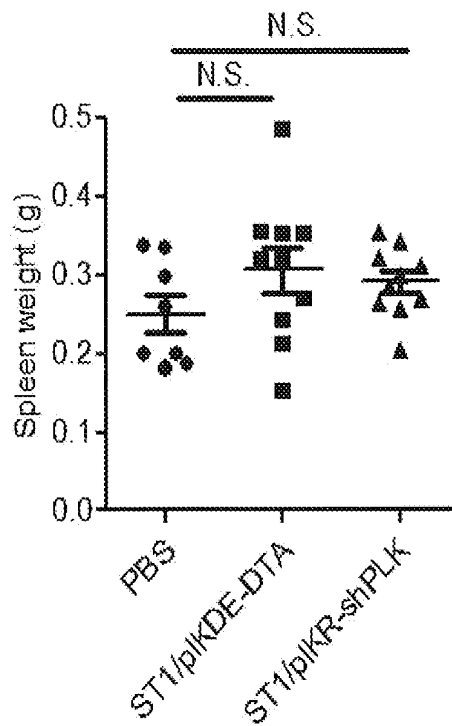


Fig. 34



Figs. 35A



Figs. 35B

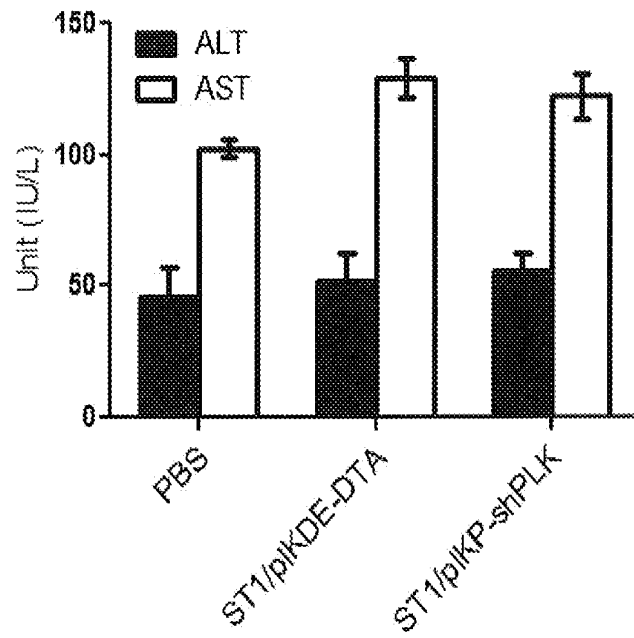


Fig. 36

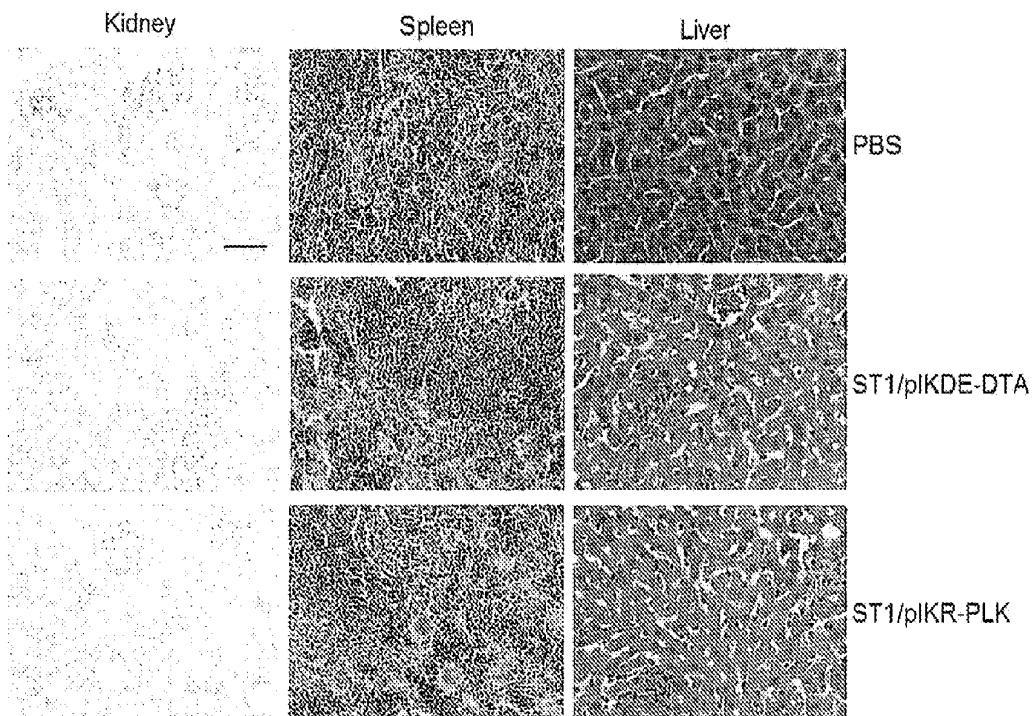


Fig. 37

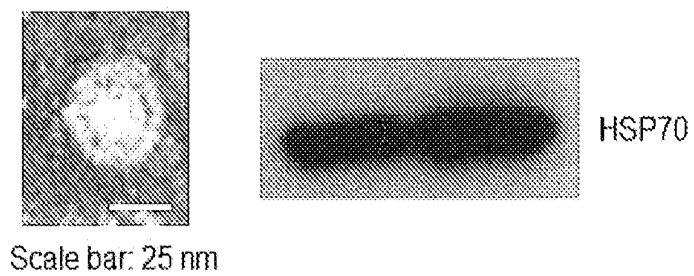
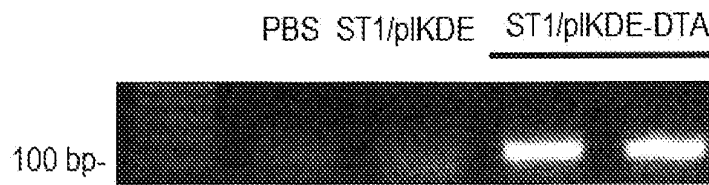
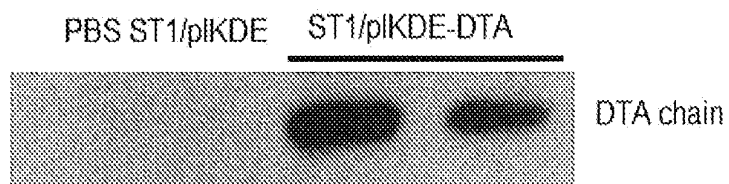


Fig. 38



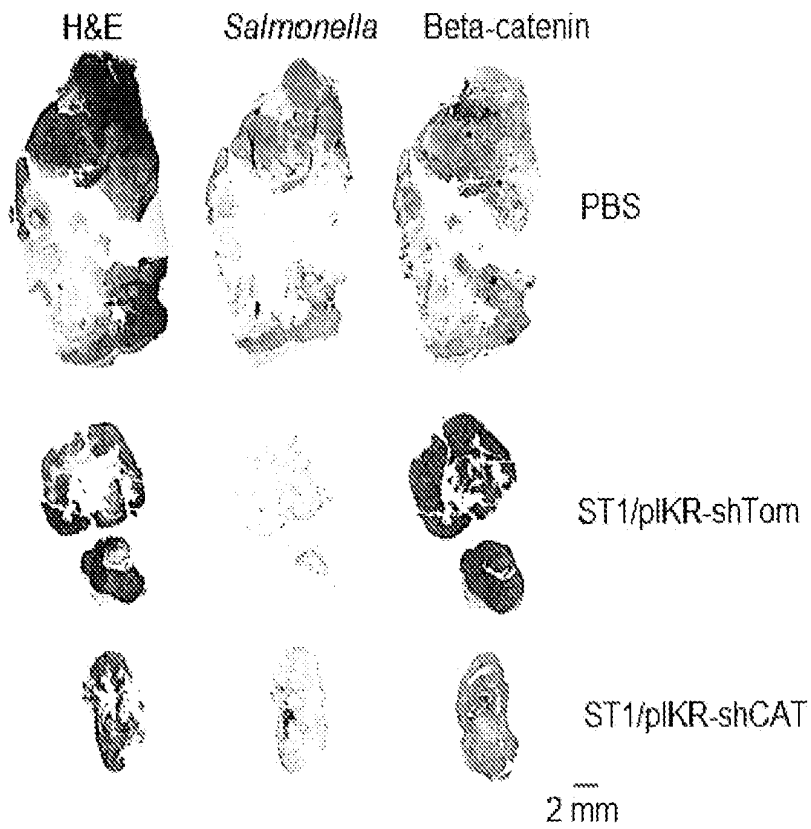
Figs. 39A



Figs. 39B



Figs. 40A



Figs. 40B

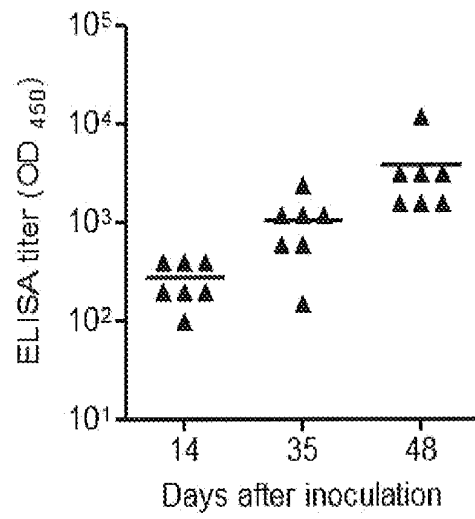


Fig. 41

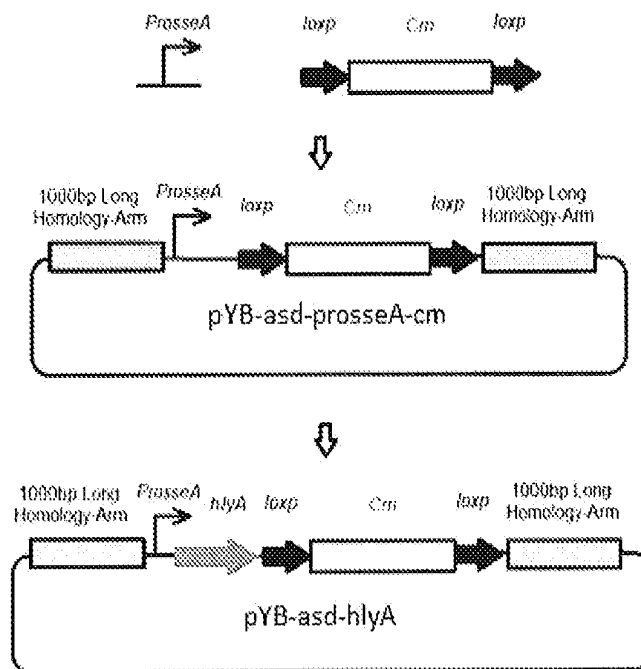


Fig. 42



Fig. 43

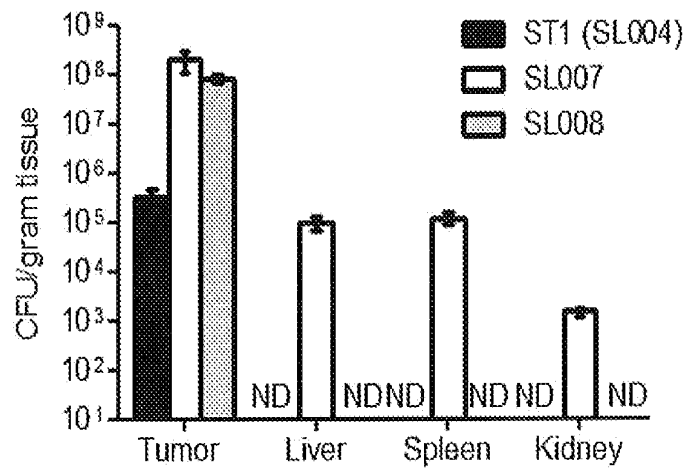


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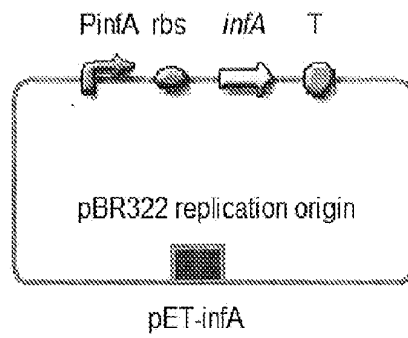


Fig. 45

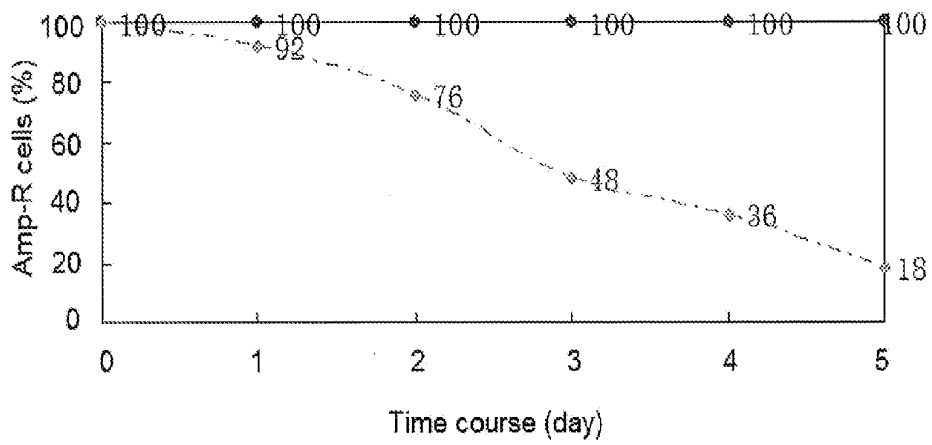


Fig. 46

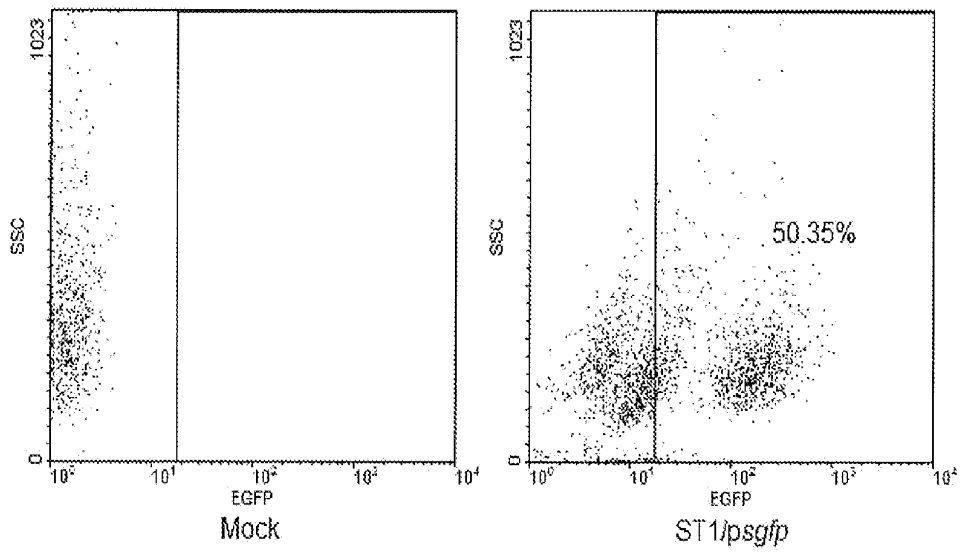
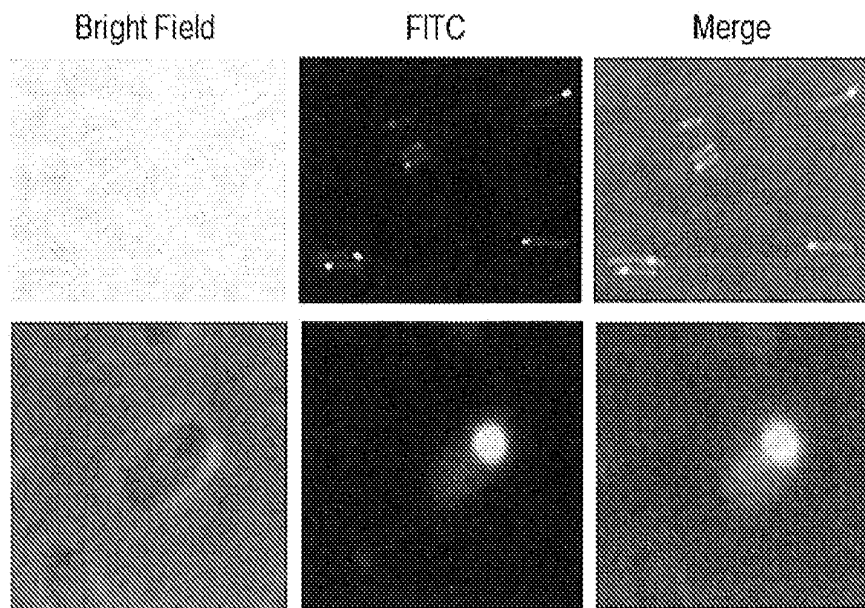
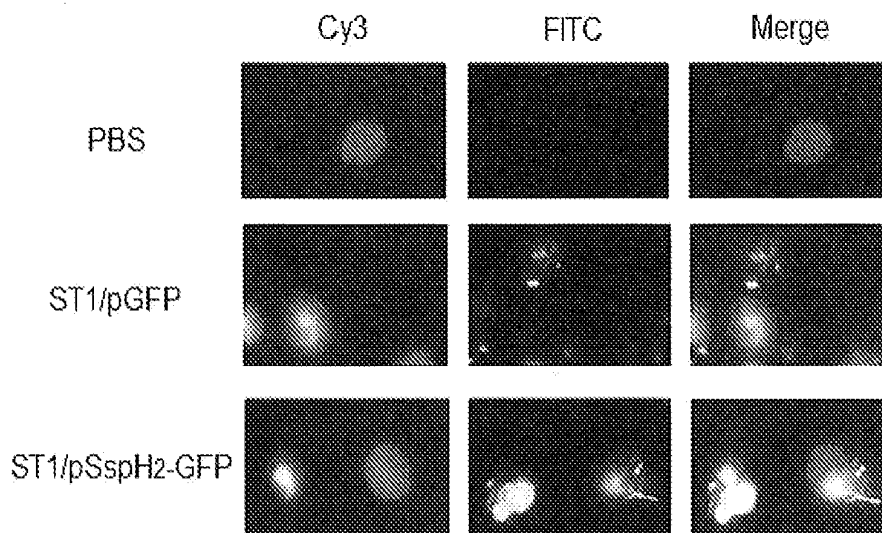


Fig. 47



Figs. 48A



Figs. 48B

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 151 TCAAGCTCTA AATCGGGGGC TCCCTTTAGG GTTCCGATTT AGTGCTTTAC
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 501 CCCCTATTTG TTTATTTTTC TAAATACATT CAAATATGTA TCCGCTCATG
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 1851 AACGACCTAC ACCGAACTGA GATACCTACA CCGTGAGCTA TGAGAAAGCG
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 1951 GTCCGAACAG GAGAGCGCAC GAGGGAGCTT CCAGGGGAA ACGCCTGGTA
 2001 TCTTTATAGT CCTGTGGGT TTCGCCACCT CTGACTTGAG CGTCGATTTT

Fig. 49A

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 2151 TCCTGCGTTA TCCCCTGATT CTGTGGATAA CCGTATTACC GCCTTTGAGT
 2201 GAGCTGATAC CGCTCGCCGC AGCCGAACGA CCGAGCGCAG CGAGTCAGTG
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 2951 ATCCGGAACA TAATGGTGCA GGGCGCTGAC TTCCCGGTTT CCAGACTTTA
 3001 CGAAACACGG AAACCGAAGA CCATTCATGT TGTGCTCAG GTCGCAGACG
 3051 TTTTGACGCA GCAGTCGCTT CACGTTGCTT CCGTATCGG TGATTCAATC
 3101 TGCTAACCAG TAAGGCAACC CCGCCAGCCT AGCCGGGTCC TCAACGACAG
 3151 GAGCACGATC ATGCGCACCC GTGGGGCCGC CATGCCGGCG ATAATGGCCT
 3201 GCTTCTCGCC GAAACGTTTG GTGGCGGGAC CAGTGACGAA GGCTTGAGCG
 3251 AGGGCGTGCA AGATTCCGAA TACCGCAAGC GACAGGCCGA TCATCGTCGC
 3301 GCTCCAGCGA AAGCGTCTT CGCCGAAAAT GACCCAGAGC GCTGCCGGCA
 3351 CCTGTCTTAC GAGTTGCATG ATAAAGAAGA CAGTCATAAG TGCGCGGACG
 3401 ATAGTCATGC CCCCGCCCCA CCGGAAGGAG CTGACTGGGT TGAAGGCTCT
 3451 CAAGGCATC GGTGAGATC CCGGTGCCTA ATGAGTGAGC TAACTTACAT
 3501 TAATTGCGTT GCGCTCACTG CCCGCTTCC AGTCGGGAAA CCTGTCTGTC
 3551 CAGCTGCATT AATGAATCGG CCAACGCGCG GGGAGAGGCG GTTTGCATAT
 3601 TGGGCGCCAG GGTGGTTTTT CTTTTCACCA GTGAGACGGG CAACAGCTGA
 3651 TTGCCCTTCA CCGCCTGGCC CTGAGAGAGT TGCAGCAAGC GGTCCACGCT
 3701 GGTTCGCCCC AGCAGGCGAA AATCCTGTTT GATGGTGGTT AACGGCGGGA
 3751 TATAACATGA GCTGTCTTCC GTATCGTCGT ATCCCCTAC CGAGATCCTT
 3801 TTAATTATTT ACAGAACTTC GGCATTATCT TGCCGGTTCA AATTACGGTA
 3851 GTGATACCCC AGAGGATTAG ATGGCCAAAG AAGACAATAT TGAAATGCAA
 3901 GGTACCGTTC TTGAAACGTT GCCTAATACC ATGTTCCGCG TAGAGTTAGA
 3951 AAACGGTCAC GTGGTTACTG CACACATCTC CCGTAAATG CGCAAAAAC
 4001 ACATCCGCAT CCTGACGGGC GACAAAGTGA CTGTTGAACT GACCCCGTAC
 4051 GACCTGAGCA AAGGCCGCAT TGTCTTCCGT AGTCGCTGAT TGTTTTACCG

Fig. 49B

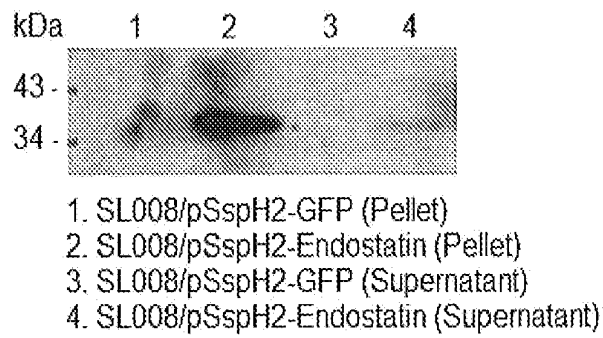
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4151 TTGCGCCCAT TGCGCCGAGC GCCATCTGAT CGTTGGCAAC CAGCATCGCA
4201 GTGGGAACGA TGCCCTCATT CAGCATTTCG ATGGTTTGTT GAAAACCGGA
4251 CATGGCACTC CAGTCGCCTT CCCGTTCCGC TATCGGCTGA ATTTGATTGC
4301 GAGTGAGATA TTTATGCCAG CCAGCCAGAC GCAGACGGC CGAGACAGAA
4351 CTTAATGGGC CCGCTAACAG CGCGATTTCG TGGTGACCCA ATGCGACCAG
4401 ATGCTCCACG CCCAGTCGGC TACCGTCTTC ATGGGAGAAA ATAATACTGT
4451 TGATGGGTGT CTGGTCAGAG ACATCAAGAA ATAACGCCGG AACATTAGTG
4501 CAGGCAGCTT CCACAGCAAT GGCATCCTGG TCATCCAGCG GATAGTTAAT
4551 GATCAGCCCA CTGACGCGTT GCGCGAGAAG ATTGTGCACC GCCGCTTTAC
4601 AGGCTTCGAC GCCGCTTCGT TCTACCATCG ACACCACCAC GCTGGCACCC
4651 AGTTGATCGG CGCGAGATTT AATCGCCGGC ACAATTTGGC ACGGCGCGTG
4701 CAGGGCCAGA CTGGAGGTGG CAACGCCAAT CAGCAACGAC TGTTTGCCCG
4751 CCAGTTGTTG TGCCACGCGG TTGGGAATGT AATTCAGCTC CGCCATCGCC
4801 GCTTCCACTT TTTCCCGCGT TTTCCGAGAA ACGTGGCTGG CCTGGFTCAC
4851 CACGCGGGAA ACGGTCTGAT AAGAGACACC GGCATACTCT GCGACATCGT
4901 ATAACGTTAC TGGTTTCACA TTCACCACCC TGAATTGACT CTCTTCGGG
4951 CGCTATCATG CCATACCGCG AAAGGTTTTG CGCCATTCGA TGGTGTCCGG
5001 GATCTCGACG CTCTCCCTTA TGCGACTCCT GCATTAGGAA GCAGCCCAGT
5051 AGTAGGTTGA GGCCGTTGAG CACCGCCGCC GCAAGGAATG GTGCATGCAA
5101 GGAGATGGCG CCCAACAGTC CCCCAGCCAC GGGGCTGCC ACCATACCCA
5151 CGCCGAAACA AGCGCTCATG AGCCCGAAGT GCGGAGCCCG ATCTTCCCCA
5201 TCGGTGATGT CGGCGATATA GCGCCAGCA ACCGCACCTG TGGCGCCGGT
5251 GATGCCGGCC ACGATGCGTC CGGCGTAGAG GATCGAGATC TCGATCCCGC
5301 GAAATTAATA CGACTCACTA TAGGGGAATT GTGAGCGGAT AACAATPCCC
5351 CTCTAGAAAT AATTTTGTTF AACTTTAAGA AGGAGATATA CCATGGCACC
5401 CTTTCATATT GGAAGCGGAT GTCTTCCCGC CACCATCAGT AATCGCCGCA
5451 TTTATCGTAT TGCCTGGTCT GATACCCCCC CTGAAATGAG TTCTGGGAA
5501 AAAATGAAGG AATTTTTTTG CTCAACGCAC CAGACTGAAG CGCTGGAGTG
5551 CATCTGGACG ATTTGTCACC CGCCGGCCGG AACGACCGGG GAGGATGTGA
5601 TCAACAGATT TGAAGTCTC AGGACGCTCG CGTATGCCGG ATGGGAGGAA
5651 AGCATTCATT CCGCCAGCA CGGGGAAAAT TACTTCTGTA TTCTGGATGA
5701 AGACAGTCAG GAGATATTGT CAGTCACCCCT TGATGATGCC GGGAACTATA
5751 CCGTAAATTG CCAGGGGTAC AGTGAAACAC ATCGCCTCAC CCTGGACACA
5801 GCACAGGGTG AGGAGGGCAC AGGACACGCG GAAGGGGCAT CCGGGTCCAT
5851 GGATTACAAG GATGACGACG ATAAGCATAT GCATACTCAT CAGGACTTTC
5901 AGCCAGTGCT CCACCTGGTG GCACTGAACA CCCCCTGTC TGGAGGCATG
5951 CGTGGTATCC GTGGAGCAGA TTTCCAGTGC TTCCAGCAAG CCCGAGCCGT
6001 GGGCTGTGCG GGCACCTTCC GGGCTTTCCT GTCCTCTAGG CTGCAGGATC
6051 TCTATAGCAT CGTGGCCCGT GCTGACCGGG GGTCTGTGCC CATCGTCAAC

6101 CTGAAGGACG AGGTGCTATC TCCCAGCTGG GACTCCCTGT TTTCTGGCTC

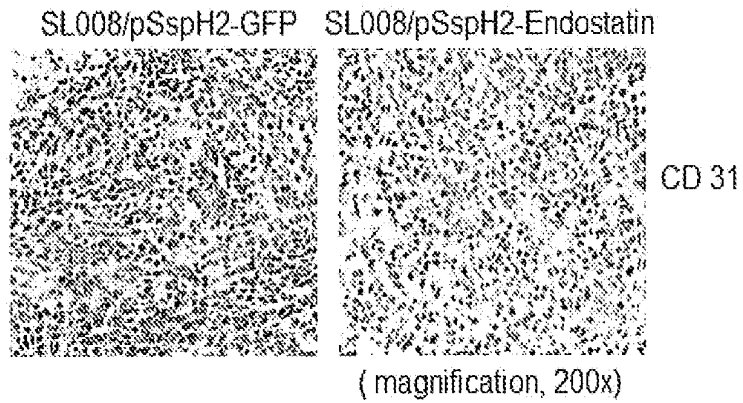
Fig. 49C

6151 CCAGGGTCAA CTGCAACCCG GGGCCCGCAT CTTTTCTTTT GACGGCAGAG
6201 ATGTCCTGAG ACACCCAGCC TGGCCGCAGA AGAGCGTATG GCACGGCTCG
6251 GACCCCACTG GCGGGAGGCT GATGGAGAGT TACTGTGAGA CATGGCGAAC
6301 TGAAACTACT GGGGCTACAG GTCAGGCCTC CTCCTGCTG TCAGGCAGGC
6351 TCCTGGAACA GAAAGCTGCG AGCTGCCACA ACAGCTACAT CGTCCTGTGC
6401 APTGAGAATA GCTTCATGAC CTC'TTCTCC AAATAACTGC AGRAGCTTGC
6451 GGCCGCACTC GAGCACCACC ACCACCACCA CTGAGATCCG GCTGCTAACA
6501 AAGCCCGAAA GGAAGCTGAG TTGGCTGCTG CCACCGCTGA GCAATAACTA
6551 GCATAACCCG TTGGGGCCTC TAAACGGGTC TTGAGGGGTT TTTTGCTGAA
6601 AGGAGGAACT ATATCCGGAT

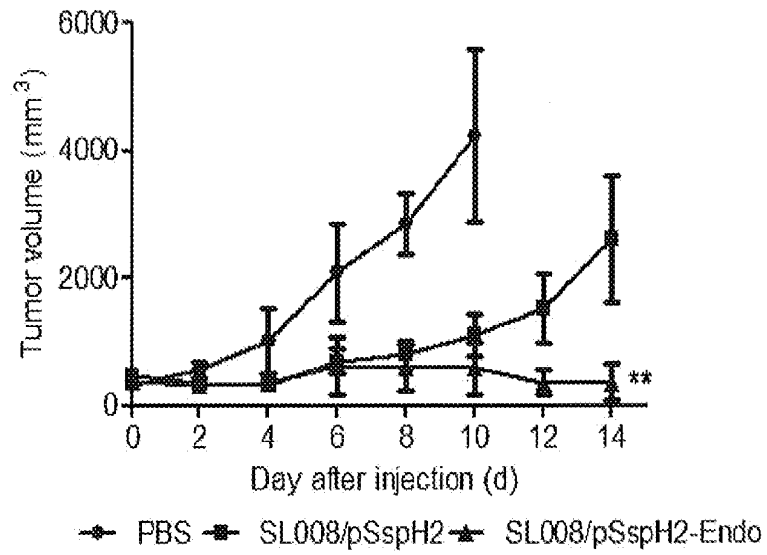
Fig. 49D



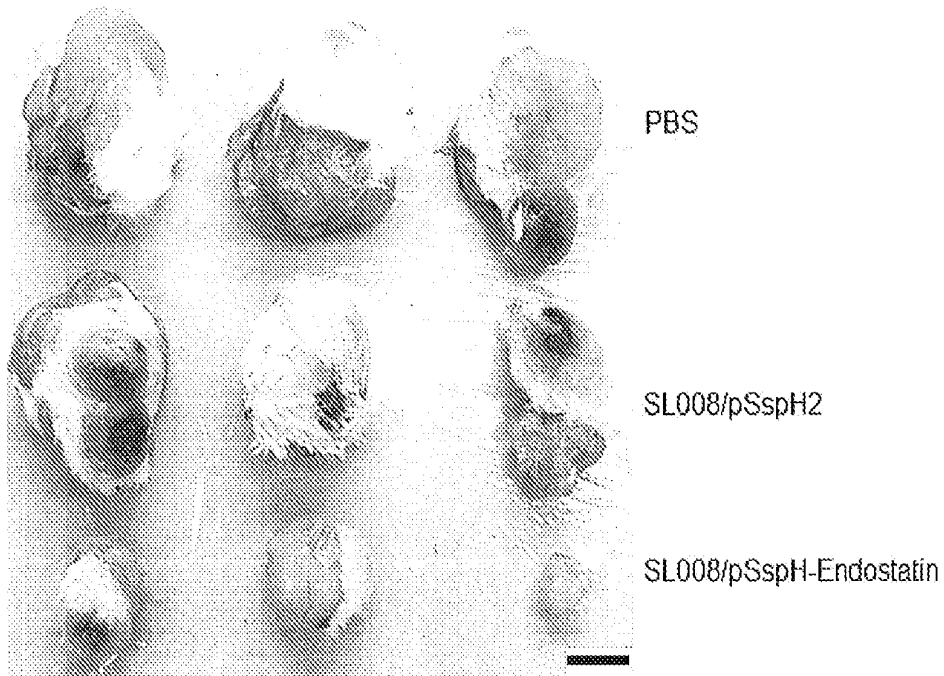
Figs. 50A



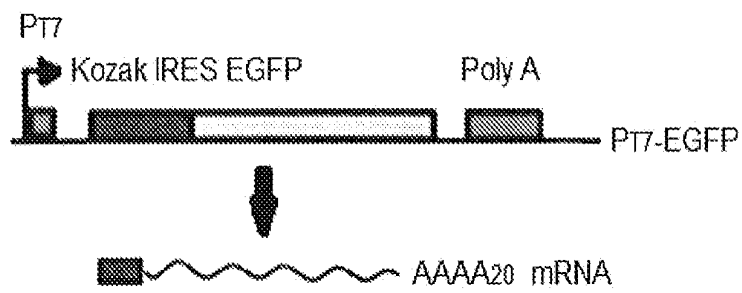
Figs. 50B



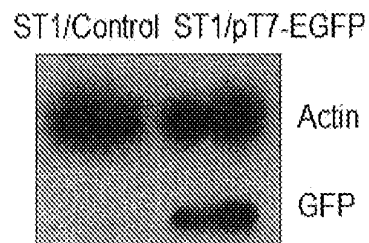
Figs. 51A



Figs. 51B



Figs. 52A



Figs. 52B

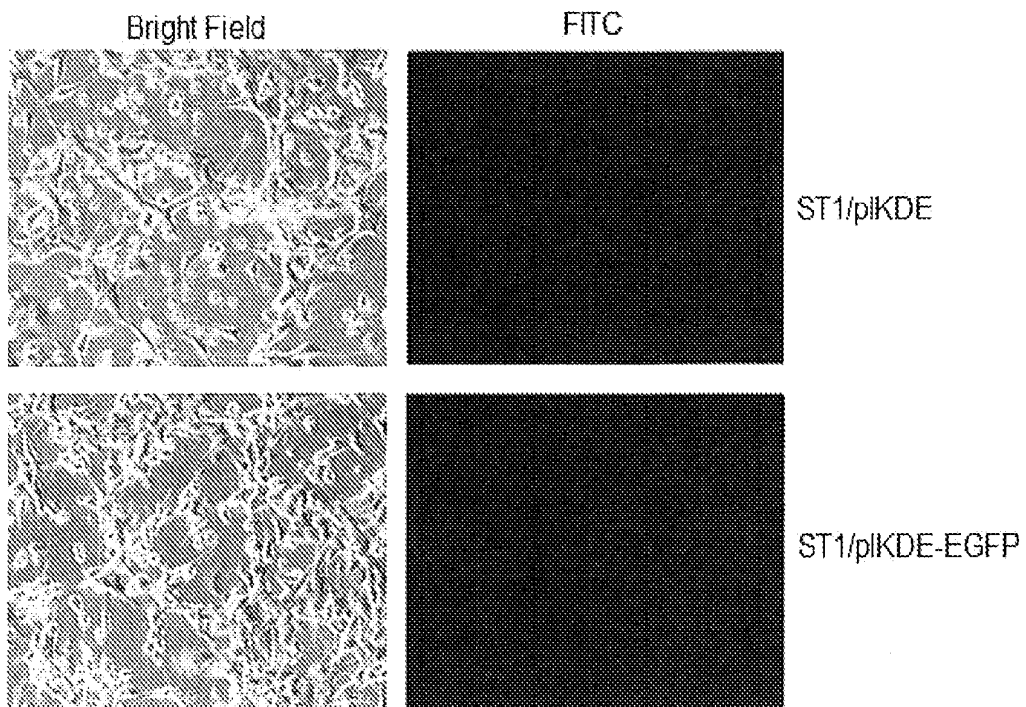


Fig. 53

1 TCAATATTGG CCATTAGCCA TATTATTCAT TGTTATATA GCATAAATCA
 51 ATATTGGCTA TTGGCCATTG CATACGTTGT ATCTATATCA TAATATGTAC
 101 ATTTATATTG GCTCATGTCC AATATGACCG CCATGTTGGC ATTGATTATT
 151 GACTAGTTAT TAATAGTAAT CAATTACGGG GTCATTAGTT CATAGCCCAT
 201 ATATGGAGTT CCGCGTTACA TAACTTACGG TAAATGGCCC GCCTGGCTGA
 251 CCGCCCAACG ACCCCCGCCC ATTGACGTCA ATAATGACGT ATGTTCCCAT
 301 AGTAACGCCA ATAGGGACTT TCCATTGACG TCAATGGGTG GAGTATTTAC
 351 GGTAACCTGC CCACTTGGCA GTACATCAAG TGTATCATAT GCCAAGTACG
 401 CCCCCTATTG ACGTCAATGA CGGTAAATGG CCCGCCCTGGC ATTTATGCCCA
 451 GTACATGACC TTATGGGACT TTCCTACTTG GCAGTACATC TACGTATTAG
 501 TCATCGCTAT TACCATGGTG ATGCGGTTTT GGCAGTACAT CAATGGGCGT
 551 GGATAGCGGT TTGACTCAGC GGGATTTCCA AGTCTCCACC CCATTGACGT
 601 CAATGGGAGT TTGTTTTGGC ACCAAAATCA ACGGGACTTT CCAAATGTG
 651 GTAACAATC CGCCCCATTG ACGCAAATGG GCGGTAGGCG TGTACGGTGG
 701 GAGGTCTATA TAAGCAGAGC TCTCTGGCTA ACTAGAGAAC CCACTGCTTA
 751 CTGGCTTATC GAAATTAATA CGACTACTA TAGGGAGACC CAAGCTGGCT
 801 AGAGCCCCTC TCCCTCCCCC CCCCCTAACG TTACTGGCCG AAGCCGCTTG
 851 GAATAAGGCC GGTGTGCGTT TGTCTATATG TTATTTTCCA CCATATTGCC
 901 GTCTTTTGGC AATGTGAGGG CCCGGAACC TGGCCCTGTC TTCTTGACGA
 951 GCATTCCTAG GGTCTTTTCC CCTCTCGCCA AAGGAATGCA AGGTCTGTTG
 1001 AATGTCGTGA AGGAAGCAGT TCCTCTGGAA GCTTCTTGAA GACAAACAAC
 1051 GTCTGTAGCG ACCCTTTGCA GGCAGCGGAA CCCCCACCT GCGCAGAGGT
 1101 GCCTCTGCGG CCAAAGCCA CGTGTATAAG ATACACCTGC AAAGGCGGCA
 1151 CAACCCAGT GCCACGTTGT GAGTTGGATA GTTGTGGAAA GAGTCAAATG
 1201 GCTCTCTCA AGCGTATTCA ACAAGGGGCT GAAGGATGCC CAGAAGGTAC
 1251 CCCATTGTAT GGGATCTGAT CTGGGGCCTC GGTGCACATG CTTTACATGT
 1301 GTTTAGTCGA GGTAAAAAA ACGTCTAGGC CCCCCGAACC ACGGGGACGT
 1351 GGTTTTCTT TGAAAAACAC GATGATAATA TGCTAGACCA CCATGGACTA
 1401 CAAAGACCAT GACGGTGATT ATAAAGATCA TGACATCGAT TACAAGGATG
 1451 ACGACGATAA GCATATGGGC GCTGATGATG TTGTTGATTC TTCTAAATCT
 1501 TTTGTGATGG AAAACTTTTC TTCGTACCAC GGGACTAAAC CTGGTTATGT
 1551 AGATTCCATT CAAAAAGGTA TACAAAAGCC AAAATCTGGT ACACAAGGAA
 1601 ATTATGACGA TGATTGGAAA GGGTTTTATA GTACCGACAA TAAATACGAC
 1651 GCTGCGGGAT ACTCTGTAGA TAATGAAAAC CCGCTCTCTG GAAAAGCTGG
 1701 AGGCGTGGTC AAAGTGACGT ATCCAGGACT GACGAAGGTT CTCGCACTAA
 1751 AAGTGGATAA TGCCGAAACT ATTAAGAAAG AGTTAGGTTT AAGTCTCACT
 1801 GAACCGTGA TGGAGCAAGT CGGAACGGAA GAGTTTATCA AAAGGTTTGA
 1851 TGATGGTGCT TCGCGTGTAG TGCTCAGCCT TCCCTTCGCT GAGGGGAGTT
 1901 CTAGCGTTGA ATATATTAAT AACTGGGAAC AGGCGAAAGC GTTAAGCGTA
 1951 GAACCTGAGA TTAATTTTGA AACCCGTGGA AAACGTGGCC AAGATGCGAT

Figs. 54A

2001 GTATGAGTAT ATGGCTCAAG CCTGTGCAGG AAATCGTGTG AGGCGATAAC
2051 TGCAGTCGAG TCTAGAGGGC CCGTTTAAAC CCGCTGATCA GCCTCGACTG
2101 TGCCCTCTAG TTGCCAGCCA TCTGTTGTTT GCCCTCCTCC CGTGCCTTCC
2151 TTGACCCTGG AAGGTGCCAC TCCCACTGTC CTTTCCTAAT AAAATGAGGA
2201 AATTGCATCG CATTGTCTGA GTAGGTGTCA TTCTATTCTG GGGGGTGGGG
2251 TGGGGCAGGA CAGCAAGGGG GAGGATTGGG AAGACAATAG CAGGCATGCT
2301 GGGGATGCGG TGGGCTCTAT GGTAAATACGA CTCACATATAG GGAGACCGCG
2351 GGCCCGGGAT CCGCCCTCTT CCTCCCTCCC CCCCTAACGT TACTGGCCGA
2401 AGCCGCTTGG AATAAGGCCG GTGTGCGTFT GTCTATATGT TATTTTCCAC
2451 CATATTGCCG TCTTTTGGCA ATGTGAGGGC CCGGAAACCT GGCCCTGTCT
2501 TCTTGACGAG CATTCTAGG GGTCTTTCCC CTCTCGCCAA AGGAATGCAA
2551 GGTCTGTGTA ATGTCTGAA GGAAGCAGTT CCTCTGGAAG CTCTTTGAAG
2601 ACAAACAACG TCTGTAGCGA CCTTTGCGAG GCAGCGGAAC CCCCCACCTG
2651 GCGACAGGTG CCTCTCGGGC CAAAAGCCAC GTGTATAAGA TACACCTGCA
2701 AAGCGGGCAC AACCCAGTG CCACGTTGTG AGTTGGATAG TTGTGGAAG
2751 AGTCAAATGG CTCTCCTCAA GCGTATTCAA CAAGGGGCTG AAGGATGCCC
2801 AGAAGGTACC CCATTGTATG GGATCTGATC TGGGGCCTCG GTGCACATGC
2851 TTTACATGTG TTTAGTCGAG GTTAAAAAA CGTCTAGGCC CCCCAGACCA
2901 CGGGGACGTG GTTTTCCTTT GAAAAACAG ATGATAATAT GGCCACAACCT
2951 AGACCACCAT GAACACGATT AACATCGCTA AGAACGACTT CTCTGACATC
3001 GAAGTGGCTG CTATCCCGTT CAACACTCTG GCTGAOCATT ACGGTGAGCG
3051 TTTAGCTCGC GAACAGTTGG CCTTGAGCA TGAGTCTTAC GAGATGGGTG
3101 AAGCACGCTT CCGCAAGATG TTTGAGCGTC AACTTAAAGC TGGTGAGGTT
3151 GCGGATAACG CTGCCGCCAA GCCTCTCATC ACTACCCCTAC TCCCTAAGAT
3201 GATTGCACGC ATCAACGACT GGTTTGAGGA AGTGAAAGCT AAGCGCGGCA
3251 AGCGCCCGAC AGCCTTCCAG TTCTTGCAAG AAATCAAGCC GGAAGCCGTA
3301 GCGTACATCA CCATTAAGAC CACTCTGGCT TGCCTAACCA GTGCTGACAA
3351 TACAACCGTT CAGGCTGTAG CAAGCGCAAT CGGTCGGGCC ATGAGGAGCG
3401 AGGCTCGCTT CCGTCTGATC CGTGACCTTG AAGCTAAGCA CTCAAGAAA
3451 AACGTTGAGG AACAACTCAA CAAGCGCGTA GGGCAGCTCT ACAAGAAAGC
3501 ATTTATGCAA GTTGTGAGG CTGACATGCT CTCTAAGGGT CTACTCGGTG
3551 GCGAGGCGTG GTCTTCGTGG CATAAGGAAG ACTCTATTCA TGTAGGAGTA
3601 CGCTGCATCG AGATGCTCAT TGAGTCAACC GGAATGGTTA GCTTACACCG
3651 CAAAATGCTT GCGTAGTAG GTCAAGACTC TGAGACTATC GAAGTCCGAC
3701 CTGAATACGC TGAGGCTATC GCAACCCGTC CAGGTGCGCT GGCTGGCATC
3751 TCTCCGATGT TCCAACCTTG CGTAGTTCCT CCTAAGCCGT GGAAGTGGAT
3801 TACTGGTGGT GGCTATTGGG CTAACGGTCC TCGTCTCTG GCGCTGGTGC
3851 GTACTCACAG TAAGAAAGCA CTGATGCGCT ACGAAGACGT TTACATGCCT
3901 GAGGTGTACA AAGCGATTAA CATTGCGCAA AACACCGCAT GGAAAATCAA
3951 CAAGAAAGTC CTAGCGGTG CCAACGTAAT CACCAAGTGG AAGCATTGTC
4001 CGGTCCAGGA CATCCCTGCG ATTGAGCGTG AAGAACTCCC GATGAAACCG

Figs. 54B

4051 GAAGACATCG ACATGAATCC TGAGGCTCTC ACCGCGTGGA AACGTGCTGC
4101 CGCTGCTGTG TACCGCAAGG ACAAGGCTCG CAAGTCTCGC CGTATCAGCC
4151 TTGAGTTCAT GCTTGAGCAA GCCAATAAGT TTGCTAACCA TAAGGCCATC
4201 TGGTTCCCTT ACAACATGGA CTGGCGCGGT CGTGTTTACG CTGTGTCAAT
4251 GTTCAACCCG CAAGGTAACG ATATGACCAA AGGACTGCTT ACGCTGGCGA
4301 AAGGTAAACC AATCGGTAAG GAAGGTTACT ACTGGCTGAA AATCCACGGT
4351 GCAAACCTGT CGGGTGTGCA TAAGGTTCCG TTCCCTGAGC GCATCAAGTT
4401 CATTGAGGAA AACCACGAGA ACATCATGGC TTGCGCTAAG TCTCCACTGG
4451 AGAACACTTG GTGGGCTGAG CAAGATTCTC CGTTCTGCTT CCTTGGCTTC
4501 TGCTTTGAGT ACGCTGGGGT ACAGCACCAC GGCCTGAGCT ATAACTGCTC
4551 CCTTCCGCTG GCGTTTGACG GGTCTTGCTC TGGCATCCAG CACTTCTCCG
4601 CGATGCTCCG AGATGAGGTA GGTGGTCGCG CGGTTAACTT GCTTCTAGT
4651 GAAACCGTTC AGGACATCTA CGGGATTGTT GCTAAGAAAG TCAACGAGAT
4701 TCTACAAGCA GACGCAATCA ATGGGACCGA TAACGAAGTA GTTACCGTGA
4751 CCGATGAGAA CACTGGTGAA ATCTCTGAGA AAGTCAAGCT GGGCACTAAG
4801 GCACTGGCTG GTCAATGGCT GGCTTACGGT GTTACTCGCA GTGTGACTAA
4851 GCGTTCAGTC ATGACGCTGG CTTACGGGTC CAAAGAGTTC GGCTTCCGTC
4901 AACAAAGTGT GGAAGATACC ATTACGCCAG CTATTGATTC CGGCAAGGGT
4951 CTGATGTTCA CTCAGCCGAA TCAGGCTGCT GGATACATGG CTAAGCTGAT
5001 TTGGGAATCT GTGAGCGTGA CGGTGCTAGC TGCGGTTGAA GCAATGAACT
5051 GGCTTAAGTC TGCTGCTAAG CTGCTGGCTG CTGAGGTCAA AGATAAGAAG
5101 ACTGGAGAGA TTCTTCGCAA GCGTTGCGCT GTGCATTGGG TAACTCCTGA
5151 TGGTTTCCCT GTGTGSCAGG AATACAAGAA GCCTATTTCAG ACGCGCTTGA
5201 ACCTGATGTT CCTCGGTCAG TTCCGCTTAC AGCCTACCAT TAACACCAAC
5251 AAAGATAGCG AGATTGATGC ACACAAACAG GAGTCTGGTA TCGCTCCTAA
5301 CTTTGTACAC AGCCAAGACG GTAGCCACCT TCGTAAGACT GTAGTGTGGG
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5401 GGTACCATTC CGGCTGACGC TCGGAACCTG TTCAAAGCAG TCGCGGAAAC
5451 TATGGTTGAC ACATATGAGT CTTGTGATGT ACTGGCTGAT TTCTACGACC
5501 AGTTCGCTGA CCAGTTGCAC GAGTCTCAAT TGGACAAAAT GCCAGCACTT
5551 CCGGCTAAAG GTAACCTGAA CCTCCGTGAC ATCTTAGAGT CGGACTTCGC
5601 GTTCGCGGAT CCAAAAAAGA AGAGAAAGGT AACTAGTCCG GCCGCTTCCC
5651 TTAGTGAGG GTTAATGCTT CGAGCAGACA TGATAAGATA CATTGATGAG
5701 FTTGGACAAA CCACAAC TAG AATGCAGTGA AAAAAATGCT TTATTTGTGA
5751 AATTTGTGAT GCTATTTGCTT TATTTGTAAC CATTATAAGC TGCAATAAAC
5801 AAGTTAACAA CAACAATTGC ATTCATTTTA TGPTTCAGGT TCAGGGGGAG
5851 ATGTGGGAGG TTTTTTAAAG CAAGTAAAC CTCTACAAAT GTGGTAAAT
5901 CCGATAAGGA TCGATCCGGG CTGGCGTAAT AGCGAAGAGG CCCGCACCGA
5951 TCGCCCTTCC CAACAGTTGC GCAGCCTGAA TGGCGAATGG ACGCGCCCTG
6001 TAGCGGCGCA PTAAGCGCGG CGGGTGTGGT GGTTCGCGC AGCGTGACCG
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Figs. 54C

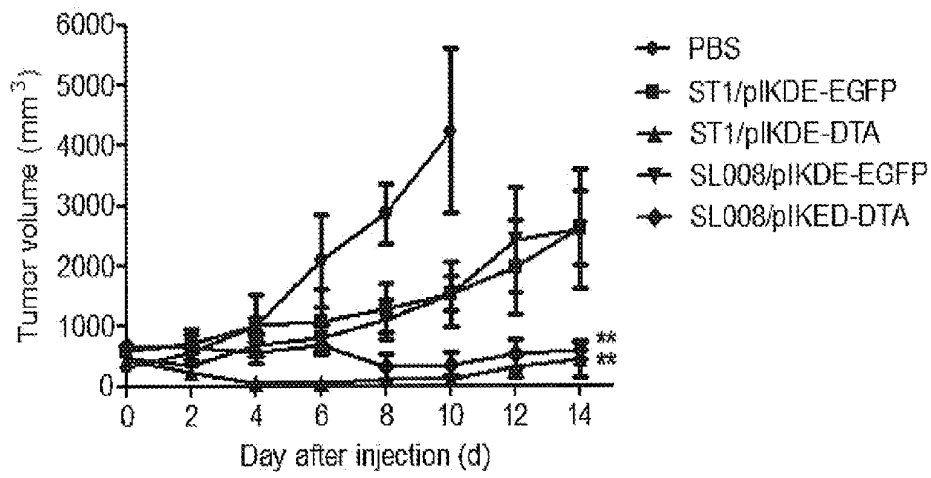
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 6301 CCAAACCTGGA ACAACACTCA ACCCTATCTC GGTCTATCTT TTTGATTTAT
 6351 AAGGGATFTT GCCGATTTTCG GCCTATTGGT TAAAAAATGA GCTGATTTAA
 6401 CAAATATTTA ACGCGAATTT TAACAAAATA TTAACGTTTA CAATTTTCGCC
 6451 TGATGCGGTA TTTTCTCCTT ACGCATCTGT GCGGTATTTT ACACCCGATA
 6501 CGCGGATCTG CGCAGCACCA TGGCCTGAAA TAACCTCTGA AAGAGGAACT
 6551 TGGTTAGGTA CCTTCTGAGG CGGAAAGAAC CAGCTGTGGA ATGTGTGTCA
 6601 GTTAGGGTGT GGAAAGTCCC CAGGCTCCCC AGCAGGCAGA AGTATGCAAA
 6651 GCATGCATCT CAATTAGTCA GCAACCAGGT GTGGAAAGTC CCCAGGCTCC
 6701 CCAGCAGGCA GAAGTATGCA AAGCATGCAT CTCAATTAGT CAGCAACCAT
 6751 AGTCCC GCCC CTAAC TCCGC CCAT CCGCC CCTA ACTCCG CCCAGTCCG
 6801 CCCATTCTCC GCCCCATGGC TGACTAATTT TTTTATTTA TGCAGAGGCC
 6851 GAGGCCGCCCT CGGCCTCTGA GCTATTCCAG AAGTAGTGAG GAGGCTTTTT
 6901 TGGAGGCCTT TTACTTATTT ACAGA ACTTC GGCATTATCT TGCCGGTTCA
 6951 AATTACGGTA GTGATACCCC AGAGGATTAG ATGGCCAAAG AAGACAATAT
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 7051 TAGAGPTAGA AAACGGTCAC GTGGTTACTG CACACATCTC CGGTAAAATG
 7101 CGCAAAAAC ACATCCGCAT CCTGACGGGC GACAAAGTGA CTGTTGAACT
 7151 GACCCCGTAC GACCTGAGCA AAGGCCGCAT TGCTTTCCGT AGTCGCTGAT
 7201 TGTTTTACCG CCTGATGGGC GAAGAGAAAG AACGAGTAAA AGGTGCGPTT
 7251 AACCGGCCTT TTGCGCGCAT GCCCGACGGC GAGGATCTCG TCGTGACCCA
 7301 TGGCGATGCC TGCTTGCCGA ATATCATGGT GGAAAATGGC CGCTTTTCTG
 7351 GATTCATCGA CTGTGGCCGG CTGGGTGTGG CGGACCGCTA TCAGGACATA
 7401 GCGTTGGCTA CCCGTGATAT TGCTGAAGAG CTTGGCGGCG AATGGGCTGA
 7451 CCGCTTCTTC GTGCTTTACG GTATCGCCGC TCCCGATTCC GAGCGCATCG
 7501 CCTTCTATCG CCTTCTTGAC GAGTCTTCT GAGCGGGACT CTGGGGTTCG
 7551 AAATGACCGA CCAAGCGACG CCCAACCTGC CATCACGATG GCCGCAATAA
 7601 AATATCTTTA TTTTCATTAC ATCTGTGTGT TGGTTTTTTG TGTGAATCGA
 7651 TAGCGATAAG GATCCCGGTA TGGTGCACCTC TCAGTACAAT CTGCTCTGAT
 7701 GCCGCATAGT TAAGCCAGCC CCGACACCCG CCAACACCCG CTGACGCGCC
 7751 CTGACGGGCT TGTCTGCTCC CGGCATCCGC TTACAGACAA GCTGTGACCG
 7801 TCTCCGGGAG CTGCATGTGT CAGAGGTTTT CACCGTCATC ACCGAAACGC
 7851 GCGAGACGAA AGGGCCTCGT GATACGCCTA TTTTATAGG TTAATGTCAT
 7901 GATAATAATG GTTTCCTAGA CGTCAGGTGG CACTTTTTCG GGAAATGTGC
 7951 GCGGAACCCC TATTTGTTTA TTTTCTAAA TACATTCAA TATGTATCCG
 8001 CTCATGAGAC AATAACCCTG ATAAATGCTT CAATAATATT GAAAAAGGAA
 8051 GAGTATGAGT ATTCAACATT TCCGTGTCGC CCTTATTCCT TTTTTTGGCG

8101 CATTGCTGCT TCCTGTTTTT GCTCACCCAG AAACGCTGGT GAAAGTAAAA

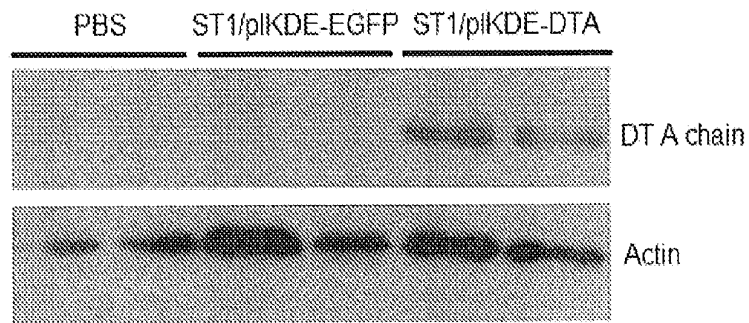
Figs. 54D

8151 GATGCTGAAG ATCAGTTGGG TGCACGAGTG GGTACATCG AACTGGATCT
 8201 CAACAGCGGT AAGATCCTTG AGAGTTTTCG CCCCAGAGAA CGTTTTCCAA
 8251 TGATGAGCAC TTTTAAAGTT CTGCTATGTG GCGCGGTATT ATCCCGTATT
 8301 GACGCCGGGC AAGAGCAACT CCGTCGCCGC ATACACTATT CTCAGAATGA
 8351 CTTGGTTGAG TACTCACCAG TCACAGAAAA GCATCTTACG GATGGCATGA
 8401 CAGTAAGAGA APTATGCAGT GCTGCCATAA CCATGAGTGA TAACACTGCG
 8451 GCCAACTTAC TTCTGACAAC GATCGGAGCA CCGAAGGAGC TAACCGCTTT
 8501 TTTGCACAAC ATGGGGGATC ATGTAACTCG CCTTGATCGT TGGGAACCGG
 8551 AGCTGAATGA AGCCATACCA AACGACGAGC GTGACACCAC GATGCCTGTA
 8601 GCAATGGCAA CAACGTTGCG CAAACTATTA ACTGGCGAAC TACTTACTCT
 8651 AGCTTCCCGG CAACAATTAA TAGACTGGAT GGAGGCGGAT AAAGTTGCAG
 8701 GACCACTTCT GCGCTCGGCC CTTCCGGCTG GCTGGTTTAT TGCTGATAAA
 8751 TCTGGAGCCG GTGAGCGTGG GTCTCGCGGT ATCATTGCAG CACTGGGGCC
 8801 AGATGGTAAG CCTTCCCGTA TCGTAGTTAT CTACACGACG GGGAGTCAGG
 8851 CAACTATGGA TGAACGAAAT AGACAGATCG CTGAGATAGG TGCCCTCACTG
 8901 ATTAAGCATT GGTAACTGTC AGACCAAGTT TACTCATATA TACTTTAGAT
 8951 TGATTTAAAA CTTCATTTTT AATTTAAAAG GATCTAGGTG AAGATCCTTT
 9001 TTGATAATCT CATGACCAA ATCCCTTAAC GTGAGTTTTT GTTCCACTGA
 9051 CCGTCAGACC CCGTAGAAAA GATCAAAGGA TCTTCTTGAG ATCCTTTTTTT
 9101 TCTGCCGTA ATCTGCTGCT TGCAAACAAA AAAACCACCG CTACCAGCGG
 9151 TGGTTTGTTT GCCGGATCAA GAGCTACCAA CTCPTTTTCC GAAGGTAACT
 9201 GGCTTCAGCA GAGCGCAGAT ACCAAATACT GTCCTTCTAG TGTAGCCGTA
 9251 GTTAGGCCAC CACTTCAAGA ACTCTGTAGC ACCGCCTACA TACCTCGCTC
 9301 TGCTAATCCT GTTACCAGTG GCTGCTGCCA GTGGCGATAA GTCGTGTCTT
 9351 ACCGGGTTGG ACTCAAGACG ATAGTTACCG GATAAGGCGC AGCGGTGGGG
 9401 CTGAACGGGG GGTTCGTGCA CACAGCCCAG CTTGGAGCGA ACGACCTACA
 9451 CCGAACTGAG ATACCTACAG CGTGAGCTAT GAGAAAGCGC CACGCTTCCC
 9501 GAAGGGAGAA AGGCGGACAG GTATCCGGTA AGCGGCAGGG TCGGAACAGG
 9551 AGAGCGCACG AGGGAGCTTC CAGGGGGAAA CGCCTGGTAT CTTTATAGTC
 9601 CTGTCGGGTT TCGCCACCTC TGACTTGAGC GTCGATTTTT GTGATGCTCG
 9651 TCAGGGGGGC GGAGCCTATG GAAAAACGCC AGCAACGCGG CCTTTTTACG
 9701 GTTCTGGGCC TTTTGCTGGC CTTTTGCTCA CATGGCTCGA CAGATCT

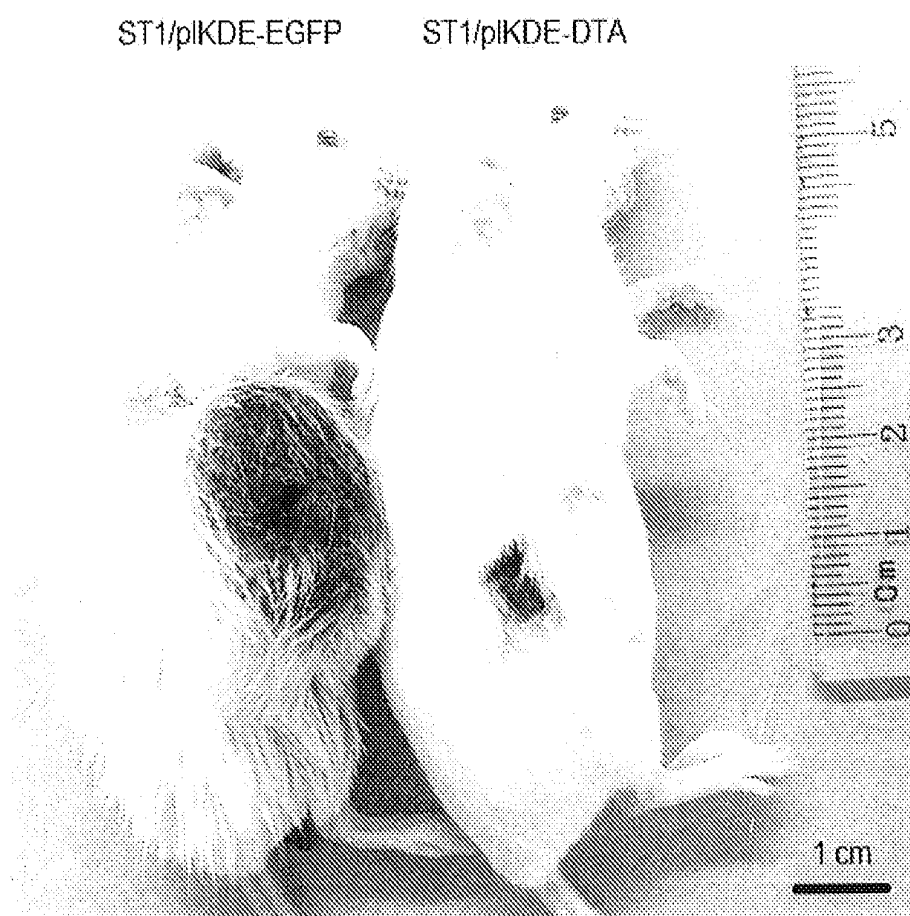
Figs. 54E



Figs. 55A



Figs. 55B



Figs. 55C

1 PCAATATFGG CCATTAGCCA TATTATTCAT TGGTTATATA GCATAAATCA
 51 ATATTGGCTA TTGGCCATTG CATACTTGT ATCTATATCA TAATATGTAC
 101 ATTTATATTG GCTCATGTCC AATATGACCG CCATGTTGGC ATTGATTATT
 151 GACTAGTTAT TAATAGTAAT CAATTACGGG GTCATTAGTT CATAGCCCAT
 201 ATATGGAGTT CCGCGTTACA TAACTTACGG TAAATGGCCC GCCTGGCTGA
 251 CCGCCCAACG ACCCCCGCCC ATTGACGTCA ATAATGACGT ATGTTCCCAT
 301 AGTAACGCCA ATAGGGACTT TCCATTGACG TCAATGGGTG GAGTATTTAC
 351 GGTAAACTGC CCACCTGGCA GTACATCAAG TGTATCATAT GCCAAGTACG
 401 CCCCCTATTG ACGTCAATGA CGGTAAATGG CCCGCCCTGGC ATTATGCCCA
 451 GTACATGACC TTATGGGACT TTCCTACTTG GCAGTACATC TACGTATTAG
 501 TCATCGCTAT TACCATGGTG ATGCGGTTTT GGCAGTACAT CAATGGGCGT
 551 GGATAGCGGT TTGACTCAGC GGGATTTCCA AGTCTCCACC CCATTGACGT
 601 CAATGGGAGT TTGTTTTGGC ACCAAAATCA ACGGGACTTT CCAAATGTC
 651 GTAACAATC CGCCCCATTG ACGCAAATGG GCGGTAGGCG TGTACGGTGG
 701 GAGGTCTATA TAAGCAGAGC TCTCTGGCTA ACTAGAGAAC CCACTGCTTA
 751 CTGGCTTATC GAAATTAATA CGACTCACTA TAGGGAGACC CAAGCTGGCT
 801 AGAGCCCCCTC TCCCTCCCCC CCCCCTAACG TTACTGGCCG AAGCCGCTTG
 851 GAATAAGGCC GGTGTGCGTT TGTCTATATG TTATTTTCCA CCATATFGCC
 901 GTCTTTTGGC AATGTGAGGG CCCGGAAACC TGGCCCTGTC TTCTTGACGA
 951 GCATTCCCTAG GGGTCTTTCC CCTCTCGCCA AAGGAATGCA AGGTCTGTGT
 1001 AATGTCTGTA AGGAAGCAGT TCCTCTGGAA GCTTCTTGAA GACAAACAAC
 1051 GTCTGTAGCG ACCCTTTGCA GGCAGCGGAA CCCCCACCT GGCGACAGGT
 1101 GCCTCTGCGG CCAAAGCCA CGTGTATAAG ATACACCTGC AAAGGCGGCA
 1151 CAACCCAGT GCCACGTGTG GAGTTGGATA GTTGTGGAAA GAGTCAAATG
 1201 GCTCTCCTCA AGCGTATTCA ACAAGGGGCT GAAGGATGCC CAGAAGGTAC
 1251 CCCATTGTAT GGGATCTGAT CTGGGGCCTC GGTGCACATG CTTTACATGT
 1301 GTTTAGTCTA GGTAAAAAA ACGTCTAGGC CCCCCGAACC ACGGGGACGT
 1351 GGTTCCTCTT TGA AAAACAC GATGATAATA TGCTAGCATT CTAGAGCCAC
 1401 CATGGGAAAC ACTCAAATCC TGGTATTGCG TCTGATTGCG ATCATTCCAG
 1451 CAAATGCAGA CAAAATCTGC CTCGGACATC ATGCCGTGTC AAACGGAAAC
 1501 AAAGTAAACA CATTAATGA AAGAGGAGTG GAAGTCGTCA ATGCAACTGA
 1551 AACAGTGGAA CGAACAAACA TCCCAGGAT CTGCTCAAAA GGGAAAAGGA
 1601 CAGTTGACCT CGGTCAATGT GGAATCCTGG GGACAATCAC TGGACCCTT
 1651 CAATGTGACC AATTCCTAGA ATTTTCAGCC GATTTAATTA TTGAGAGGCG
 1701 AGAAGGAAGT GATGTCTGTT ATCCTGGGAA ATTCGTGAAT GAAGAAGCTC
 1751 TGAGGCAAAT TCTCAGAGAA TCAGGCGGAA TTGACAAGGA AGCAATGGGA
 1801 TTCACATACA GTGGAATAAG AACTAATGGA GCAACCAGTG CATGTAGGAG
 1851 ATCAGGATCT TCATCTATG CAGAAATGAA ATGGCTCCTG TCAAACACAG
 1901 ATAATGCTGC ATTCCCGCAG ATGACTAAGT CATATAAAAA TACAAGAAAA
 1951 AGCCCAGCTC TAATAGTATG GGGGATCCAT CATTCGGTAT CAACTGCAGA
 2001 GCAAACCAAG CTATATGGGA GTGGAAACAA ACTGGTGACA GTPGGGAGTT
 2051 CTAATTATCA ACAATCTTTT GTACCGAGTC CAGGAGCGAG ACCACAAGTT

Figs. 56A

2101 AATGGTCTAT CTGGAAGAAT TGACTTTCAT TGGCTAATGC TAAATCCCAA
 2151 TGATACAGTC ACTTTCAGTT TCAATGGGGC TTTCATAGCT CCAGACCGTG
 2201 CAAGCTTCCT GAGAGGAAAA TCTATGGGAA TCCAGAGTGG AGTACAGGTT
 2251 GATGCCAATT GTGAAGGGGA CTGCCATCAT AGTGGAGGGA CAATAATAAG
 2301 TAACTTGCCA TTTCAGAACA TAGATAGCAG GGCAGTTGGA AAATGTCCGA
 2351 GATATGTTAA GCAAAGGAGT CTGCTGCTAG CAACAGGGAT GAAGAATGTT
 2401 CCTGAGATTC CAAAGGGAAG AGGCCTATTT GGTGCTATAG CGGGTTTCAT
 2451 TGAAAATGGA TGGGAAGGCC TAATTGATGG TTGGTATGGT TTCAGACATC
 2501 AAAATGCACA GGGAGAGGGA ACTGCTGCAG ATTACAAAAG CACTCAATCG
 2551 GCAATTGATC AAATAACAGG AAAATTAAAC CGGCTTATAG AAAAAACCAA
 2601 CCAACAATPT GAGTTGATAG ACAATGAATT CAATGAGGTA GAGAAGCAAA
 2651 TCGGTAATGT GATAAATTGG ACCAGAGATT CTATAACAGA AGTGTGGTCA
 2701 TACAATGCTG AACTCTTGGT AGCAATGGAG AACCAGCATA CAATGATCT
 2751 GGCTGATTC AAAATGGACA AACTGTACGA ACGAGTGAAA AGACAGCTGA
 2801 GAGAGAATGC TGAAGAAGAT GGCACTGGTT GCTTTGAAAT ATTTACAAG
 2851 TGTGATGATG ACTGTATGGC CAGTATTAGA AATAACACCT ATGATCACAG
 2901 CAAATACAGG GAAGAGGCAA TGCAAAATAG AATACAGATT GACCCAGTCA
 2951 AACTAAGCAG CCGCTACAAA GATGTGATAC TTTGGTTTAG CTTCCGGGCA
 3001 TCATGTTTCA TACTTCTAGC CATTGTAATG GGCCTTGTCT TCATATGTGT
 3051 AAAGAATGGA AACATGCGGT GCACTATTTG TATATAAGTT GAAGCACTAA
 3101 CTGCAGTCGA GTCTAGAGGG CCGGTTTAAA CCCGCTGATC AGCCTCGACT
 3151 GTGCCTTCTA GTTGCCAGCC ATCTGTTGTT TGCCCTCCC CCGTGCCTTC
 3201 CTTGACCCTG GAAGGTGCCA CTCCCCTGT CCTTTCCTAA TAAAATGAGG
 3251 AAATTGCATC GCATTGTCTG AGTAGGTGTC ATTCTATTCT GGGGGGTGGG
 3301 GTGGGGCAGG ACAGCAAGGG GGAGGATTGG GAAGACAATA GCAGGCATGC
 3351 TGGGGATCGG GTGGGCTCTA TGGTAATACG ACTCACTATA GGGAGACCGC
 3401 GGGCCCGGGA TCCGCCCTC TCCCTCCCC CCCCTAACG TTACTGGCCG
 3451 AAGCCGCTTG GAATAAGGCC GGTGTGCGTT TGTCTATATG TTATTTCCA
 3501 CCATATTGCC GTCTTTTGGC AATGTGAGGG CCCGGAACC TGGCCCTGTC
 3551 TTCTTGACGA GCATTCCTAG GGGTCTTTC CCTCTCGCA AAGGAATGCA
 3601 AGGTCTGTG AATGTCTGA AGGAAGCAGT TCCTCTGGAA GCTTCTTGAA
 3651 GACAAACAAC GTCTGTAGCG ACCCTTTGCA GGCAGCGGAA CCCCCACCT
 3701 GCGCACAGGT GCCTCTGCGG CCAAAGCCA CGTGTATAAG ATACACCTGC
 3751 AAAGCGGGCA CAACCCAGT GCCACGTTGT GAGTTGGATA GTTGTGAAA
 3801 GAGTCAAATG GCTCTCCTCA AGCGTATTCA ACAAGGGGCT GAAGGATGCC
 3851 CAGAAGGTAC CCCATTGTAT GGGATCTGAT CTGGGGCTC GGTGCACATG
 3901 CTTTACATGT GTTTAGTCGA GGTAAAAAA ACGTCTAGGC CCCCCAACC
 3951 ACGGGGACGT GGTTCCTT TGA AAAACAC GATGATAATA TGGCCACAAC
 4001 TAGACCACCA TGAACACGAT TAACATCGCT AAGAACGACT TCTCTGACAT
 4051 CGAACTGGCT GCTATCCCGT TCAACACTCT GGCTGACCAT TACGGTGAGC
 4101 GTTAGCTCG CGAACASTG GCCCTTGAGC ATGAGTCTTA CGAGATGGGT

4151 GAAGCACGCT TCCGCAAGAT GTTTGAGCGT CAACTTAAAG CTGGTGAGGT

Figs. 56B

4201 TGCGGATAAC GCTGCCGCCA AGCCTCTCAT CACTACCCTA CTCCTAAGA
4251 TGATTGCACG CATCAACGAC TGGTTTGAGG AAGTGAAAGC TAAGCGCGGC
4301 AAGGCCCCGA CAGCCTTCCA GTTCTGCAA GAAATCAAGC CGGAAGCCGT
4351 AGCGTACATC ACCATTAAGA CCACTCTGGC TTGCCTAACC AGTGCTGACA
4401 ATACAACCGT TCAGGCTGTA GCAAGCGCAA TCGGTGGGC CATTGAGGAC
4451 GAGGCTCGCT TCGGTGATAT CCGTGACCTT GAAGCTAAGC ACTTCAAGAA
4501 AAACGTTGAG GAACAACCTCA ACAAGCGCGT AGGGCACGTC TACAAGAAAG
4551 CATTATGCA AGTTGTGAG GCTGACATGC TCTTAAGGG TCTACTCGGT
4601 GCGGAGGCGT GGTCTTCGTG GCATAAGGAA GACTCTATTC ATGTAGGAGT
4651 ACGCTGCATC GAGATGCTCA TTGAGTCAAC CGGAATGGTT AGCTTACACC
4701 GCCAAAATGC TGGCGTAGTA GGTCAAGACT CTGAGACTAT CGAACTCGCA
4751 CCTGAATACG CTGAGGCTAT CGCAACCCGT GCAGGTGCGC TGGCTGGCAT
4801 CTCTCCGATG TTCCAACCTT GCGTAGTTC TCCTAAGCCG TGGACTGGCA
4851 TTACTGGTGG TGGCTATTGG GCTAACGGTC GTCGTCTCTT GCGCTGGTG
4901 CGTACTCACA GTAAGAAAGC ACTGATGCGC TACGAAGACG TPTACATGCC
4951 TGAGGTGTAC AAAGCGATTA ACATTGCGCA AAACACCGCA TGGAAAATCA
5001 ACAAGAAAGT CCTAGCGGTC GCCAACGTAA TCACCAAGTG GAAGCATTGT
5051 CCGGTGAGG ACATCCCTGC GATTGAGCGT GAAGAACTCC CGATGAAACC
5101 GGAAGACATC GACATGAATC CTGAGGCTCT CACCGCGTGG AAACCTGCTG
5151 CCGCTGCTGT GTACCGCAAG GACAAGGTC GCAAGTCTCG CCGTATCAGC
5201 CTTGAGTTCA TGCTTGAGCA AGCCAATAAG TTTGCTAACC ATAAGGCCAT
5251 CTGGTTCCCT TACAACATGG ACTGGCGCGG TCGTGTTTAC GCTGTGCAA
5301 TGTTCAACCC GCAAGGTAAC GATATGACCA AAGGACTGCT TACGCTGGCG
5351 AAAGGTAAAC CAATCGGTA GGAAGGTTAC TACTGGCTGA AAATCCACGG
5401 TGCAAACCTGT GCGGGTGTG ATAAGGTTCC GTTCCCTGAG CGCATCAAGT
5451 TCATTGAGGA AAACCACGAG AACATCATGG CTTGGCTAA GTCTCCACTG
5501 GAGAACAACCTT GGTGGGCTGA GCAAGATTCT CCGTTCTGCT TCCTTGCGTT
5551 CTGCTTTGAG TACGCTGGGG TACAGCACCA CGGCCTGAGC TATAACTGCT
5601 CCCTCCGCT GCGGTTTGAC GGTCTTGCT CTGGCATCCA GCACCTCTCC
5651 GCGATGCTCC GAGATGAGGT AGGTGGTCCG GCGGTTAACT TGCTTCTAG
5701 TGAAACCGTT CAGGACATCT ACGGGATTGT TGCTAAGAAA GTCAACGAGA
5751 TTCTACAAGC AGACGCAATC AATGGGACCG ATAACGAAGT AGTTACCGTG
5801 ACCGATGAGA ACACTGGTGA AATCTCTGAG AAAGTCAAGC TGGGCACTAA
5851 GGCCTGGCT GGTCAATGGC TGGCTTACGG TGTTACTCGC AGTGACTACTA
5901 AGCGTTCAGT CATGACGCTG GCTTACGGGT CCAAAGAGTT CCGCTTCCGT
5951 CAACAAGTGC TGGAAGATC CATTCAGCCA GCTATTGATT CCGGCAAGGG
6001 TCTGATGTTT ACTCAGCCGA ATCAGGCTGC TGGATACATG GCTAAGCTGA
6051 TTTGGGAATC TGTGAGCGTG ACGGTGGTAG CTGCGGTTGA AGCAATGAAC
6101 TGGCTTAAAGT CTGCTGCTAA GCTGCTGGCT GCTGAGGTCA AAGATAAGAA
6151 GACTGGAGAG ATTCTTCGCA AGCGTTGCGC TGTGCATTGG GTAACCTCTG

6201 ATGGTTTCCC TGTGTGGCAG GAATACAAGA AGCCTATCA GACGCGCTTG
6251 AACCTGATGT TCCTCGGTCA GTTCCGCTTA CAGCCTACCA TTAACACCAA

Figs. 56C

6301 CAAAGATAGC GAGATTGATG CACACAAACA GGAGTCTGGT ATCGCTCCTA
6351 ACTTTGTACA CAGCCAAGAC GGTAGCCACC TTCGTAAGAC TGTAGTGTGG
6401 GCACACGAGA AGTACGGAAT CGAATCTTTT GCACTGATTC ACGACTCCTT
6451 CGGTACCATT CCGGCTGACG CTGCCAACCT GTTCAAAGCA GTGCGCGAAA
6501 CTATGGTGA CACATATGAG TCTTGTGATG TACTGGCTGA TTTCTACGAC
6551 CAGTTOGCTG ACCAGTTGCA CGAGTCTCAA TTGGACAAA TGCCAGCACT
6601 FCCGGCTAAA GGTAACCTGA ACCTCCGTGA CATCTTAGAG TCGGACTTCG
6651 CGTTCGCGGA TCCAAAAAAG AAGAGAAAGG TAACTAGTGC GGCCGCTTCC
6701 CTTTAGTGAG GGTAAATGCT TCGAGCAGAC ATGATAAGAT ACATTGATGA
6751 GTTTGGACAA ACCACAACCTA GAATGCAGTG AAAAAAATGC TTTATTTGTG
6801 AAAPPTGTGA TGCTATFGCT TTATTGTAA CCATTATAAG CTGCAATAAA
6851 CAAGTTAACA ACAACAATTG CATTCAATTT AIGTTTCAGG TTCAGGGGGA
6901 GATGTGGGAG GTTTTTTAAA GCAAGTAAA CCTCTACAAA TGTGGTAAAA
6951 TCCGATAAGG ATCGATCCGG GCTGGCGTAA TAGCGAAGAG GCCCGCACCG
7001 ATCGCCCTTC CCAACAGTTC CGCAGCCTGA ATGGCGAATG GACGCGCCCT
7051 GTAGCGGCGC APTAAGCGCG GCGGGTGTGG TGGTTACGCG CAGCGTGACC
7101 GCTACACTTG CCAGCGCCCT AGCGCCCGCT CCTTTCGCTT TCTTCCCTTC
7151 CTTTCTCGCC ACGTTCGCCG GCTTTCCTCG TCAAGCTCTA AATCGGGGCG
7201 FCCCTTAGG GTTCCGATTT AGAGCTTTAC GGCACCTCGA CCGCAAAAA
7251 CTTGATTTGG GTGATGGTTC ACGTAGTGG CCATCGCCCT GATAGACGGT
7301 TTTTCGCCCT TTGACGTTGG AGTCCACGTT CTTAATAGT GGACTCTTGT
7351 TCCAAACTGG AACACACTC AACCCATCT CGGTCTATTC TTTGATTTA
7401 TAAGGGATTT TGCCGATTC GGCCTATTGG TTAATAAATG AGCTGATTTA
7451 ACAAAATATT AACCGGAATT TTAACAAAAT ATTAACGTTT ACAATTTCCG
7501 CTGATGCGGT ATTTCTCCTT TACGCATCTG TCGGGTATTT CACACCGCAT
7551 ACGCGGATCT GCGCAGCACC ATGGCCTGAA ATAACCTCTG AAAGAGGAAC
7601 TTGGTTAGGT ACCTTCTGAG GCGGAAAGAA CCAGCTGTGG AATGTGTGTC
7651 AGTTAGGGTG TGGAAAGTCC CCAGGCTCCC CAGCAGGCAG AAGTATGCAA
7701 AGCATGCATC TCAATTAGTC AGCAACCAGG TGTGGAAAGT CCCCAGGCTC
7751 CCCAGCAGGC AGAAGTATGC AAAGCATGCA TCTCAATTAG TCAGCAACCA
7801 TAGTCCCGCC CCTAACTCCG CCCATCCCGC CCCTAACTCC GCCAGTTCC
7851 GCCCATCTC CGCCCCATGG CTGACTAATT TTTTTTATTT ATGCAGAGGC
7901 CGAGGCCGCC TCGCCCTCTG AGCTATFCCA GAAGTAGTGA GGAGGCTTTT
7951 TTGGAGGCTT TTTACTTATT TACAGAACTT CGGCATTATC TTGCCGGTTC
8001 AAATTACGGT AGTGATACCC CAGAGGATTA GATGGCCAAA GAAGACAATA
8051 TTGAAATGCA AGGTACCGTT CTTGAAACGT TGCCTAATAC CATGPTCCGC
8101 GTAGAGTTAG AAAACGGTCA CGTGGTACT GCACACATCT CCGGTAAAAT
8151 GCGCAAAAC TACATCCGCA TCCTGACGGG CGACAAAGTG ACTGTTGAAC
8201 TGACCCCGTA CGACCTGAGC AAAGGCCGCA TTGTCTCCG TAGTCGCTGA

8251 TTGTTTTACC GCCTGATGGG CGAAGAGAAA GAACGAGTAA AAGGTCCGTT
 8301 TAACCGGCCT TTTGCCGCGA TGCCCGACGG CGAGGATCTC GTCGTGACCC
 8351 AFGCGCATGC CTGCTTGCCG AATATCATGG TGGAAAATGG CCGCTTTTCT

Figs. 56D

8401 GGATTCATCG ACTGTGGCCG GCTGGGTGTG GCGGACCGCT ATCAGGACAT
 8451 AGCGTTGGCT ACCCGTGATA TTGCTGAAGA GCTTGGCGGC GAATGGGCTG
 8501 ACCGCTTCCT CGTGCTTTAC GGTATCGCCG CTCCCGATTC GCAGCGCATC
 8551 GCCTTCTATC GCCTTCTTGA CGAGTTCTTC TGAGCGGGAC TCTGGGGTTC
 8601 GAAATGACCG ACCAAGCGAC GCCCAACCTG CCATCACGAT GGCCGCAATA
 8651 AATATCTTT ATTTTCATTA CATCTGTGTG TTGGTTTTTT GTGTGAATCG
 8701 ATAGCGATAA GGATCCGCGT ATGGTGCAC TCACTACAA TCTGCTCTGA
 8751 TGCCGCATAG TTAAGCCAGC CCCGACACC GCCAACACC GCTGACCGCG
 8801 CCTGACGGGC TTGTCTGCTC CCGGCATCCG CTTACAGACA AGCTGTGACC
 8851 GTCTCCGGGA GCTGCATGTG TCAGAGGTTT TCACCGTCAT CACCGAAACG
 8901 CGCGAGACGA AAGGGCCTCG TGATACGCCT ATTTTTATAG GTTAATGTCA
 8951 TGATAATAAT GGTTCCTTAG ACGTCAGGTG GCACTTTTCG GGGAAATGTG
 9001 CGCGGAACCC CTATTTGTTT ATTTTCTAA ATACATTCAA ATATGTATCC
 9051 GCTCATGAGA CAATAACCC TATAAATGCT TCAATAATAT TGAAAAGGA
 9101 AGAGTATGAG TATTCAACAT TTCCGTGTG CCGTTATPCC CTTTTTTGCG
 9151 GCATTTTGCC TTCCTGTTTT TGCTCACCCA GAAACGCTGG TGAAGTAAA
 9201 AGATGCTGAA GATCAGTTGG GTGCACGAGT GGGTTACATC GAACTGGATC
 9251 TCAACAGCGG TAAGATCCTT GAGAGTTTT CCCCCGAAGA ACGTTTTCCA
 9301 ATGATGAGCA CTTTTAAAGT TCTGCTATGT GCGCGGTAT TATCCCGTAT
 9351 TGACCCGGG CAAGAGCAAC TCGGTGCGCG CATACTAT TCTCAGAATG
 9401 ACTTGGTTGA GFACTACCA GTCACAGAAA AGCATCTTAC GGATGGCATG
 9451 ACAGTAAGAG AATTATGCAG TGCTGCCATA ACCATGAGTG ATAACACTGC
 9501 GGCCAACTTA CTTCTGACAA CGATCGGAGG ACCGAAGGAG CTAACCGCTT
 9551 TTTTGCACAA CATGGGGGAT CATGTAACTC GCCTTGATCG TTGGGAACCG
 9601 GAGCTGAATG AAGCCATACC AAACGACGAG CGTGACACCA CGATGCCTGT
 9651 AGCAATGGCA ACAACGTTGC GCAACTATT AACTGGCGAA CTACTFACTC
 9701 TAGCTTCCCG GCAACAATTA ATAGACTGGA TGGAGGCGGA TAAAGTTGCA
 9751 GGACCACTTC TGCGCTCGGC CCTTCCGGCT GGCTGGTTTA TTGCTGATAA
 9801 ATCTGGAGCC GGTGAGCGTG GGTCTCGCGG TATCATTGCA GCACTGGGGC
 9851 CAGATGGTAA GCCCTCCCCT ATCGTAGTTA TCTACACGAC GGGGAGTCAG
 9901 GCAACTATGG ATGAACGAAA TAGACAGATC GCTGAGATAG GTGCCTCACT
 9951 GATTAAGCAT TGGTAAGTGT CAGACCAAGT TTACTCATAT ATACTTTAGA
 10001 TTGATTTAAA ACTTCATTTT TAATTTAAAA GGATCTAGGT GAAGATCCTT
 10051 TTTGATAATC TCATGACCAA AATCCCTTAA CGTGAGTTTT CGTTCCACTG
 10101 AGCGTCAGAC CCCGTAGAAA AGATCAAAGG ATCTTCTTGA GATCCTTTTT
 10151 TTCTGCGCGT AATCTGCTGC TTGCAAACAA AAAAACCACC GCTACCAGCG
 10201 GTGGTTTGTG TGCCGGATCA AGAGCTACCA ACTCTTTTTT CGAAGGTAAC
 10251 TGGCTTCAGC AGAGCGCAGA TACCAAATAC TGTCTTCTA GTGTAGCCGT

10301 AGTTAGGCCA CCACTTCAAG AACTCTGTAG CACCGCCTAC ATACCTCGCT
10351 CTGCTAATCC TGTACCAGT GGCTGCTGCC AGTGGCGATA AGTCGTGTCT
10401 TACCGGGTTG GACTCAAGAC GATAGTTACC GGATAAGGCG CAGCGGTCGG
10451 GCTGAACGGG GGGTTCGTGC ACACAGCCCA GCTTGGAGCG AACGACCTAC

Figs. 56E

10501 ACCGAACTGA GATACCTACA GCGTGAGCTA TGAGAAAGCG CCACGCTTCC
10551 CGAAGGGAGA AAGGCGGACA GGTATCCGGT AAGCGGCAGG GTCGGAACAG
10601 GAGAGCGCAC GAGGGAGCTT CCAGGGGGAA ACGCCTGGTA TCTTTATAGT
10651 CCTGTCGGGT TTCGCCACCT CTGACTTGAG CGTCGATTTT TGTGATGCTC
10701 GTCAGGGGGG CGGAGCCTAT GGAAAACGC CAGCAACGCG GCCTPTTTAC
10751 GGTTCCTGGC CTTTGTCTGG CCTTTTGCTC ACATGGCTCG ACAGATCT

Figs. 56F

1 TCAATATTGG CCATTAGCCA TATTATTCAT TGGTTATATA GCATAAATCA
 51 ATATTGGCTA TTGGCCATTG CATACGTTGT ATCTATATCA TAATATGTAC
 101 APTTAFATTG GCTCATGTCC AATATGACCG CCATGTTGGC ATTGATTATT
 151 GACTAGTTAT TAATAGTAAT CAATTACGGG GTCATTAGTT CATAGCCCAT
 201 ATATGGAGTT CCGCGTTACA TAACTTACGG TAAATGGCCC GCCTGGCTGA
 251 CCGCCCAACG ACCCCCGCCC ATFGACGTCA ATAATGACGT ATGTTCCCAT
 301 AGTAACGCCA ATAGGGACTT TCCATTGACG TCAATGGGTG GAGTATTIAC
 351 GGTAACCTGC CCACTTGGCA GTACATCAAG TGTATCATAT GCCAAGTCCG
 401 CCCCCTATTG ACGTCAATGA CGGTAAATGG CCCGCCCTGGC ATTATGCCCA
 451 GTACATGACC TTACGGGACT TTCCTACTTG GCAGTACATC TACGTATTAG
 501 TCATCGCTAT TACCATGGTG ATGCGGTTTT GGCAGTACAT CAATGGGCGT
 551 GGATAGCGGT TTGACTCACG GGGATTTCCA AGTCTCCACC CCATTGACGT
 601 CAATGGGAGT TTGTTTTGGC ACCAAAATCA ACGGGACTTT CCAAAATGTC
 651 GTAACAACCTC CGCCCCATTG ACGCAAATGG GCGGTAGGCG TGTACGGTGG
 701 GAGGTCTATA TAAGCAGAGC TCTCTGGCTA ACTAGAGAAC CCACTGCTTA
 751 CTGGCTTATC GAAATTAATA CGACTCACTA TAGGGAGACC CAAGCTGGCT
 801 AGAGCCCTC TCCCTCCCC CCCCTAACG TFACTGGCCG AAGCCGCTTG
 851 GAATAAGGCC GGTGTGCGTT TGTCTATATG TTATTTTCCA CCATATTGCC
 901 GTCTTTTGGC AATGTGAGGG CCCGGAAACC TGGCCCTGTC TTCTTGACGA
 951 GCATTCTAG GGTCTTTCC CCTCTCGCCA AAGGAATGCA AGGTCTGTTG
 1001 AATGTCTGA AGGAAGCAGT TCCTCTGGAA GCTTCTTGAA GACAAACAAC
 1051 GTCTGTAGCG ACCCTTTGCA GGCAGCGGAA CCCCCACCT GCGCAGAGGT
 1101 GCCTCTGCGG CCAAAAGCCA CGTGTATAAG ATACACCTGC AAAGGCGGCA
 1151 CAACCCAGT GCCACGTTGT GAGTTGATA GTTGTGAAA GAGTCAAATG
 1201 GCTCTCCTCA AGCGTATTCA ACAAGGGGCT GAAGGATGCC CAGAAGGTAC
 1251 CCCATTGTAT GGGATCTGAT CTGGGGCCTC GGTGCACATG CTTTACATGT
 1301 GTTTAGTCGA GGTAAAAAA ACGTCTAGGC CCCCCGAACC ACGGGGACGT
 1351 GGTTCCTT TGAAAAACAC GATGATAATA TGCTAGCGAA TTCTGCAGAT
 1401 ATCCAGCACA GTGGCGGCCG CTCGAGTCTA GACCACCATG AACACGATTA
 1451 ACATCGCTAA GAACGACTTC TCTGACATCG AACTGGCTGC TATCCCGTTC
 1501 AACACTCTGG CTGACCATTA CGGTGAGCGT TTAGCTCGCG AACAGTTGGC
 1551 CCTTGAGCAT GAGTCTTACG AGATGGGTGA AGCACGCTTC CGCAAGATGT
 1601 TTGAGCGTCA ACTTAAAGCT GGTGAGGTTG CGGATAACGC TGCCGCCAAG
 1651 CCTCTCATCA CTACCCTACT CCCTAAGATG ATGACAGCA TCAACGACTG
 1701 GTTTGAGGAA GTGAAAGCTA AGCGCGGCAA GCGCCGACA GCCTTCCAGT

1751 TCCTGCAAGA AATCAAGCCG GAAGCCGTAG CGTACATCAC CATTAAAGACC
 1801 ACTCTGGCTT GCCTAACCAG TGCTGACAAT ACAACCGTTC AGGCTGTAGC
 1851 AAGCGCAATC GGTTCGGCCA TTGAGGACGA GGCTCGCTTC GGTCGTATCC
 1901 GTGACCTTGA AGCTAAGCAC TTCAAGAAAA ACGTTGAGGA ACAACTCAAC
 1951 AAGCGCGTAG GGCACGTCTA CAAGAAAGCA TTTATGCAAG TTGTCGAGGC

Figs. 57A

2001 TGACATGCTC TCTAAGGGTC TACTCGGTGG CGAGGCGTGG TCTTCGTGGC
 2051 ATAAGGAAGA CTCTATTCAT GTAGGAGTAC GCTGCATCGA GATGCTCATT
 2101 GAGTCAACCG GAATGGTTAG CTTACACCGC CAAAATGCTG GCGTAGTAGG
 2151 TCAAGACTCT GAGACTATCG AACTCGCACC TGAATACGCT GAGGCTATCG
 2201 CAACCCGTGC AGGTGCGCTG GCTGGCATCT CTCCGATGTT CCAACCTTGC
 2251 GTAGTTCCTC CTAAGCCGTG GACTGGCATT ACTGGTGGTG GCTATTGGGC
 2301 TAACGGTCGT CGTCCCTCTG CGCTGGTGGG TACTCACAGT AAGAAAGCAC
 2351 TGATGCGCTA CGAAGACGTT TACATGCCCTG AGGTGTACAA AGCGATTAAC
 2401 ATTGCGCAAA ACACCGCATG GAAAATCAAC AAGAAAGTCC TAGCGGTGCG
 2451 CAACGTAATC ACCAAGTGGG AGCATTGTCC GGTGAGGAC ATCCCTGCGA
 2501 TTGAGCGTGA AGAACTCCCG ATGAAACCGG AAGACATCGA CATGAATCCT
 2551 GAGGCTCTCA CCGCGTGGAA ACGTGTGCC GCTGTGTGT ACCGCAAGGA
 2601 CAAGGCTCGC AAGTCTCGCC GTATCAGCCT TGAGTTCATG CTTGAGCAAG
 2651 CCAATAAGTT TGCTAACCAT AAGGCCATCT GGTTCCTTA CAACATGGAC
 2701 TGGCGCGGTC GTGTTTACGC TGTGTCAATG TTCAACCCGC AAGGTAACGA
 2751 TATGACCAA GGACTGCTTA CGCTGGCGAA AGGTAAACCA ATCGGTAAGG
 2801 AAGGTTACTA CTGGCTGAAA ATCCACGGTG CAAACTGTGC GGGTGTGAT
 2851 AAGGTTCCTG TCCCTGAGCG CATCAAGTTC ATTGAGGAAA ACCACGAGAA
 2901 CATCATGGCT TCGCTAAGT CTCCACTGGA GAACACTTGG TGGGCTGAGC
 2951 AAGATTCTCC GTTCTGTTC CTTGCGTTC TCTTTGAGTA CGCTGGGGTA
 3001 CAGCACCAGC GCCTGAGCTA TAACTGCTCC CTTCCGCTGG CGTTTGACGG
 3051 GTCTTGCTCT GGCATCCAGC ACTTCTCCGC GATGCTCCGA GATGAGGTAG
 3101 GTGGTCCCGC GGTTAACCTG CTTCCTAGTG AAACCGTTCA GGACATCTAC
 3151 GGGATTGTTG CTAAGAAAGT CAACGAGATT CTACAAGCAG ACGCAATCAA
 3201 TGGGACCGAT AACGAAGTAG TTACCGTGAC CGATGAGAAC ACTGGTGAAA
 3251 TCTCTGAGAA AGTCAAGCTG GGCATAAGG CACTGGCTGG TCAATGGCTG
 3301 GCTTACGGTG TTACTIONCAG TGTACTAAG CGTTCAGTCA TGACGCTGGC
 3351 TTACGGGTCC AAAGAGTTCG GCTTCCGTCA ACAAGTGTCTG GAAGATACCA
 3401 TTCAGCCAGC TATTGATTC GGCAGGGGTC TGATGTTTAC TCAGCCGAAT
 3451 CAGGCTGCTG GATACATGGC TAAGCTGATT TGGGAATCTG TGAGCGTGAC
 3501 GGTGGTAGCT GCGGTTGAAG CAATGAACTG GCTTAAGTCT GCTGCTAAGC
 3551 TGCTGGCTGC TGAGGTCAAA GATAAGAAGA CTGGAGAGAT TCTTCGCAAG
 3601 CGTTGCGCTG TGCATTGGGT AACTCCTGAT GGTTCCTCTG TGTGGCAGGA

3651 ATACAAGAAG CCTATTCAGA CGCGCTTGAA CCTGATGTTC CTCGGTFCAGT
3701 TCCGCTTACA GCCTACCATT AACACCAACA AAGATAGCGA GATTGATGCA
3751 CACAAACAGG AGTCTGGTAT CGCTCCTAAC TTTGTACACA GCCAAGACGG
3801 TAGCCACCTT CGTAAGACTG TAGTGTGGGC ACACGAGAAG TACGGAATCG
3851 AATCTTTTGC ACTGATTAC GACTCCTTCG GTACCATTCC GGCTGACGCT
3901 GCGAACCTGT TCAAAGCAGT GCGCGAAACT ATGTTGACA CATATGAGTC
3951 TTGTGATGTA CTGGCTGATT TCTACGACCA GTTCGCTGAC CAGTTGCACG
4001 AGTCTCAATT GGACAAAATG CCAGCACTTC CGGCTAAAGG TAACTTGAAC

Figs. 57B

4051 CTCCGTGACA TCTTAGAGTC GGACTTCGGG TTCGCGGATC CAAAAAGAA
4101 GAGAAAGGTA ACTAGTGC GG CCGCTTCCCT TTAGTGAGGG TTAATGCTTC
4151 GAGCAGACAT GATAAGATAC APTGATGAGT TTGGACAAAC CACAAC TAGA
4201 ATGCAGTGAA AAAAAAGCTT TATTTGTGAA ATTTGTGATG CTATTGCTTT
4251 ATTTGTAACC ATTATAAGCT GCAATAACA AGTTAACAAC AACAAATTGCA
4301 TTCATTTTAT GTTTCAGGTT CAGGGGGAGA TGTGGGAGST TTTTAAAGC
4351 AAGTAAAACC TCTACAAATG TGGTAAAATC CGATAAGGAT CGATCCGGGG
4401 TGGCGTAATA GCGAAGAGGC CCGCACCGAT CGCCCTTCCC AACAGTTGCG
4451 CAGCCTGAAT GCGAATGGA CCGGCCCTGT AGCGCGCAT TAAGCGCGGC
4501 GGTGTGGTG GTTACGCGCA GCGTGACCGC TACACTTGCC AGCGCCCTAG
4551 CGCCGCTCC TTTCCGTTTC TTCCCTTCCCT TTCTCGCCAC GTTCCGCGGC
4601 TTTCCCGTCC AAGCTCTAAA TCGGGGGCTC CCTTAGGGT TCCGATTTAG
4651 AGCTTTACGG CACCTCGACC GCAAAAAACT TGATTTGGGT GATGGTTCAC
4701 GTAGTGGGCC ATCGCCCTGA TAGACGGTTT TTCGCCCTTT GACGTTGGAG
4751 TCCACGTTCT TTAATAGTGG ACTCTTGTTC CAACTGGAA CAACACTCAA
4801 CCCTATCTCG GTCTATTCTT TTGATTTATA AGGGATTTTG CCGATTTCCG
4851 CCTATTGGTT AAAAAATGAG CTGATTTAAC AAATATTTAA CGCGAATTTT
4901 AACAAAATAT TAACGTTTAC AATTTCGCCT GATGCGGTAT TTTCTCCTTA
4951 CGCATCTGTG CCGTATTTCA CACCGCATAC GCGGATCTGC GCAGCACCAT
5001 GGCTGAAAT AACCTCTGAA AGAGGAACTT GGTTAGGTAC CTTCTGAGGC
5051 GGAAAGAACC AGCTGTGGAA TGTGTGTCAG TTAGGGTGTG GAAAGTCCCC
5101 AGGCTCCCCA GCAGGCAGAA GTATGCAAAG CATGCATCTC AATTAGTCAG
5151 CAACCAGGTG TGGAAAGTCC CCAGGCTCCC CAGCAGGCAG AAGTATGCAA
5201 AGCATGCATC TCAATTAGTC AGCAACCATA GTCCCGCCCC TAACTCCGCC
5251 CATCCCGCCC CTAACTCCGC CCAGTTCCGC CCATTCTCCG CCCCATGGCT
5301 GACTAATTTT TTTTATTTAT GCAGAGGCCG AGGCCGCCCTC GGCTCTGAG
5351 CTATCCAGA AGTAGTGAGG AGGCTTTTTT GGAGGCCTAG GCTTTTGCAA
5401 AAAGCTTGAT TCTTCTGACA CAACAGTCTC GAACTTAAGG CTAGAGCCAC
5451 CAGATTGAA CAAGATGGAT TGCACGCAGG TTCTCCGGCC GCTTGGGTGG
5501 AGAGGCTATT CCGCTATGAC TGGGCACAAC AGACAATCGG CTGCTCTGAT
5551 GCCCGCGTGT TCCGGCTGTC AGCGCAGGGG CGCCCGGTTT TTTTGTCAA
5601 GACCGACCTG TCCGGTCCCC TGAATGAACT GCAGGACGAG GCAGCGCGGC
5651 TATCGTGGCT GGCCACGACG GCGTTCCTT GCGCAGCTGT GCTCGACGTT

5701 GTCACTGAAG CGGGAAGGGA CTGGCTGCTA TTGGGCGAAG TGCCGGGGCA
5751 GGATCTCCTG TCATCTCACC TTGCTCCTGC CGAGAAAGTA FCCATCATGG
5801 CTGATGCAAT GCGGCGGCTG CATACGCTTG ATCCGGCTAC CTGCCCATTC
5851 GACCACCAAG CGAAACATCG CATCGAGCGA GCACGTACTC GGATGGAAGC
5901 CGGTCTTGTC GATCAGGATG ATCTGGACGA AGAGCATCAG GGGCTCGCGC
5951 CAGCCGAACT GTTCGCCAGG CTCAAGGCGC GCATGCCCGA CCGCGAGGAT
6001 CTCGTCGTGA CCCATGGCGA TGCCCTGCTG CCGAATATCA TGGTGGAAAA
6051 TGGCCGCTTT TCTGGATTCA TCGACTGTGG CCGGCTGGGT GTGGCGGACC
6101 GCTATCAGGA CATAGCGTTG GCTACCCGTG ATATTGCTGA AGAGCTTGGC

Figs. 57C

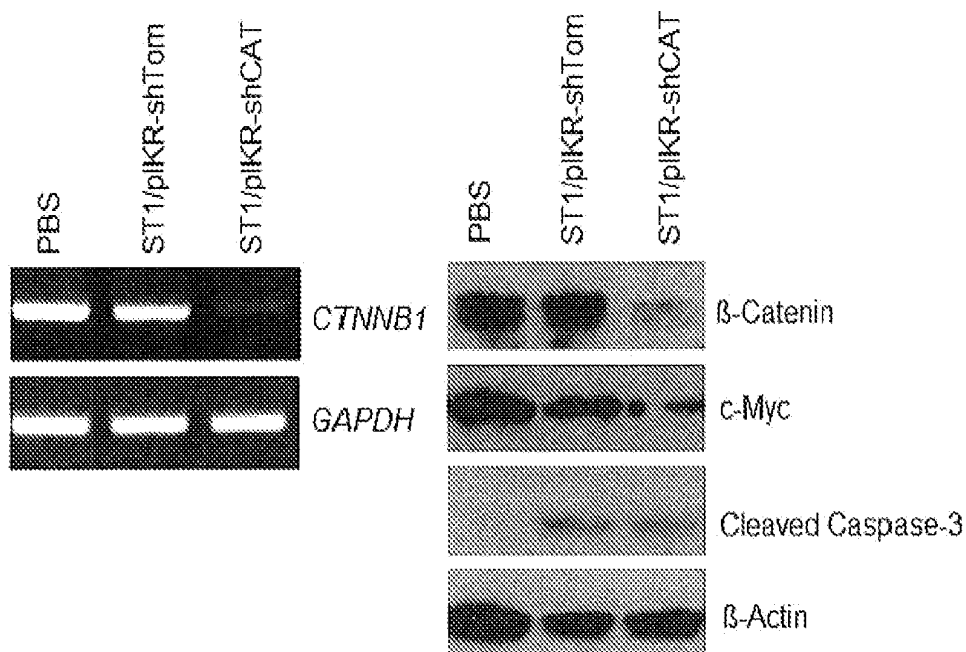
6151 GCGAATGGG CTGACCGCTT CCTCGTGCTT TACGSTATCG CCGCTCCCGA
6201 TTCGCAGCGC ATCGCCTTCT ATCGCCTTCT TGACGAGTTC TTCTGAGCGG
6251 GACTCTGGGG TTCGAAATGA CCGACCAAGC GACGCCAAC CTGCCATCAC
6301 GATGGCCGCA ATAAATATC TTTATTTTCA TTACATCTGT GTGTTGGTTT
6351 TTTGTGTGAA TCGATAGCGA TAAGATCCG CGTATGCTGC ACTCTCAGTA
6401 CAATCTGCTC TGATGCCGCA TAGTTAAGCC AGCCCCGACA CCCGCCAAC
6451 CCCGCTGACG CGCCCTGACG GGCTTGCTCG CTCCCGCAT CCGCTTACAG
6501 ACAAGCTGTG ACCGTCTCCG GGAGCTGCAT GTGTCAGAGG TTTTCACCGT
6551 CATCACGAA ACGCGCGAGA CGAAAGGGCC TCGTGATACG CCTATTTTTA
6601 TAGGTTAATG TCATGATAAT AATGGTTTCT TAGACGTCAG GTGGCACTTT
6651 TCGGGGAAAT GTGCGCGGAA CCCCTATTTG TTTATTTTTC TAAATACATT
6701 CAAATATGTA TCCGCTCATG AGACAATAAC CCTGATAAAT GCTTCAATAA
6751 TATTGAAAAA GGAAGAGTAT GAGTATTCOA CATTCCGTG TCGCCCTTAT
6801 TCCCTTTTTT GCGGCATTTT GCCTTCCTGT TTTTGCTCAC CCAGAAACGC
6851 TGGTGAAGT AAAAGATGCT GAAGATCAGT TGGGTGCACG AGTGGGTTAC
6901 ATCGAAGTGG ATCTCAACAG CGGTAAGATC CTTGAGAGTT TTCGCCCCGA
6951 AGAACGTTTT CCAATGATGA GCACTTTTAA AGTTCGTCTA TGTGGCGCGG
7001 TATTATCCCG TATTGACGCC GGGCAAGAGC AACTCGGTG CCGCATAAC
7051 TATTCTCAGA ATGACTTGGT TGAGTACTCA CCAGTCACAG AAAAGCATCT
7101 TACGGATGGC ATGACAGTAA GAGAATTATG CAGTGCTGCC ATAACCATGA
7151 GTGATAACAC TGCGGCCAAC TTACTTCTGA CAACGATCGG AGGACCGAAG
7201 GAGCTAACCG CTTTTTTGCA CAACATGGGG GATCATGTAA CTCGCCTTGA
7251 TCGTTGGGAA CCGGAGCTGA ATGAAGCCAT ACCAAACGAC GAGCGTGACA
7301 CCACGATGCC TGTAGCAATG GCAACAACGT TGCGCAACT ATTAAGTGGC
7351 GAACTACTTA CTCTAGCTTC CCGCAACAA TTAATAGACT GGATGGAGGC
7401 GGATAAAGTT GCAGGACCAC TTCTGCGCTC GGCCCTTCCG GCTGGCTGGT
7451 TTATTGCTGA TAAATCTGGA GCGGCTGAGC GTGGGTCTCG CCGTATCATT
7501 GCAGCACTGG GGCCAGATGG TAAGCCCTCC CGTATCGTAG TTATCTACAC
7551 GACGGGAGT CAGGCAACTA TGGATGAACG AAATAGACAG ATCGCTGAGA
7601 TAGGTGCCCT ACTGATTAAG CATTGGTAAC TGTCAGACCA AGTTTACTCA
7651 TATATACTTT AGATTGATTT AAAACTTCAT TTTTAATTTA AAAGGATCTA
7701 GGTGAAGATC CTTTTTGATA ATCTCATGAC CAAAATCCCT TAACGTGAGT

7751 TTTCGTTCCA CTGAGCGTCA GACCCCGTAG AAAAGATCAA AGGATCTTCT
7801 TGAGATCCTT TTTTCTGCG CGTAATCTGC TGCTTGCAAA CAAAAAACC
7851 ACCGCTACCA GCGGTGGTTT GPTTGCCGGA TCAAGAGCTA CCAACTCTTT
7901 TTCCGAAGGT AACTGGCTTC AGCAGAGCGC AGATACCAA TACTGTCTTT
7951 CTAGGTAGC CGTAGTTAGG CCACCACTTC AAGAACTCTG TAGCACCGCC
8001 TACATACCTC GCTCTGCTAA TCCTGTTACC AGTGGCTGCT GCCAGTGGCG
8051 ATAAGTCGTG TCTTACCGGG TTGGACTCAA GACGATAGTT ACCGGATAAG
8101 GCGCAGCGGT CGGGCTGAAC GGGGGGTTTCG TGCACACAGC CCAGCTTGA
8151 GCGAACGACC TACACCGAAC TGAGATACCT ACAGCGTGAG CTATGAGAAA

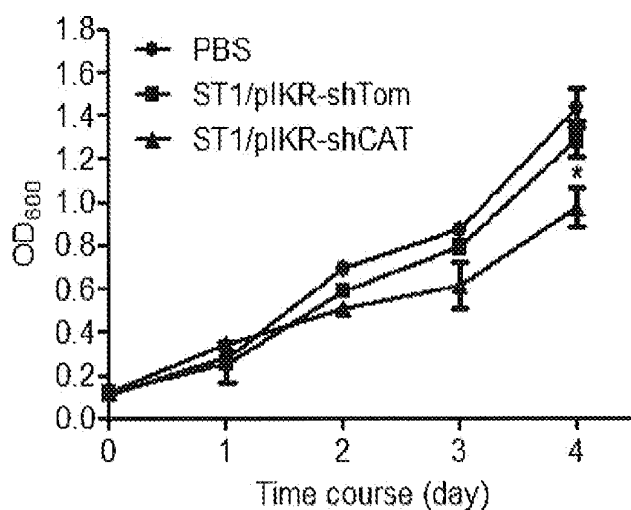
Figs. 57D

8201 GCGCCACGCT TCCCGAAGGG AGAAAGGCGG ACAGGTATCC GGTAAGCGGC
8251 AGGGTCGGAA CAGGAGAGCG CACGAGGGAG CTTCCAGGGG GAAACGCCTG
8301 GTATCTTTAT AGTCCTGTCG GPTTTCGCCA CCTCTGACTT GAGCGTCGAT
8351 TTTTGTGATG CTCGTCAGGG GGGCGGAGCC TATGGAAAAA CGCCAGCAAC
8401 GCGGCCTTTT TACGGTTCCT GGCCTTTTGC TGGCCTTTTG CTCACATGGC
8451 TCGACAGATC TAATACGACT CACTATAGGA GACAGGATCC AGCTGATATT
8501 GATGGACAGT TCAAGAGACT GTCCATCAAT ATCAGCTTTG TCGACTAGCA
8551 TAACCCCTTG GGGCCTCTAA ACGGGTCTTG AGGGGTTTTT TGAGATCT

Figs. 57E



Figs. 58A



Figs. 58B

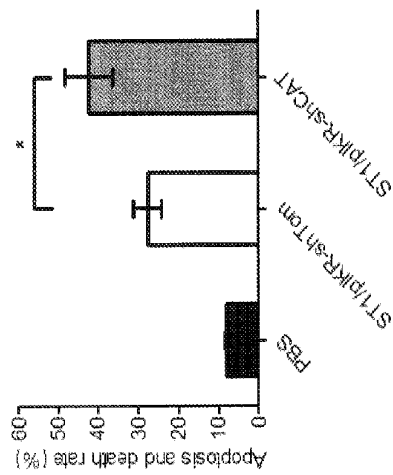
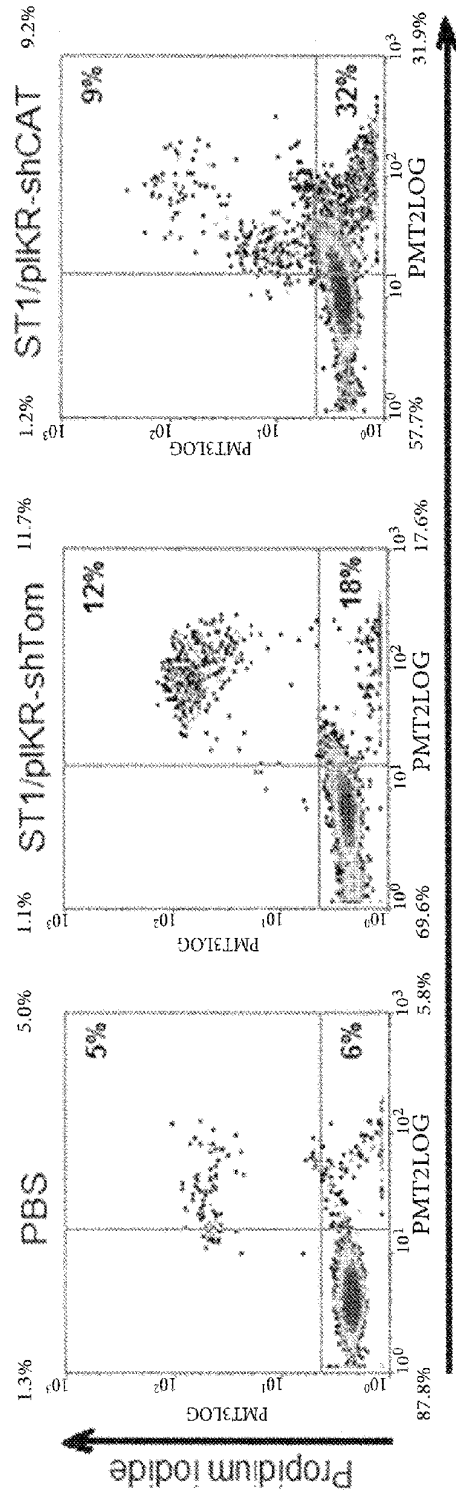
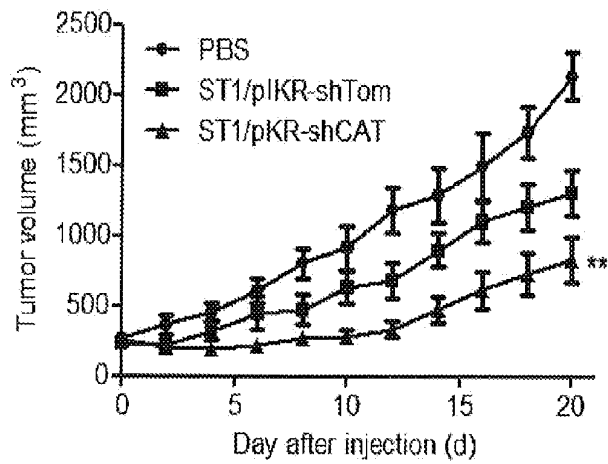
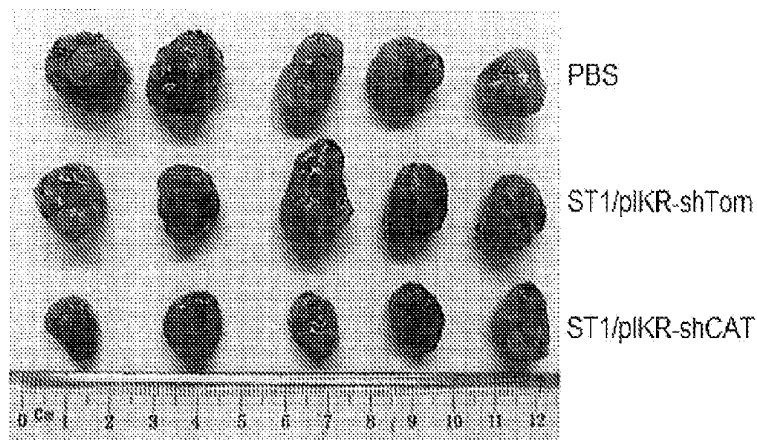


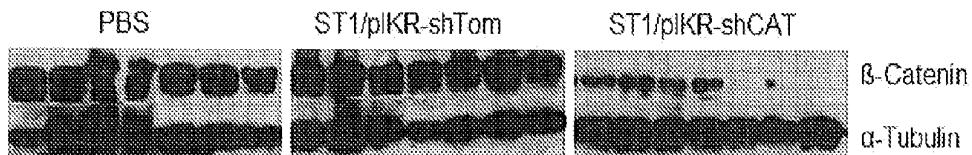
Fig. 58C



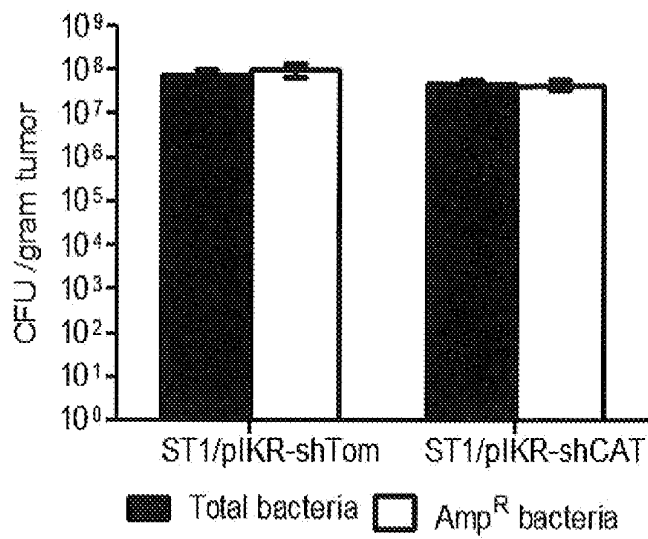
Figs. 59A



Figs. 59B



Figs. 59C



Figs. 59D

THERAPEUTIC DELIVERY AND EXPRESSION SYSTEM, METHODS AND USES THEREOF

1. FIELD OF THE INVENTION

[0001] Disclosed herein is a targeted delivery and expression system of multiple therapeutic molecules. Therapeutic molecules include cellular components such as RNA, DNA, proteins or any combination of at least two of the cellular components. In an embodiment, the delivery and expression system is an inter-kingdom expression system. The system provided herein has great potential to overcome delivery limitations because of their biocompatibility, low toxicity, small size, stable plasmid maintenance, targeted delivery ability, efficient gene transfer, feasible cargoes and low costs. Provided herein are methods and compositions having preventive, diagnostic and therapeutic applications against cancer and infectious diseases. Described herein is a method of treatment of cancer or tumor using a modified bacteria or composition comprising the modified bacteria. In certain embodiments, the method of prevention or treatment of tumor/cancer or influenza or other infectious diseases is in combination with other treatment approaches. In certain embodiments, the treatment against cancer or tumor is chemotherapy, radiation therapy, gene therapy, surgery or a combination thereof. Described herein are vectors, cells comprising the vectors. Also described herein are therapeutic and prophylactic compositions comprising the modified bacteria. In certain embodiments, the therapeutic and prophylactic compositions contain a purified form of the modified bacteria. In certain embodiments, the therapeutic and prophylactic compositions do not contain other strains of microorganisms. In one aspect, the modified bacteria harboring therapeutic cargoes grow within a tumor/cancer, retarding its growth. In one aspect, the tumor/cancer is a solid tumor/cancer. In one aspect, the modified bacteria are rapidly eliminated from normal tissues. In another aspect, the modified bacteria are excellent carriers for vaccine antigens from other bacteria, viruses, parasites and tumors, being able to stimulate strong host immune responses against the corresponding antigens.

2. BACKGROUND OF THE INVENTION

[0002] The low costs, fast production, diverse natural and modified tropism profiles, high packaging capacity, coupled with their immunological tolerance in target organs and relative ease of control in the case of adverse events, make bacterial-mediated delivery an attractive alternative to gastrointestinal, respiratory, urogenital tracts and solid tumors. Therapeutic benefits of bacteria including attenuated *Salmonella* spp., *Shigella* spp., *Bacillus Calmette-Guerin* (BCG), *Y. enterocolitica*, *Lactobacillus* spp. and non-pathogenic *E. coli*, have been observed in vaccination against infectious disease, gene therapy against cancer, and topical delivery of antibodies and immunomodulatory cytokines in inflammatory bowel disease. These encouraging but pre-clinical studies justify further development of bacteria as a therapeutic vector against many types of pathology.

[0003] In cancer therapy, reducing problems such as inadequate tumor targeting, inefficient penetration of a tumor by a drug, toxicity to normal cells and limited lifetimes of therapeutic agents is a major target of research (Minchinton and Tannock 2006). Microbial organisms, which can act as natural anti-cancer agents and can be modified to enhance their

therapeutic capabilities, are useful in overcoming many of the problems of conventional treatments (Forbes 2010). Most studies use attenuated bacteria to decrease the virulence (Hoffman 2011) and augment their natural cytotoxicity with vectors designed to deliver an agent to the tumor. Strategies for bacteria-based treatment have included delivering prodrugs that are activated in the tumor (Barbe, Van Mellaert et al. 2006), producing a cytotoxic protein in the bacteria (Zhang, Man et al. 2010), delivery of DNA expression vectors and short hairpin RNA (shRNA) (Xiang, Fruehauf et al. 2006).

[0004] Oncolytic bacteria have a long/checkered history in cancer therapy and are perceived as safe but ineffective. Many challenges remain in effectively utilizing bacteria as antitumor agents and, to date, clinical trials of bacterial mediated therapy have had modest results (Cunningham and Nemunaitis 2001). One factor relates to the virulence of the bacteria and the effects of the host immune response to their presence (Westphal, Leschner et al. 2008). Other factors are the efficiency of intracellular delivery of multiple agents to target cells, instability of the vector carrying plasmid leading to its loss in the bacteria, inefficient transport of expression plasmids to the tumor cell nucleus and ensuring an optimal dose level of therapeutic agents. Thus, new methods are needed for the development of a novel class of bacterial vectors to treat mammals.

[0005] In immunotherapy/vaccination, delivery of eukaryotic plasmid-encoded antigens into macrophages, T cells and/or dendritic cells by live attenuated bacteria is has been used to stimulate cellular and humoral immune responses against bacterial toxin, virus and tumor antigens, but not very successfully. As an adjuvant for recruitment of innate immunity, pathogen-associated molecular patterns of these vaccine strains can amplify the immune response. When using invasive recombinant bacterial vectors for delivery of DNA vaccines, at least five problems should be solved: (a) plasmid loss (b) low frequency of host internalization (c) bacteria-induced apoptosis/pyroptosis (d) low efficacy of gene transfer and (e) transient gene expression.

[0006] A better understanding of the mechanism of preferential tropism profiles and of host-vector interactions as well as the advent of modern techniques for genetic manipulation have accelerated the development of novel synthetic bacterial systems for clinical testing. A 'long-homology arm' strategy which is able to increase both accuracy and efficiency of genetic engineering technology in gram-negative strains has previously been reported. This robust and efficient method is powerful for multiple chromosome insertions or deletions to engineer bacteria for targeted delivery and expression of multiple therapeutic molecules and/or antigens in the target cells.

3. SUMMARY OF THE INVENTION

[0007] In view of the foregoing, it is an object of the present invention to provide modified bacteria to treat cancers and infectious diseases, thereby overcoming various deficiencies and shortcoming of the prior art, including those outlined above. Provided herein is a gene expression system that has an inter-kingdom dual expression (IKDE) of antitumor agents and recombinant antigens, in the nucleus or cytoplasm of eukaryotic cells, with priming and maintenance of the system in the bacteria. Also provided herein are modified bacteria comprising one or more of the following characteristics: (a) deletion of the amino acid biosynthesis-related gene and/or stress response gene for further attenuation; (b) muta-

tion of the biofilm-producing gene to preclude biofilm formation; (c) placing essential genes with a tightly regulated control to program cell lysis; (d) development of a balanced-lethal host-vector system; (e) incorporation of cholesterol-dependent cytolysin gene to enhance endosomal escape.

[0008] In an embodiment, the modified bacteria is a facultative anaerobic. In certain embodiment, the modified bacterium is a Gram-negative bacterium. In certain embodiments, the facultative anaerobic, includes, but not limited to *Salmonella typhimurium*. In certain embodiment, the essential gene is, for example, a gene for aspartate-semialdehyde dehydrogenase (“asd”). In certain embodiments, asd is operatively linked and is under the control of a hypoxia-conditioned promoter. In certain embodiments, the normal functions of the bacteria are not compromised by the deletion or mutation of any of its genes. In certain embodiments, the modified bacteria are *Salmonella typhimurium*, *Salmonella choleraesuis*, *Salmonella enteritidis* and *S. typhimurium*, *Escherichia coli*, *Escherichia coli* K-12, *Escherichia coli* O157:H7, *Shigella*, *Shigella dysenteriae*, *Shigella flexneri*, *Shigella boydii*, *Shigella sonnei*, *Yersinia*, *Yersinia pestis*, *Yersinia pseudotuberculosis* and *Yersinia enterocolitica*.

[0009] Described herein is a method of enhancing the safety and tumor-targeted ability of bacterial pathogens by using recombinant DNA techniques. Provided herein is a method of making the modified bacterial vector. Examples of such genetic mutations include, but are not limited to: (a) deletion of the amino acid biosynthesis-related gene, such as *aroA*, *gua*, *thy*, *leu* and *arg* gene, to make the bacteria more susceptible to nutrient shortage, particularly in the intracellular compartment where they will die due to lack of nutrients; (b) mutation of the biofilm-producing gene, such as *csgD* (curli synthesis), *adrA* (cellulose synthesis) and *gmd* (colonic acid synthesis), to enhance intracellular gene transfer and make the bacteria more sensitive to antibiotics; (c) placing an essential gene “asd” (coding for aspartate-semialdehyde dehydrogenase) with a tightly regulated control to make the bacteria only survive in the necrotic/hypoxic core of solid tumors; (d) deletion of the stress response gene, such as *htrA*, *recA* and *lisp* gene; (e) relocating an essential gene “*infA*” (coding for translation initiation factor IF-1) to the therapeutic plasmid and (f) incorporation of the gene coding for Listeriolysin O to break the *Salmonella*-containing vesicles.

[0010] Described herein is a tumor-targeting bacterial vector for prokaryotic-eukaryotic delivery and expression with multiple genetic mutations and a method of making the vector. The obligate anaerobic *Salmonella* strain YB1 with hypoxia regulatory control can only survive within the tumor in animals (Yu, Yang et al. 2012). The naturally occurring antitumor activity of facultative anaerobic bacteria is further enhanced by genetic manipulations. In a preferred embodiment, an engineered strain ST1, was generated in the *Salmonella* 7207 strain background using the λ Red-mediated ‘long homology arm’ recombination technology. Firstly, T7 RNA polymerase (T7 RNAP) gene was integrated into the *gmd* chromosomal locus to provide an initial RNAPs source for activating the system and enable a high transcription of RNA or exogenous gene driven by P_{T7} . Removal of *gmd* gene encoding GDP-D-mannose dehydratase precludes the synthesis of colonic acid, thereby interfering with the ability of *Salmonella* to produce biofilms and enhance intracellular presence in vivo. Secondly, an essential gene *asd* was replaced by a pore-forming listeriolysin O gene *hlyA*, which

encodes a bacterial factor required for an efficient breakdown of lysosomal membrane after phagocytosis and then release the exogenous protein as well as DNA/mRNA. Subsequently, the *asd* gene with anaerobic control was cloned back at the *htrA* gene locus. Double mutation (*asd* and *htrA*) made bacteria to grow only under anaerobic conditions while being disabled for growth in normal tissues. The final step was to relocate a small essential gene *infA* (encoding for translation initiation factor 1) from chromosome to plasmid for stable plasmid maintenance. In certain embodiments, the modified bacteria include, but not limited to *Salmonella* spp., *Escherichia coli*, *Shigella* spp., *Bacillus Calmette-Guerin* (BCG), *Listeria monocytogenes*, *Yersinia enterocolitica*, *Mycobacterium*, *Streptococcus* spp., and *Lactobacillus* spp.

[0011] The modified tumor-targeted bacteria provided herein are not lethally toxic. The modified bacteria exclusively grow inside the tumor hypoxic/necrotic zone. In one embodiment, the modified bacterium is ST1. Controlled growth of ST1 lowers the risk for sepsis in the clinical setting. In mouse models, ST1 is replication-incompetent in normal organs and amplified within tumors to as high as 10^8 cfu per gram tissue. These high titers can enhance the therapeutic effects, as the high amount therapeutic molecules generated and delivered by bacteria. Furthermore, undesirable property of forming biofilms by bacteria at high concentrations poses a serious problem in the clinics and restricts the intracellular residence of the bacteria in tumors which may impair their potential to act as gene transfer vehicles {Crull, 2011 #954}. However, these problems are circumvented by the present disclosed system which allows more efficient and safe usage of bacterial vectors in the animals.

[0012] Provided herein is a bacterial vector that is superior to previous bacterial and viral systems in term of in vivo stability. In the previous studies, all the bacterial delivery systems used prokaryotic plasmids to express the helper proteins for invasion or cellular entry into the target host cells (Souders, Verch et al. 2006). In comparison, provided herein, the bacterial factors involved in tumor fitness, intracellular delivery and expression were integrated into the chromosome. It made some improvements in reducing metabolic burden, enhancing stability, safe concern, tight regulation of gene expression and high efficacy of gene transfer. Furthermore, due to the fact the efficiency and the duration of expression of nucleic acids-based products are mainly dependent on the amount of plasmid DNA or RNA delivered. A *Salmonella* *infA*⁺ vector/*infA*⁻ host maintenance system was developed to maintain the therapeutic vectors at high copies in vivo. This adaptation helps to maintain the plasmid in suitable strains in the absence of selective antibiotics.

[0013] The modified bacterial vectors useful for practicing the method disclosed herein is capable of entering tumor cells and localizing to the cytoplasm. Such bacterial vectors are endocytosed and pass through the endocytic membrane and are ultimately processed by the targeted cells for generation of therapeutic factors. The non-virulent bacteria described herein have invasive properties (or are modified to have invasive properties) and enter a mammalian host cell through various mechanisms. In one embodiment, *Salmonella* can invade cells by a trigger behavior including massive membrane ruffling and actin cytoskeleton reorganization, which are stimulated by a series of bacterial effector proteins translocated by a type III secretion system (Murli, Watson et al. 2001). After adhesion and entry, the modified bacteria could escape from the host-cell vacuole and replicate in the cyto-

plasm by integrating a cytolysin expression cassette under an in vivo inducible promoter from *Salmonella* Pathogenicity Island 2. In some embodiments, the bacterium can stay alive for various times in the said cells and may continue to amplify protein or genetic materials. The exogenous molecules can then be released from the bacterium into the cell by leakage. In certain embodiments of the invention, the bacterium can also replicate in the eukaryotic cell. In a preferred embodiment, bacterial replication does not kill the said cell. In certain embodiments, the bacteria deliver therapeutic factors, which includes, but are not limited to, for example, functional protein, DNA, RNA or a combination of at least two factors.

[0014] Provided herein is a feasible shuttle for introducing multiple therapeutic factors including, but not limited to, proteins, DNA and translation-competent mRNA, into the mammalian cells. For the RNA delivery, in a preferred embodiment, the translation-competent mRNA with poly A tail adapted for eukaryotic translation by linking a viral ribosome entry site sequence before the encoding sequence, which is driven by T7 RNAPs in the bacterial carriers. Upon intracellular entry, the mRNAs are efficiently released from the disrupted bacteria and immediately translated in the eukaryotic cytoplasm. However, the exogenous gene expression level directly dependent on the amount of the released mRNA and RNAPs. One approach for enhancing gene expression is the use of an autocatalytic cytoplasmic expression system mediated by phage RNAPs, which is based on the previous observation that a plasmid-based gene expression system including T7 RNAP amplification circuit could increase the expression level (Finn, Lee et al. 2004). However, for the plasmid delivery system, it is more difficult due to the presence of nuclear barriers. Only a small proportion of intact plasmid DNA can be transported into the nucleus through the nuclear pore complex for transcription. To overcome this main barrier and facilitate gene expression, provided herein is a combined prokaryotic-eukaryotic (inter-kingdom) dual expression (IKDE) system, containing a T7 RNAP-based cytoplasmic expression system as well as a well-known nuclear system. Following in vitro infection, the expression efficiency of this dual expression with a T7 autogene-based positive feedback loop is more than two orders of magnitude higher than that of the standard nuclear expression system (cytomegalovirus (CMV)-based). Having established the feasibility of an inter-kingdom delivery and expression mediated by the modified bacteria as described in the Examples, the present disclosure addressed the issue of gain and loss-of-function genetics in a therapeutic scenario.

[0015] Using tumor-killing bacteria may be the cheapest way of treating cancer. A more potent synergistic antitumor effect might be obtained by the intrinsic toxicity of bacteria plus the inter-kingdom expression of versatile tumor specific therapeutic factors. In one embodiment, the modified bacteria express one or more therapeutic bioactive polypeptide or protein, preferably oncolytic proteins, negative regulators of tumor angiogenesis tumor-specific antigens and immune modulating factors (such as cytokines) in the extracellular environment and/or inside the target cells. In certain embodiments, the modified bacteria are effective in raising an anti-tumor response in mammals. Provided herein are methods comprising introducing at least one modified bacterium, comprising a prokaryotic vector, said vector comprising one or more DNA molecules encoding one or more proteins, and one *infA* locus, wherein said proteins include prodrug-converting enzymes, cytokines, bacterial toxins and angiogenic inhibitors with

high oncolytic abilities. In a certain embodiment, as a protein delivery vehicle, *Salmonella* ST1 encoding the extracellular domain of TNF-related apoptosis-inducing ligand (TRAIL) expressed cytokines directly in the extracellular environment or in the tumor cells through a surface display or *Salmonella* type III secretion system without the requirement of bacterial lysis. The soluble domain of TRAIL can trigger rapid apoptosis in a board range of human cancer cells independent of their p53 status, but not in most normal cells. Unlike traditional chemotherapy drugs, TRAIL-expressing ST1 can selectively grow inside tumors and continuously release the biologically active cytokines in situ at high concentration, thereby achieving maximal killing effects while sparing systemic cytotoxicity. In an embodiment, the delivery of biologically active cytokines by ST1 led to strong inhibitions of tumor development in the MDA-MB-231 xenograft tumor model. Live attenuated ST1-mediated protein delivery is potentially superior to inactivated preparations owing to: (1) their ability to induce cell-mediated immunity in addition to cytokine response; (ii) effectiveness after single-dose administration; (iii) low production cost; (iv) the ease of manufacturing and (v) as carriers for the delivery of active proteins to the solid tumors.

[0016] In an embodiment, the modified bacteria are live attenuated. In an embodiment, the modified bacteria are used as a vector to transfer DNA encoding therapeutic proteins through an inter-kingdom dual expression system, wherein said proteins include bacterial toxins, cytokines, prodrug-converting enzymes, angiogenic inhibitors, tumor cell-expressed antigens and immunomodulatory mediators. In a preferred embodiment, the modified bacterium is ST1. ST1 transferred with one or more DNA molecules encoding therapeutic proteins delivers nucleic acids to achieve tumor-targeted expression. In an embodiment, DT-A gene, encoding the catalytic fragment of diphtheria toxin, was cloned to inter-kingdom expression plasmid pIKDE. In an embodiment, the inter-kingdom system (bacteria and plasmid vector) has been modified to include the deletion of the initiation factor 1 and GDP-mannose dehydrogenase encoding gene, replacement with a hypoxia-controlled *asd* gene expression cassette, integration of T7 RNAP and cytolysin expression cassette, carrying a plasmid pIKDE-DTA with one or more of the following characteristics: (i) a reasonably small size (9.7 kb); (ii) an origin of replication responding for a high copy number; (iii) *E. coli infA* locus allowing in vivo plasmid maintenance; (iv) T7 RNAP autogene cassette which can amplify the polymerases after cytoplasmic entry; (v) insertion of an excellent DNA nuclear targeting sequence—a 72-bp element of the SV40 enhancer for enhancing nuclear import; (vi) suicide gene expression driven by both P_{CMV} (for nuclear expression) and P_{T7} (for cytoplasmic expression). It is the first report to date describing the design and use of such expression system. By taking advantage of the tumor-targeted and oncolytic effects of ST1 and the translation inhibition activity of DT A chain, a single dose of ST1/pIKDE-DTA induced remarkable shrinkage of solid tumors and greatly reduced the risk of death with no gross sign of toxicity in both immune deficient and competent mice models. In one embodiment, the pIKDE-DTA comprises the sequence as indicated in FIGS. 54A-E, or a derivative, variant of the sequence which is within the ability of one of skilled in the art.

[0017] In an embodiment, the bacterial vectors are used for RNA interference (RNAi) therapy. An inter-kingdom RNAi interference system has been developed by combining

prokaryotic and eukaryotic transcription of shRNA. In certain embodiments, the system has one or more of the following improvements: (1) high tumor-targeting characteristics; (2) in vivo plasmid maintenance; (3) combination of prokaryotic and eukaryotic transcription of the shRNA. In a preferred embodiment, ST1-mediated inter-kingdom RNAi can transfer RNAi effectors between bacteria and mammals. The transcription of shRNA and microRNA precursor can be achieved in the bacterial cells as well as through eukaryotic transcription machinery in the cytosol of the infected tumor cells. Specifically, shRNA targets against oncogenes or tumor-related factors, such as vascular endothelial growth factor (VEGF), kinesin spindle protein (KSP), human epidermal growth factor receptor 2 (HER2), V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (KRAS), β -catenin, signal transducer and activator of transcription 3 (STAT3), transforming growth factor β -activated kinase 1 (TAK1), and polo-like kinase 1 (PLK1), can be transcribed from an RNAi plasmid pIKR-shRNA by T7 RNAPs inside the ST1 cells. Following phagosome disruption, these shRNAs will be released within the shRNA-encoding plasmids in the target host cells. Subsequent transcription of shRNAs by mammalian cells takes place in the cytoplasm. Then a series of RNAi processing steps will occur sequentially in mammals. In a certain embodiment, oligonucleotides encoding shRNA against no actual target tdTomato and a key mediator of Wnt/ β -catenin pathway β -Catenin, cell cycle-associated protein (PLK1) genes were inserted into the multiple cloning sites, generating pIKR-shTom (vector control), pIKR-shCAT and pIKR-shPLK. In one embodiment, the pIKR-shCAT comprises the sequence as indicated in FIGS. 58A-E or a derivative, variant of the sequence. These plasmids contain a shRNA expression cassette including P_{T7}, shRNA sequence and T7 terminator and a T7 RNAP autogene expression cassette. After intracellular entry, ST1 harboring pIKR-shRNA could trigger a high-level shRNA expression ($\sim 10^4$ copies/ng RNA) in the cytoplasm, which elicits an efficient initiation of RNAi. In certain embodiments, substantial silencing and its knockdown efficiency achieved was comparable or superior to previous results that have reported silencing levels ranging between $\sim 50\%$ and 90% (Zhao, L'Abbe et al. 2005; Strillacci, Griffoni et al. 2010). Compared to the previously reported bacterial-mediated trans-kingdom RNAi (Xiang, Fruehauf et al. 2006), in vivo studies suggested that ST1-mediated inter-kingdom RNAi achieves a stronger silencing ability by the combination of prokaryotic and eukaryotic transcription and reduce total adverse effects. Systematic administration of ST1 carrying pIKR-shCAT or pIKR-shPLK induced a specific and effective silencing of targeted proteins in the tumor and retarded its growth without apparent side effects. In some embodiments, the disease or disorder can be, but is not limited to, a disease or disorder characterized by an increased expression (DNA, RNA or protein).

[0018] In an embodiment, bacterially activated exosomes, and in particular naturally derived exosomes comprising therapeutic factors, which are prepared according to the methods disclosed herein. Cytotoxic exosomes which have been loaded by the bacterial cells described herein include, but are not limited to one or more protein or peptides, RNA species, DNA species or thereof. In certain embodiments, the RNA content includes one or more RNA species, such as, but not limited to, mRNA, microRNA, siRNA, shRNA, or a combination thereof.

[0019] The invention further provides methods of eliciting an effective immune response in a subject by administering an attenuated *Salmonella* or composition of the invention to a subject. By encapsulation of the DNA/mRNA/microRNA and shRNA vaccine expressing the hemagglutinin (HA) gene of H7N9 influenza virus in the modified nonreplicative *Salmonella* by intraperitoneal injection, it is sufficient to elicit immune response against HA. The vaccine strain constructed by the invention can be subcultured stably in vitro with selection pressure or not, which is proved by CFU tests. Furthermore, DNA vaccine delivery may benefit from the auto-amplified expression system, resulting in improved immunogenicity effect against the vectored antigens. The inventive vaccine is advantageous over current inactivated or live attenuated vaccines, as updating of the vaccine requires only the replacement of the encoding sequence with the new virus.

[0020] Provided herein is a modified bacterial vector. The modified bacterial vector is a tumor-targeted delivery vehicle of antitumor molecules and as a therapeutic 'factory' for feasible drug production to enhance tumor regression. More importantly, these engineered bacteria carrying different cargoes were well tolerated in animal models. In a preferred embodiment, *Salmonella* ST1 harboring inter-kingdom expression vectors, which are able to replicate within the tumors, and are useful for inhibiting the growth and/or reducing the tumor volume of sarcomas, carcinomas, lymphomas or other solid tumors, including, but not limited to, breast cancer, prostate cancer, cervical cancer, uterine cancer, lung cancer, ovarian cancer, testicular cancer, thyroid cancer, astrocytoma, glioma, pancreatic cancer, stomach cancer, liver cancer, colon cancer, and melanoma.

[0021] This invention provides multivalent bacterial vectors that can be used either prophylactically or therapeutically for the treatments of patients by using the bacteria harboring multiple cargoes generated by the methods described herein. Also described herein are therapeutic and prophylactic compositions comprising the modified bacteria. Such a composition can be administered alone or as an adjunct to other therapy selected from gene therapy, chemotherapy, radiation, immunotherapy and/or other therapeutics or vaccines. In certain embodiments, the therapeutic and prophylactic compositions contain a purified form of the modified bacteria. In certain embodiments, the therapeutic and prophylactic compositions do not contain other strains of microorganisms. Also disclosed are methods of preventing and treating tumor and/or cancer and infectious diseases using the system provided herein.

[0022] Other features and advantages of the disclosure will be apparent from the following detailed description and claims.

[0023] Also described herein is a kit comprising the modified bacteria and a pharmaceutically acceptable carrier.

4. BRIEF DESCRIPTION OF THE FIGURES

[0024] FIGS. 1A-B Generation of a tumor-targeting *Salmonella* strain ST1 for delivery and expression of multiple therapeutic factors. (A) Schematic diagram of the creation process of ST1 strain. (B) PCR confirmation of the accurate insertion of the four genes at their respective loci. The forward primers were positioned outside the homologous region. Reverse primers were positioned within the heterogeneous regions.

[0025] FIGS. 2A-B Integration of T7 RNAP expression cassette into the *gmd* locus resulted in the generation of T7

RNAP-expressing *Salmonella* strains with weak biofilm-forming ability. (A) Western blot showing the expression of T7 RNAP (99 kDa) in SL7207 strain and its mutants using mouse monoclonal antibody against T7 RNAP. (B) Colonies on the Congo Red agar plates incubated at 30° C. Wild-type *S. typhimurium* 7207 strain exhibit the typical biofilm, i.e. the 'rdar' morphotype in vitro; while *gmd* knockout strains (SL001 and ST1) were no longer able to form the biofilm.

[0026] FIGS. 3A-B The conversion of SL7207 to the anaerobic ST1 prevented bacterial killing of the mice. (A) Strains SL7207 and ST1 were grown on LB agar plates under aerobic (+O₂) or anaerobic (-O₂) conditions for 24 h at 37° C. (B) Kaplan-Meier survival curves of mice receiving ST1 or wild-type stain 7207 at a dose of 5×10⁷ cells/mouse.

[0027] FIGS. 4A-B Examination of the tumor-targeting potential of ST1 in immunocompromised mice and bacterial colonization in the hypoxic core of solid tumors. (A) Preferential accumulation of ST1 within the tumors after one intravenous injection. Bacterial counts in the different organs were determined on day 7, 14 and 21 post infections. Measurements are from three independent experiments, and the error bars represent the s.e.m. (ND stands for not detected). (B) Composite images were generated for the whole tumor to observe macroscopic bacterial colonization. Immunohistochemical staining identified regions of the necrotic area (left), hypoxic region (middle) and *Salmonella* accumulation (right).

[0028] FIG. 5 Bacterial counts per cell from Gentamicin protection assay in MDA-MB-231 cells at 2 and 4 h post infection at an MOI of 200:1.

[0029] FIG. 6 Fluorescence-activated cell sorting was used to demonstrate EGFP expression on day 2 following bacterial infections. Dot plot representation of tumor cells infected with ST1/pEGFP-C1 (plasmid DNA delivery) or ST1/pT7-EGFP (functional mRNA delivery) showing green fluorescence.

[0030] FIG. 7 CFU tests of ST1/pcDNA3.1-infA (high-copy) and ST1/pET32a-infA (low-copy) were performed at 3 weeks after intravenous injections into tumor-bearing mice. Tumor lysate was homogenized in PBS and plated onto agar plates with or without ampicillin selection to determine the counts of recombinant and total bacteria, respectively. Results are expressed as mean±s.e.m. (n=5).

[0031] FIG. 8 Schematic diagram of ST1-mediated inter-kingdom dual expression (IKDE) system. The activation of this system requires cytoplasmic delivery of both pIKDE-EGFP containing a reporter gene driven by P_{T7} and P_{CMV} and an initial source of T7 RNAP or its mRNA.

[0032] FIG. 9 Theoretical steps of ST1-mediated inter-kingdom expression. (1) In the bacterial cytosol, T7 RNAPs bind to the P_{T7} on the plasmid (circle) and then mediate the efficient transcription of mRNA (curve). (2) Upon intracellular delivery, functional mRNAs will be translated into T7 RNAP (triangle) or EGFP (circle) in the cytoplasm, where an initial source of T7 RNAPs can transcribe T7 RNAPs (through a positive feedback loop) and reporter gene mRNA form pIKDE-EGFP. (3) A small percentage of DNA will enter the nucleus, where the transcription machinery will generate stable transcripts through the nuclear system.

[0033] FIG. 10 Schematic representations of the expression plasmids and the RT-PCR detection of EGFP mRNA in the bacterial hosts using an anchor gene specific primer.

[0034] FIG. 11 EGFP expression in the human MDA-MB-231 cells at 5 h after ST1/pIKDE-EGFP infection.

[0035] FIG. 12 Expression kinetics of EGFP in the cells infected with ST1 harboring the indicated vectors (at an MOI of 200:1) were determined at 24, 48 and 72 h post infections. ST1 carrying an IKDE system leads to a rapid and high-level transgene expression.

[0036] FIG. 13 Therapeutic effects of ST1 harboring different therapeutic factors on cell viability and apoptosis in human MDA-Mb-231 cells. MDA-MB-231 cells were incubated with ST1 harboring the indicated vectors (at an MOI of 200:1). Cell viability and Annexin-V/propidium iodide staining analysis of apoptotic cells following the indicated treatments were assessed at 48 h post infections. Data represent the values of triplicates (means±s.e.m.).

[0037] FIG. 14 Diagram of soluble TRAIL expression and translocation through bacterial surface display or *Salmonella* type III secretion system.

[0038] FIG. 15 Expression of Lpp_ompA_sTRAIL fusion protein was observed in the outer membrane of ST1 transformed with pLpp_ompA_sTRAIL by immunoblot analysis. Samples were loaded with equal total protein content.

[0039] FIG. 16 Human MDA-MB-231 cells were incubated with type III secretion system competent ST1 strain or a translocation-defective strain carrying pSspH2-sTRAIL. Then the expression of SspH2-sTRAIL in the bacterial-associated and host cell fractions was examined. Actin was used as a loading control.

[0040] FIG. 17 Immunofluorescent staining of cytokine soluble TRAIL expression and the presence of *Salmonella* in a solid tumor treated with ST1/pLpp_ompA_sTRAIL or ST1/pSspH2-sTRAIL. Scale bar, 50 μm.

[0041] FIGS. 18A-B Systemic administration of sTRAIL-expressing ST1 significantly suppresses the MDA-MB-231 tumor growth without apparent weight loss. (A) Tumor growth curves after receiving with PBS, ST1/control, ST1/pLpp_ompA_sTRAIL or ST1/pSspH2-sTRAIL during a 20-day observation. (B) Body weights of tumor-bearing mice receiving the indicated treatments.

[0042] FIG. 19 Distribution and tumor colonization of ST1/pSspH2-sTRAIL in tumor-bearing nude mice. Different organs were homogenized and analyzed for the presence of bacteria. ND stands for not detected. Shown is the mean CFU per gram tissues plus s.e.m.

[0043] FIG. 20 Biodistribution of ST1 in the 4T1 tumor-bearing mice. Female BALB/c mice (n=3 per group) bearing 4T1 mammary tumors received a single intravenous injection of 2×10⁷ cfu of ST1. Bacterial accumulation in the tumors, lungs, spleens, livers, kidneys and hearts were determined 7 and 14 days later. Bars correspond to mean±s.e.m. (n=3). ND stands for not detected.

[0044] FIGS. 21A-B In vivo validation of ST1-mediated functional gene transfer. (A) Time course of DT A chain expression in tumors after ST1/pIKDE-DTA injections. (B) Transcript levels of nuclear and cytoplasmic DT-A transcripts (mean±s.e.m., n=5) were measured by quantitative real-time RT-PCR.

[0045] FIG. 22 Intracellular DT A chain expression was detected by immunostaining. Scale bar, 25 μm. Target gene expression was detected in the ST1/pIKDE-DTA treated tumors, but was absent in the vector control group.

[0046] FIGS. 23A-B Detection of DT A chain expression in the ST1/pIKDE-DTA treated tumors by western blot (A) and immunohistochemistry (B) on day 21 after intravenous injection. (B) Composite images of a whole tumor infected with

ST1/pIKDE-DTA stained to visualize bacteria (left) and DTA chain (right). Scale bar, 1 mm.

[0047] FIGS. 24A-B In situ DT A chain expression induced massive cell death in ST1/pIKDE-DTA treated tumors on day 3 following systemic injection. (A) Apoptosis determined by TUNEL staining. The tumor sections were stained doubly with DAPI and TUNEL and visualized under a fluorescence microscope with 200 \times magnification. Scale bar, 50 μ m. (B) Relative % of the TUNEL-positive cells was determined in four fields for each group.

[0048] FIGS. 25A-C BALB/c mice with 4T1 breast tumors (6-8 mm in diameter) received intravenous injections of PBS, ST1/pIKDE-EGFP or ST1/pIKDE-DTA (2×10^7 cfu/mouse) (n=5 to 8 per group). (A) Tumor volumes were measured every other day after a single injection. Values are expressed as mean \pm s.e.m. (B) Representative photomicrographs of the tumors treated with ST1/pIKDE-EGFP or ST1/pIKDE-DTA at the endpoint. (C) Bright field imaging and H&E staining of the lungs from 4T1 tumor-bearing mice treated as described above. Scale bars, 1 cm for bright field imaging; 250 μ m for H&E staining. Each dot represents the number of nodules per mouse. Horizontal bars indicate the mean values in each group.

[0049] FIG. 26 Kaplan-Meier survival curves of 4T1 tumor-bearing mice administered with the indicated treatments.

[0050] FIGS. 27A-C ST1/pIKDE-DTA administration potently suppressed tumor growth in the MDA-MB-231 tumor-bearing nude mice. Tumor volumes were measured every other day over 20 days after a single injection. (A) Tumor growth curve for breast tumors received the indicated treatments. Values are expressed as mean \pm s.e.m. (n=5). (B) Kaplan-Meier survival curves of the tumor-bearing mice injected intravenously with either ST1/pIKDE-EGFP or ST1/pIKDE-DTA, and PBS. A significant improvement in survival was found in ST1/pIKDE-DTA treated mice compared with controls (n=12). (C) Representative photomicrographs of tumor-free mice after ST1/pIKDE-DTA treatment.

[0051] FIG. 28 Schematic diagram of ST1-mediated inter-kingdom RNAi. After successful entry and rupturing the phagosomal membrane, all the bacterial content, including genetic materials (shRNAs and eukaryotic expression vectors encoding shRNA) and proteins, can reach to the cytoplasm of tumor cells. Only a finite amount of T7 RNAP and its mRNA could be released into the cytoplasm. Regardless, this should provide enough polymerases to trigger further T7 RNAP mRNA transcription in the cytoplasm via P_{T7} through a positive feedback loop. After endocytosis, a small portion of plasmid DNA can be transferred to the nucleus where the transcription of T7 RNAP can be initiated at a constitutive P_{CMV} promoter. The accumulation of T7 RNAPs driven by dual (cytoplasmic and nuclear) expression system results in a subsequent transcription of shRNA from pIKR-shRNA by mammalian cells takes place in the cytoplasm. Then a series of RNAi processing steps will occur sequentially in mammals.

[0052] FIGS. 29A-B ST1-mediated combined inter-kingdom RNAi in vivo via specific cleavage of mRNA. Analysis of gene silencing effects in tumors at week 3 following treatments. (A) Quantitative real-time RT-PCR analysis of the reduction of PLK1 mRNA after the indicated treatments. (B) In vivo 5'-RACE analysis of RNA extracted tumors confirmed the presence of specific cleaved product (414 bp).

[0053] FIGS. 30A-B Targeted knockdown of protein expression by ST1-mediated RNAi. (A) Western blot analysis for the target protein expression in the MDA-MB-231 tumors as indicated. (B) Representative histopathologic and immunohistochemical staining of target protein on tumor sections as indicated. Scale bar, 50 μ m.

[0054] FIG. 31 ST1-mediated RNAi does not upregulate the expression of OSA1 in the tumor-bearing mice. At the endpoint, the OSA1 mRNA levels in the indicated groups were determined by quantitative real-time RT-PCR. The levels of OSA1 mRNA were normalized to that of GAPDH mRNA, and the relative mRNA levels in the ST1/pIKR-shTom and ST1/pIKR-shPLK treated tumors were expressed as a ratio to that in PBS group. Values are mean \pm s.e.m. (n=3).

[0055] FIGS. 32A-B Incorporation of T7 RNAP autogene cassette enhances the silencing efficiency. (A) The target protein levels treated with ST1 carrying shRNA expression plasmid with (ST1/pIKR-shPLK) or without T7 RNAP autogene (ST1/pIKR^{AT7P}-shPLK) were compared at week 3 following injections. (B) shRNA levels in ST1/pIKR-shPLK and ST1/pIKR^{AT7P}-shPLK treated mice were measured by quantitative stem-loop RT-PCR. Results are referred as mean \pm s.e.m. of 3 to 5 mice.

[0056] FIG. 33 Tumor growth curves of mice receiving with PBS, ST1/pIKR-shTom or ST1/pIKR-shPLK treatments.

[0057] FIG. 34 Representative results of tumor sections immunostained for expression of endothelial cell marker CD31 and TUNEL assay for detecting cell apoptosis. Scale bar, 25 μ m.

[0058] FIGS. 35A-B Body weights (A) and spleen weights (B) of mice receiving with PBS, ST1/pIKDE-DTA or ST1/pIKR-shPLK treatments.

[0059] FIG. 36 Serum ALT and AST levels (mean \pm s.e.m., n=3) in mice receiving the indicated treatments at the endpoint.

[0060] FIG. 37 Spleen, liver and kidney were fixed in 4% PFA and embedded in paraffin blocks. Tissue sections were stained using hematoxylin-eosin for light microscopic examination. Histopathological changes in the kidney, spleen and liver of the mice received bacterial treatments were examined. No apparent damages were found in any of the organs in either treatment group. Scale bar, 50 μ m.

[0061] FIG. 38 Electron microscopy of exosome and western blot analysis of exosome-specific HSP70 protein.

[0062] FIGS. 39A-B Presence of tumoricidal protein in the exosome-like microvesicles derived from the mice treated with ST1/pIKDE-DTA. (A) Isolated RNA from vesicles was used for RT-PCR detection. A specific PCR product was detected corresponding to DT-A transcript. (B) Validation of the presence of DT A chain by western blot. Exosomal proteins from the tumors were loaded onto a 12% acrylamide gel and probed with anti-DT A chain antibody. Exosomes derived from ST1/pIKDE-DTA treated tumors were positive for DT A chain.

[0063] FIGS. 40A-B (A) Presence of shRNA against CTNNB1 in the exosomes isolated from tumors. Gel electrophoresis analysis confirmed the presence of shRNA against CTNNB1 in the exosomes from tumors infected with ST1/pIKR-shCAT but not in ST1/pIKR-shTom group. (B) Hematoxylin and eosin stain and immunohistochemical analyses on tumor sections. Excised breast tumors from tumor-bearing mice on day 20 were fixed in 4% paraformaldehyde and embedded in paraffin. Serial 5- μ m sections were subjected to

hematoxylin and eosin staining, immunohistochemical assay with *Salmonella* and β -Catenin antibody. Shown are low-power field examples of tumor sections from mice treated intravenously with PBS, ST1/pIKR-shTom or ST1/pIKR-shCAT. All images were acquired at $\lambda 40$ magnification using Nikon microscope. Low magnification overviews (Scale bar, 2 mm).

[0064] FIG. 41 Results from ELISA experiments showing HA-specific IgG responses raised by the ST1/pIKDE-HA. 10^7 bacteria were used to prime and each mouse were given three boosts on day 14, 21 and 28; all by i.p. Blood sera from infected mice were collected on day 14, 28 and 35 for ELISA analysis.

[0065] FIG. 42 The steps of constructing long homology-arm recombination vectors. Firstly, 1000 bp long homology arms targeting *asd* gene were cloned into the plasmid pYB-*asd*. Secondly, *PsseA* and chloramphenicol resistance gene (*cat*) were amplified and cloned into pYB-*asd*-*PsseA*-*cat*. Finally, *hlyA* gene was cloned into pYB-*asd*-*PsseA*-*cat* to generate plasmid pYB-*asd*-*hlyA*.

[0066] FIG. 43 HE-stained (left) and anti-*Salmonella* immunohistochemical stained (right) paraffin sections of CT26 tumor on day 14 p.i. with SL008 cells. Low magnification overviews, black bar correspond to 1 mm.

[0067] FIG. 44 Balb/c mice with CT26 colon tumor received temporal vein injections of ST1 (SL004), SL007 or SL008. Mice were euthanized on day 14 and liver, spleen and tumor tissues were collected and homogenized and bacterial accumulation evaluated. In ST1 (SL004) (black), SL007 (white) or SL008 (gray) treated mice, CFU counts per gram organs are shown as mean \pm s.e.m. (n=3).

[0068] FIG. 45 Construction of plasmid pET32a-*infA* for maintaining bacterial survival after *infA* deletion. The *infA* expression cassette including its promoter and terminator region was cloned from *E. coli* MG1655.

[0069] FIG. 46 Stability determination of plasmid pET32-*infA* in vitro. Bacterial cells were routinely cultured by daily sub-culturing without antibiotic supplement. The number of plasmid-carrying cells was determined by replica plating onto LB agar plates with ampicillin. Numbers indicate the proportion of plasmid-harboring cells recovered at different time points. Filled line: *infA*- strain ST1 harboring pET32-*infA*, Broken line: Parental strain SL003 transformed with pET32-*infA*.

[0070] FIG. 47 EGFP positive cells were directly measured by FACS analysis. Human MDA-MB-231 cancer cells treated with PBS alone were defined as mock controls. 2000 cells were acquired. Dot plot representation of percentage of mammalian cells infected with ST1/*psgfp* showing green fluorescence.

[0071] FIGS. 48A-B Surface display and secretion expression of reporter proteins by ST1. (A) ST1/pLpp_ompA_GFP was cultured and directly photographed by fluorescent microscopy. (B) Secretory expression of recombinant protein SspH2 (1-142aa)-GFP by ST1/pSspH2-GFP in vitro. The tdTomato-expressing MDA-MB-231 cells were infected with ST1 harboring pGFP or pSspH2-GFP for expressing fluorescent marker. The intracellular location of GFP was examined by fluorescent microscopy.

[0072] FIGS. 49A-D The predicted sequence of pSspH2-Endostatin.

[0073] FIGS. 50A-B Secretion of SspH2-Endostatin fusion proteins from SL008/pSspH2-Endostatin inhibits angiogenesis. (A) The cell lysate and cultured medium of SL008/

pSspH2-Endostatin were positive for SspH2-Endostatin by western blot analysis. (B) Inhibition of tumor angiogenesis was estimated by CD31 immunohistochemical analysis. (Magnification, 200 \times).

[0074] FIGS. 51 A-B Anti-tumor effects in the immune-competent mice with aggressive CT26 colon tumors. Tumor-bearing mice treated with Endostatin-expressing SL008. Tumor growth curve (A) and actual size (B) of mice received with the indicated treatments.

[0075] FIGS. 52 A-B (A) Diagram of EGFP mRNA transcription in ST1/pT7-EGFP. (B) Western blot analysis of EGFP expression at 48 h post infection with ST1 harboring functional mRNA.

[0076] FIG. 53 Fluorescence microscopy analysis of the cells at 48 h post infection with ST1/pIKDE or ST1/pIKDE-EGFP.

[0077] FIGS. 54A-E The predicted sequence of pIKDE-DTA.

[0078] FIGS. 55A-C Effects of ST1/pIKDE-DTA and SL008/pIKDE-DTA in CT26-bearing mice. (A) Tumor growth curves for CT26 tumors receiving the indicated treatments. Values are expressed as mean \pm s.e.m. (B) Intratumoral expression of DT A chain was detected by western blot. (C) Representative photomicrographs of ST1/pIKDE-EGFP and ST1/pIKDE-DTA treated mice at the endpoint.

[0079] FIGS. 56A-F The predicted sequence of pIKDE-HA.

[0080] FIGS. 57A-E The predicted sequence of pIKR-shCAT.

[0081] FIGS. 58A-C ST1/pIKR-shCAT could elicit a potent and specific gene silencing and induce massive cell death in MDA-MB-231 cancer cells. (A) ST1/pIKR-shCAT infection decreased β -Catenin and its downstream gene expression and activated Caspase-3 expression. ST1 harboring the control vector cannot interfere with β -Catenin expression. (B) MTT analysis of cell viability (over 0-96 h) following PBS treatment, treatment with ST1/pIKR-shTom or ST1/pIKR-shCAT. The absorbance of each well at wavelength 600 nm was measured by an ELISA reader. (C) Annexin-V and propidium iodide staining analysis of cell death following the indicated treatments. Flow cytometry indicated cell fractions undergoing cell death (Annexin-V positive, propidium iodide positive) and early apoptosis (Annexin-V positive, propidium iodide negative). Data shown as mean \pm s.e.m. of three separate experiments.

[0082] FIGS. 59A-D Effects of ST1/pIKR-shCAT injection on MDA-MB-231 breast tumors. Tumor growth curves (A) and actual sizes (B) receiving the indicated treatments. (C) Western blot analysis of β -Catenin and α -Tubulin (internal control) expression in tumors at the end point. (D) Tumors were collected from 5 animals on day 20, homogenized and plated onto agar plates with or without ampicillin selection to determine the counts of recombinant and total bacteria, respectively. Values are expressed as mean \pm s.e.m.

5. DETAILED DESCRIPTIONS

[0083] Since *Salmonella* is closely related to the *Escherichia* genus and has a broad host range, its genomic information is clear and share many common features with *E. coli*. Compared to gram-positive bacteria (e.g. *Clostridium*), *Salmonella* is easier to perform genetic manipulations. It survives and proliferates within cells; therefore it can deliver genetic materials in the targeted cells. For example, it may directly deliver ectopic mRNA and utilize the translation

machinery of host cells to synthesize the corresponding exogenous proteins. On the other hand, since it is facultative anaerobic, it is easy to culture it in vitro and then send them to target hypoxic region within tumors. More important, genetically attenuated *Salmonella* vectors provide additional safety as they can be readily controlled or eliminated from the human body by the application of ciprofloxacin in case of serious sepsis and can avoid (random) genomic integration {Crull, 2011 #955}. Thus, *Salmonella* can serve as both bacterial “weapon” and “vector” in research and medication.

[0084] In the process of utilizing live attenuated *Salmonella* as a carrier in cancer therapy or DNA vaccination, safety, stability and delivery efficiency are the most important issue, which can be solved by deleting virulent genes and inserting functional genes. For example, by placing an essential gene *asd* under a hypoxia controlled genetic circuit, *S. typhimurium* strain SL7207 was engineered to an obligate anaerobic *Salmonella* strain YB1. YB1 could only survive inside the tumor, but were totally cleared from other normal tissues. However, its curative ability needs to be further improved. Description of YB1 is in pending U.S. patent application Ser. No. 13/871,716, filed Apr. 26, 2013, the content of which is incorporated by reference in its entirety.

[0085] Therefore, the present disclosure provides a superior modified bacterial strain that has improved curative ability. Provided herein is a modified bacteria comprising one or more of the following characteristics: (a) deletion of one amino acid biosynthesis-related gene *aroA*; (b) mutation of *gmd* gene to preclude the biofilm formation; (c) placing an essential gene aspartate-semialdehyde dehydrogenase (“*asd*”) with a tightly hypoxic control; (d) deletion of the stress response gene *htrA*; (e) introduction of an *infA*⁺ (cloned from *E. coli* MG1655 strain) plasmid in *infA*⁻ mutant makes the bacterial strain plasmid-dependent and (f) integration of the *hlyA* gene coding for Listeriolysin O (LLO) under the regulation of *Salmonella* pathogenicity island II promoter into the genome. In certain embodiments, the modified bacteria comprise two, three, four or five of the above-identified characteristics. In one embodiment, the modified bacteria comprise all of the above-identified characteristics.

[0086] In one embodiment, the modified bacterium comprises a vector comprising T7 RNAP and an essential gene under a tightly hypoxic regulation. In one embodiment, the T7 RNAP is under the control of *lac* promoter. In one embodiment, the essential gene is *asd*. In one embodiment, the FNR related anaerobic capable promoter *PpepT* controls *asd* transcription while an aerobic promoter, *PsodA*, facilitates transcription of antisense *asd* that blocks any leakage of *asd* expression under aerobic conditions. In one embodiment, the modified bacterium is ST1.

[0087] In one embodiment, the modified bacterium comprises a vector comprising a transgene under the transcription regulation by *P_{T7}* and *P_{CMV}* and a T7 RNAP autogene expression cassette. In one embodiment, the transgene and the T7 RNAP comprise a viral ribosome binding site (*IRES_{EMCV}*). In one embodiment, the transgene expresses therapeutic molecules including RNA, DNA and proteins. In one embodiment, the transgene express cytokine. In one embodiment, the cytokine is cytokine sTRAIL. In one embodiment, the transgene expresses a fusion protein. In one embodiment, the transgene is a toxin. In one embodiment, the toxin is DT-A. In one embodiment, the transgene is an antigen. In one embodiment, the antigen is Influenza A virus (A/Shanghai/4664T/2013(H7N9)) hemagglutinin (HA). In one embodiment, the

modified bacteria comprise a vector with chromosomal *infA*, *aroA* and *gmd* deletion and site-specific integration of T7 RNAP and *hlyA* gene. In certain embodiments, the vector comprises one or more of the following: (i) less than 10 kb of sequences; (ii) an origin of replication; (iii) *E. coli infA* locus; (iv) T7 RNAP autogene cassette; (v) a 72-bp element of the SV40 enhancer for enhancing nuclear entry and (vi) therapeutic gene expression driven by both *P_{CMV}* and *P_{T7}*. In one embodiment, the vector is ST1.

[0088] In one embodiment, the transgene expresses an oligonucleotide encoding a shRNA or a microRNA precursor. In one embodiment, the shRNA is against a cell cycle-associated protein polo-like kinase 1 (“PLK1”). In one embodiment, the shRNA is against a key intracellular signal transducer beta-catenin (“CTNNB1”) in the Wnt signaling pathway. In one embodiment, the microRNA precursor is tumor suppressor microRNA *let-7*.

[0089] Described herein is a method of making the modified bacteria. The method comprises one or more of the following steps: (a) deletion of the amino acid biosynthesis-related gene, such as *aroA*, *gua*, *thy*, *leu* and *arg* gene; (b) mutation of the genes required for biofilm formation on the surface of epithelial cells, such as *csgD*, *adrA* and *gmd*; (c) placing an essential gene *asd* with a tightly hypoxic control; (d) construction of a balanced-lethal system in which the *infA* gene of *E. coli* MG1655 strain was designed to be introduced in a plasmid that complements an *infA* mutation in the chromosome of the *Salmonella* strain; (e) deletion of the stress response gene, such as *htrA*, *recA* and *hsp* gene and (f) integration of a pore-forming cytolysin gene under the control of an in vivo-inducible promoter.

[0090] Described herein is a tumor-targeting vector for prokaryotic-eukaryotic delivery and expression and a method of making the vector. In a preferred embodiment, an engineered strain ST1, was generated in the *Salmonella* 7207 strain background using the λ Red-mediated ‘long homology arm’ recombination technology. In certain embodiments, the method comprises one or more of the following steps: (i) integration of a T7 RNA polymerase (T7 RNAP) gene into the *gmd* chromosomal locus; (ii) mutation or deletion of *gmd* gene encoding GDP-mannose 4,6-dehydratase; (iii) replacement of an essential gene, such as *asd* with a pore-forming listeriolysin O gene such as *hlyA*; (iv) the essential gene such as *asd* gene with anaerobic control, for example, an essential gene expression cassette comprising an essential gene cloned behind a hypoxia-inducible promoter in the sense orientation as well as a aerobic promoter in the antisense orientation is added at the *htrA* gene locus; (v) double mutation of *asd* and *htrA*; and (vi) relocate a small essential gene such as *infA* (encoding for translation initiation factor 1) from chromosome to plasmid.

[0091] In certain embodiments, the modified bacteria include, but not limited to *Salmonella*, *Escherichia coli*, *Shigella*, *Bacillus Calmette-Guerin* (BCG), *Listeria monocytogenes*, *Yersinia*, *Mycobacterium*, *Streptococcus*, and *Lactobacillus*. In certain embodiments, the modified bacteria are *Salmonella typhimurium*, *Salmonella choleraesuis*, *Salmonella enteritidis* and *S. typhimurium*, *Escherichia coli*, *Escherichia coli* K-12, *Escherichia coli* O157:H7, *Shigella*, *Shigella dysenteriae*, *Shigella flexneri*, *Shigella boydii*, *Shigella sonnei*, *Yersinia*, *Yersinia pestis*, *Yersinia pseudotuberculosis* and *Yersinia enterocolitica*.

[0092] Provided herein is a method of producing exosomes comprising one or more protein or peptides, mRNA, shRNA,

microRNA or a combination therefore. The method comprises the steps of infecting a host animal with ST1 that comprises a vector expressing a transgene; and isolating exosomes from said host animals.

5.1 Generation and Functional Assays of Tumor-Targeted Delivery and Expression Vector ST1

[0093] ST1 strain was engineered from the auxotrophic *S. typhimurium* 7207 strain through a series of genetic manipulations (FIG. 1, Table 1). The SL7207 stain has the following genotype: *S. typhimurium* 2337-65 derivative hisG46, DEL407 [aroA::Tn10 (Tcs)]. First, the integration of T7 RNAP expression cassette into the *grad* chromosomal locus leads to a moderate, stable level of T7 RNAP in the bacterial cells (FIG. 2A) and an inability to form colanic acid (FIG. 2B). T7 RNAP-mediated transcription within bacterial hosts triggered the expression of reporter gene downstream of the P_{T7} . Next, by placing the essential gene *asd* under a tightly hypoxic regulation at the *htrA* locus, the lethal toxicity of parental strain has been totally removed while the targeted capability significantly increased without compromising the normal functions (FIG. 3 & FIG. 4A). ST1 is accumulated in tumors and other organs during a 3-week observation (FIG. 4A). A large number of bacteria accumulate within the solid tumors, achieving about 10^8 - 10^9 cfu/gram tissues. Lack of nonspecific accumulation in the liver and other organs is a major improvement over most current bacterial systems. In contrast, ST1 was gradually eliminated from healthy tissues. Biodistribution study of normal organs on 21 day post-infection also showed that the bacteria were barely detectable in

mouse blood, lung, heart, liver, spleen, kidney and lymph node, which further showed that this bacterial vector is replication-incompetent in normal organ tissues. A more detailed examination of the distribution of ST1 inside the tumors revealed that the bacteria were resisted to the hypoxic regions (Hyperxyprobe-1 labeled) (FIG. 4B). As shown herein, after intravenous administration of ST1 into tumor-bearing animals, the bacteria are dispersed throughout the body, but only those that encounter the hypoxia/necrotic regions of the tumor can survive and amplify.

[0094] ST1 can target the solid tumors and invade into the targeted cells (FIG. 5). Subsequently, the bacteria break the endosomal compartment with the helper protein LLO and release the multiple components into the cytosol of the targeted cells. For the first time, the phagosome-disrupting ST1 directly deliver both plasmid DNA and translation-competent mRNA with IRES_{ECMP} structure driven by T7 RNAPs into the cytosol, leading to model gene (EGFP) expression (FIG. 6). To address the plasmid instability issue, an *infA*⁺ vector/*infA*⁻ host maintenance system was developed. Colony-forming unit (CFU) tests suggested that both high-copy-number (*pUC* origin) and low-copy-number plasmid (*CoE1* origin) in the ST1 was stable at 3 weeks in vivo (FIG. 7), while those in its paternal strain were disappearing within 48 h. The high-copy-number plasmids were still maintained (474.4 ± 35.4 copies/cell) as long as 3 weeks in vivo. The advantages of this host/plasmid *Salmonella* stability system based on *infA* gene include no cross-feeding effect, small vector size, feasibility, avoiding antibiotics and antibiotic resistance genes.

TABLE 1

Bacterial strains and plasmids used		
Strains and plasmids	Relevant genotype	Reference or source
<i>S. typhimurium</i>		
SL7207	<i>S. typhimurium</i> 2337-65 derivative hisG46, DEL407 [aroA::Tn10(Tc-s)]; wild type	Lab stock
SL001	SL7207 Δ gmd::T7 RNAP	This study
SL002	SL7207 Δ gmd::T7RNAP; Δ asd::PsseA-hlyA	This study
SL003	SL7207 Δ gmd::T7 RNAP; Δ asd::PsseA-hlyA; Δ htrA::cat-PpepT-asd-sodA	This study
SL004 (ST1)	SL7207 Δ gmd::T7 RNAP; Δ asd::PsseA-hlyA; Δ htrA::cat-PpepT-asd-sodA; Δ infA::tetR	This study
SL005	SL7207 Δ gmd::T7 RNAP; Δ invA	This study
SL006	SL7207 Δ gmd::T7 RNAP; Δ asd::PsseA-hlyA; Δ infA::cat	This study
SL007	SL7207 Δ gmd::T7 RNAP; Δ htrA::PsseA-hlyA; Δ infA::cat	This study
SL008	SL7207 Δ gmd::T7 RNAP; Δ asd::PsseA-hlyA; Δ htrA::PpepT-asd-sodA; Δ infA::tetR	This study
Plasmid		
pBSK-cat	Ap ^R ; Cm ^R ; pBSK derivative with loxp-cat-loxp fragment	This study
pYB-asd-hlyA	Ap ^R ; Cm ^R ; pBSK-cat derivative with long homology arms of <i>asd</i> sites; P _{sseA} -hlyA-cat	This study
pYB-htrA-asd	Ap ^R ; Cm ^R ; pBSK derivative with long homology arms of <i>htrA</i> sites; cat-PpepT-asd-sodA	This study
pYB-infA-tetR	Ap ^R ; Tet ^R ; pBSK derivative with long homology arms of <i>infA</i> sites; <i>infA</i> locus from <i>E. coli</i> MG1655 strain	This study
pEGFP-C1	Km ^R ; cloning vector	Clontech
pET32a-infA	Ap ^R ; pET32a (+) derivative with <i>infA</i> locus from <i>E. coli</i> MG1655 strain	This study
pcDNA3.1-infA	Ap ^R ; pcDNA3.1(+) derivative with <i>infA</i> locus	This study
pT7-EGFP	Ap ^R ; pET32-infA derivative with P _{T7} -IRES-kozak-EGFP-pA ₂₀	This study

TABLE 1-continued

Bacterial strains and plasmids used		
Strains and plasmids	Relevant genotype	Reference or source
pSE1	Ap ^R ; pcDNA3.1(+) derivative with P _{CMV} -IRES-EGFP	This study
pSE2	Ap ^R ; pcDNA3.1(+) derivative with P _{T7} -IRES-EGFP	This study
pSE3	Ap ^R ; pcDNA3.1(+) derivative with P _{CMV/T7} -IRES-EGFP	This study
pIKDE-EGFP	Ap ^R ; pcDNA3.1(+) derivative with EGFP expression cassette driven by P _{CMV/T7} dual promoter and T7 RNAP autogene cassette	This study
pIKDE	Ap ^R ; pIKDE-EGFP derivative without EGFP gene	This study
pIKDE-DTA	Ap ^R ; pcDNA3.1(+) derivative with DT-A expression cassette driven by P _{CMV/T7} dual promoter and T7 RNAP autogene cassette	This study
pIKDE-Endo	Ap ^R ; pcDNA3.1(+) derivative with Endostatin expression cassette driven by P _{CMV/T7} dual promoter and T7 RNAP autogene cassette	This study
pIKDE-HA	Ap ^R ; pcDNA3.1(+) derivative with Influenza A virus hemagglutinin (HA) expression cassette driven by P _{CMV/T7} dual promoter and T7 RNAP autogene cassette	This study
pIKDE-PEA	Ap ^R ; pcDNA3.1(+) derivative with PEA (II + III) expression cassette driven by P _{CMV/T7} dual promoter and T7 RNAP autogene cassette	This study
pIKDE-shepherdin	Ap ^R ; pcDNA3.1(+) derivative with Shepherdin expression cassette driven by P _{CMV/T7} dual promoter and T7 RNAP autogene cassette	This study
pIKDE-sTRAIL	Ap ^R ; pcDNA3.1(+) derivative with soluble TRAIL expression cassette driven by P _{CMV/T7} dual promoter and T7 RNAP autogene cassette	This study
pLpp_ompA_GFP	Ap ^R ; pET32-infA derivative with Lpp_ompA fragment with GFP	This study
pSspH2-GFP	Ap ^R ; pET32-infA derivative with SspH2_(1-142aa)_FLAG fragment with GFP	This study
pLpp_ompA_sTRAIL	Ap ^R ; pET32-infA derivative with Lpp_ompA fragment with sTRAIL	This study
pSspH2-sTRAIL	Ap ^R ; pET32-infA derivative with SspH2_(1-142aa)_FLAG fragment with sTRAIL	This study
pSspH2-Endostatin	Ap ^R ; pET32-infA derivative with SspH2_(1-142aa)_FLAG fragment with mouse Endostatin	This study
pIKR-shTom	Ap ^R ; pcDNA3.1(+)-infA derivative with T7 RNAP autogene cassette driven by P _{CMV/T7} dual promoter and shRNA cassette against tdTomato	This study
pIKR-shPLK	Ap ^R ; pIKR-shTom derivative with shRNA sequence against human PLK1	This study
pIKR-shCAT	Ap ^R ; pIKR-shTom derivative with shRNA sequence against human CTNNB1	This study
pIKR-shTAK1	Ap ^R ; pIKR-shTom derivative with shRNA sequence against human TAK1	This study
pIKR-let-7	Ap ^R ; pIKR-shTom derivative with human let-7a miRNA	This study
TRIP	Ap ^R ; transkingdom RNA interference plasmid	(Xiang, Fruehauf et al. 2006)
TRIP-shCAT	Ap ^R ; TRIP derivative with shRNA sequence against CAT	(Xiang, Fruehauf et al. 2006)
TRIP-shCAT-infA	Ap ^R ; TRIP-shCAT derivative with infA locus	This study

5.2 ST1 Carrying an Inter-Kingdom Dual Expression (IKDE) System Leads to a Rapid and High-Level Transgene Expression

[0095] Although ST1 is able to localize the cell cytoplasm and efficiently release genetic materials, one obstacle most likely still hindering DNA delivery is the nuclear trafficking. Here, a novel IKDE system is provided, including a T7 RNAP-based cytoplasmic expression system as well as the nuclear system. The activation of transgene expression was based on an inter-kingdom interaction of bacteria and host cells (FIG. 8). First, plasmid pIKDE-EGFP was constructed (FIG. 9), which contains a transgene expression cassette

under the transcriptional regulation by both P_{T7} (cytoplasmic) and P_{CMV} (nuclear) through a dual expression system and a T7 RNAP autogene-based cytoplasmic expression cassette. Furthermore, the insertion of a ribosome binding site (IRE-S_{EMCV}) allows for the cap-independent translation of cytoplasmic transcripts driven by T7 RNAPs.

[0096] Next, kinetics of the reporter expression was tested after ST1-mediated delivery of an IKDE system versus that of plasmid DNA and/or translation-competent mRNA in the post-infection period (FIG. 10). The EGFP expression resulting from 'pre-made' translation-competent mRNA released by ST1/pIKDE-EGFP occurred as early as 5 h after infection (FIG. 11), whereas EGFP expression after delivery of plas-

mid DNA pSE3 (P_{CMV} -IRES-EGFP) was only observed 24-48 h post infection (p.i.). The DNA/RNA dual delivery by ST1/pSE3 ($P_{CMV/IT7}$ -IRES-EGFP) led to a higher expression level compared to a single delivery system, evidencing that ST1-mediated delivery of eukaryotic plasmid DNA plus translation-competent mRNA significantly enhanced ST1-mediated transfection efficiency. In the time course, EGFP expression in ST1/pIKDE-EGFP maintained at the highest levels at all time points, resulting in a >50-fold and 10-fold increase in the average gene expression compared to a standard nuclear and DNA/RNA dual delivery system, respectively (FIG. 12). This indicated that the incorporation of an autogene expression cassette can maintain a stable and continuous cytoplasmic expression of a gene of interest through a self-amplifying regeneration mechanism for the polymerases. It is the first report to date describing the design and use of such combined inter-kingdom expression system in gene therapy.

5.3 In Vitro Screening of Potential Drugs by ST1-Mediated Inter-Kingdom Gene Transfer and RNAi System

[0097] We are capable of having far more effective in vitro and in vivo screening methods. Using our synthetic inter-kingdom expression platform, the intracellular expression of proteins and small interfering RNAs can be achieved by ST1-mediated gene transfer and RNAi. We sought to assess the therapeutic effects of promising candidates (Table. 2) on human cancers by in vitro screening. ST1 harboring different therapeutic candidates (e.g. protein, DNA or RNA, either individually or in combination) were added to the medium and released multiple cargos into the cells. The effects of the therapeutic factors were detected by measuring or monitoring physiological events such as cell death, proliferation or disturbances in signal transduction pathways. Here, cell viabilities and apoptosis rates were measured to rapidly evaluate anti-tumor factors, including protein, shRNA and microRNA on human breast cancer MDA-MB-231 cells. Polypeptide DT-A and shRNA against PLK1 were most effective in suppressing growth and killing abilities (FIG. 13).

TABLE 2

Potential candidates tested by ST1-mediated inter-kingdom system in vitro		
Drug candidates	Description	Functions
EGFP	Enhanced GFP	Fluorescent protein
DTA	Diphtheria toxin fragment A	Protein synthesis inhibitor
Endostatin	type XVIII collagen's C-terminal fragment	Angiogenesis inhibitor
PEA (II + III)	Pseudomonas exotoxin A domain	Protein synthesis inhibitor
Shepherdin	Shepherdin (79-87 aa)	Peptidomimetic antagonist of the complex between Hsp90 and survivin
sTRAIL	TRAIL'S soluble domain	Apoptotic inducer
shTom	shRNA against dtTomato	No actual target
shCAT	shRNA against human β -Catenin	Wnt signaling pathway inhibitor and metastatic inhibitor
shHer-2	shRNA against human Her-2	Pro-apoptotic inducer and cell growth inhibitor
shPLK	shRNA against human Polo-like kinase 1	Pro-apoptotic inducer and cell growth inhibitor

TABLE 2-continued

Potential candidates tested by ST1-mediated inter-kingdom system in vitro		
Drug candidates	Description	Functions
shTAK1	shRNA against human TAK1	Wnt signaling pathway inhibitor and apoptotic inducer
let-7	Human let-7a micro RNA	Cell cycle, proliferation, and apoptosis regulator

5.4 A Host/Plasmid System Based on infA that is not Dependent on Antibiotics and Antibiotic Resistance Genes for Stable Plasmid Maintenance

[0098] The present invention provides a method for plasmid maintenance, the method comprising: providing expression plasmids comprising the plasmid maintenance systems described herein and encoding for a protein of interest, said expression plasmids having copy numbers which vary from low copy number (1~10 copies per cell) to medium copy number (15~20 copies per cell) to high copy number (up to 100's of copies per cell); transforming bacterial live vectors with such expression plasmids; and testing for stabilities in vivo (FIG. 7). This system takes advantage of the phenotype of the *infA*⁻ mutant, which can not synthesize translation initiation factor 1. A complementation plasmid with a functional copy of the *infA* gene from *E. coli* MG1655 was constructed providing a translation initiation factor source and thus allowing growth of the auxotrophic bacterial strain. Interestingly, *E. coli* *infA* expression cassette succeeded to complement *infA*⁻ mutant *S. typhimurium*. Plasmid constructs based on this backbone could therefore be selected and maintained in culture without addition of antibiotics. We demonstrate the plasmids carrying an *infA* gene complemented the phenotype of the *infA*⁻, and that therapeutic plasmids carrying this selectable marker were maintained faithfully both in vitro and in an animal system in the absence of selection pressure (FIG. 7, also see Example 6.5). The main advantages of *infA* targeting include minimal metabolic burden and no cross-feeding effect.

5.5 Engineered Tumor-Targeting Bacterial Vector ST1 Expressing Active Cytokines Leads to Delayed Tumor Growth

[0099] Provided herein is a method of delivery of active anti-tumor proteins. In accordance with this invention therapeutic proteins are introduced into tumor cells via a bacterial vector comprising a nucleic acid sequence encoding for a therapeutic gene. Unlike traditional chemotherapy drugs, as a carrier for generating heterogenous therapeutic proteins, ST1 can selectively grow inside solid tumors and continuously release the biologically active proteins in situ at high regional concentration, thereby achieving maximal killing effects while sparing systemic cytotoxicity. Special delivery systems in *Salmonella* carriers such as surface display or secretion of therapeutic proteins were shown to be advantageous for eliciting antitumor responses. FIG. 14 presented two strategies to express cytokine sTRAIL. One is to deliver the therapeutic proteins through a surface display system by fusing with *E. coli* *lpp_ompA* (46-159) hybrid protein. Another one is to secrete them via *Salmonella* type III secretion system in the intracellular space or inside the tumor cells.

[1000] To examine whether the soluble TRAIL fusion protein could target to surface, the outer-membrane fraction of ST1/pLpp_ompA_sTRAIL bacterial cells was isolated by ultracentrifugation. One single band migrating at a molecular

mass of the expected size of the monomeric form of the Lpp_ompA (46-159) fusion protein was detected by western blot (FIG. 15). Another plasmid pSspH2-sTRAIL was constructed to express chimeric proteins. In this plasmid, 1-142 amino acids from protein SspH2, which are recognized as the secretion signal for *Salmonella* type III secretion system, were fused to a soluble TRAIL encoding sequence. The correct plasmid was transformed into ST1 by electroporation. ST1/pSspH2-sTRAIL was exposed to MDA-MB-231 cells and the presence of SspH2-sTRAIL in the culture medium and the translocated fraction were confirmed by western blot. ST1 secreted the fusion proteins into the cytosol of target cells through the type III secretion system; while a type III secretion-defective (\square_{invA}) failed to translate (FIG. 16). To study the bacterial colonization and distribution of sTRAIL inside the tumors, immunohistochemistry assays on tumor sections were carried out. As shown in FIG. 17, the presence of sTRAIL was detected in tumor specimens, indicating that sTRAIL-expressing ST1 successfully expressed exogenous cytokines in vivo.

[0101] After validation of protein expression, the tumor inhibitory effects were examined in a nude mouse model. Tumor volumes were monitored by a two-dimensional caliper measurement. As indicated in FIG. 18A, tumors in the PBS treated group grew exponentially; increasing 10-fold during the observed period. Tumor growth was continuously reduced during the first week post-injection in the sTRAIL-expressing ST1 treated groups, and the difference between ST1/pLpp_ompA_sTRAIL or ST1/pSspH2-sTRAIL with vector controls was significant ($P < 0.05$). ST1 alone had slight anti-cancer effect on breast tumors; with ~25% inhibition on day 20. The mean tumor volume was reduced by approximately 70% after treatments with the sTRAIL-expressing ST1, which created a sTRAIL-enriched tumor microenvironment, leading to a more potent suppression effect that that achieved by ST1 treatment alone. During the treatments, the animals in bacterially treated groups showed a transient weight loss (FIG. 18B). However, the observed weight loss was totally reversible after several days post injections. ST1 gradually disappeared in normal tissues after intravenous administration with no significant side effects (FIG. 19). Gross appearances and behaviors of mice provided no signs of systemic toxicity.

5.6 Suppression of Tumor Growth and Metastasis by ST1-Mediated Expression of Therapeutic Genes

[0102] In vitro results encourage us to determine whether ST1 could trigger a high level expression of therapeutic genes in vivo. In a certain embodiment, DT-A gene, encoding the catalytic fragment of diphtheria toxin, was cloned into plasmid pIKDE. The bacteria-vector system consists of the *Salmonella* ST1 with chromosomal infA and gmd deletion, integration of T7 RNAP and LLO expression cassette, and tightly anaerobic control of survival, carrying a plasmid pIKDE-DTA with the following features: (1) a reasonably small size (9.7 kb); (2) an origin of replication responding for a high copy number; (3) *E. coli* infA locus allowing in vivo plasmid maintenance; (4) T7 RNAP autogene cassette which can amplify the polymerases after cytoplasmic entry; (5) a 72-bp element of the SV40 enhancer and (6) the suicide gene was fused in frame with the Kozak sequence and inserted into down-stream of the CMV/T7 combinational promoter. It is

the first report to date describing the design and use of such a T7 RNAP autogene-based nuclear/cytoplasmic dual expression system.

[0103] The therapeutic efficiency of ST1/pIKDE-DTA was tested in a metastatic breast cancer model. To do so, 4T1 mouse tumor cells were implanted into the mammary fat pad of immune-competent, syngeneic BALB/c hosts. The 4T1 tumors are highly malignant and often lead to death because of metastasis, rapid growth rate and limited treatment options. Biodistribution experiments in the immune-competent mice confirmed that the bacteria were specifically internalized by primary tumors and metastatic nodules (FIG. 20). In animals that received ST1/pIKDE-DTA treatment, tumor-specific DT A chain expression increased gradually over the course of several weeks (FIG. 21A). After 3 weeks, all the mice have been sacrificed and primary tumors have been harvested. First, total RNA was reverse transcribed by using DT-A specific reverse anchor primer. $92.7 \pm 1.7\%$ transcripts in cells were driven by T7 RNAP-based cytoplasmic expression system (FIG. 21B). Next, immunocytochemistry followed by indirect immunofluorescence and DAPI staining on tumor sections revealed the definitive intracellular presence of bacterial toxins (red) in the cytosol of ST1/pIKDE-DTA (green) infected cells, but not in ST1/pIKDE infected counterparts (FIG. 22). Western blot (FIG. 23A) and immunohistochemical analysis also confirmed the intracellular expression of DT A chain (FIG. 23B). The spatial distribution of ST1 and DT A chain in tumor sections revealed the therapeutic proteins diffused around the bacteria and some molecules have been found to be transferred to the viable rim (FIG. 23B), which contributed to extensive tumor cytolytic abilities. Relative to vector control, the in situ expression of DT-A triggered by ST1/pIKDE-DTA caused significant cell death ($P = 0.022$) in a short time (at 3 days) after treatments (FIG. 24).

[0104] In vivo antitumor effect of ST1-mediated expression of DT A chain was evaluated in terms of tumor growth and survival rate. Systemic delivery of ST1/pIKDE-DTA potentially reduced growth of primary tumors (FIG. 25A, B) and pulmonary metastases (FIG. 25C) in mouse models using multidrug-resistant murine tumors, whereas ST1/pIKDE-EGFP showed a slight inhibitory effect. A single dose of 5×10^7 ST1/pIKDE-DTA resulted in turning tumor into a crusty mass and enabled the complete survival of mice bearing aggressive tumors (FIG. 26). Similar results were also obtained in the study of the MDA-MB-231 xenograft model. Mice bearing established tumors ($\sim 250 \text{ mm}^3$) were dosed once with 100 μl PBS, ST1/pIKDE or ST1/pIKDE-DTA. In the PBS treated group, the tumors grew rapidly and exceeded a mean of 2500 mm^3 at day 24, while nearly 90% of tumor burden was inhibited in the ST1/pIKDE-DTA treated mice, with a mean volume of $274 \pm 66.0 \text{ mm}^3$ at the same time point (FIG. 27A). Medium survival of ST1/pIKDE-DTA treated mice is significantly longer than either the empty vector treated mice (44 days) or untreated controls (41 days), with an increase in the 60-day survival from 0% to 75% ($P < 0.001$) (FIG. 27B). After ST1/pIKDE-DTA injection, 25% tumors (3 of 12) were totally eliminated with breast tumors, and the animals remained cancer-free and survived till the 2-month observation stopped (FIG. 27C). Taken together, ST1/pIKDE-DTA treatment was effective in tumor shrinkage and greatly reduced the risk of death by tumor development.

5.7 ST1-Mediated an Enhanced Inter-Kingdom RNAi

[0105] ST1 packaged with shRNA-encoding plasmid DNA has knockdown effects in human cancer xenografts. The theoretic steps implemented for inter-kingdom RNAi were shown in FIG. 28. First, the oligonucleotides encoding shRNA against no actual target tdTomato and a cell cycle-associated protein polo-like kinase 1 (PLK1) gene which express in most human tumors (Liu, Lei et al. 2006) was inserted to generate pIKR-shTom and pIKR-shPLK. The targeting sequence of human Plk1 (GenBank accession no NM_005030, term id. 34147632) is AGATCACCTCCTTAAATATT, corresponding to the coding regions of positions 1424 to 1444. After transformation, a high amount of shRNA (5.9 ± 0.6 pg/ng total RNA) was detected in the bacterial host. Subsequently, the MDA-MB-231 xenograft model was established and treated with PBS, ST1/pIKR-shTom or ST1/pIKR-shPLK. The targeted protein and mRNA expression were examined at 3 weeks following injections. PLK1 transcript level in tumors treated with ST1/pIKR-shPLK was $75.5 \pm 11.5\%$ lower than the controls treated with the saline buffer ($P=0.002$) and $62.5 \pm 18.6\%$ lower than in mice injected with vector control ($P=0.015$) (FIG. 29A). The presence of sequence-specific 5' RACE-PCR cleavage products also confirmed a sustained RNAi-mediated mechanism of action up to 3 weeks after a single dose (FIG. 29B). A dramatic reduction of tumor-related gene expression in tumors with ST1/pIKR-shPLK at protein levels was also confirmed by western blot (FIG. 30A) and immunohistochemical assay (FIG. 30B). No induction in interferon-inducible gene OAS1 (encoding 2',5'-oligoadenylate synthetases) ($P=0.42$, $n=3$) was detected in ST1/pIKR-shTom or ST1/pIKR-shPLK treated mice (FIG. 31), suggesting cytokine induction was not responsible for the observed effects.

[0106] The incorporation of T7 RNAP autogene cassette is designed to maintain a high transcription level in the mammalian system, which was confirmed by quantitative RT-PCR (159.1 ± 67.4 copies/ng RNA). To determine whether the T7 RNAP-based cytoplasmic expression system elicits vector specific shRNA transcription in the transformed *Salmonella* as well as in the bacterially infected host cells, the gene-silencing activity of ST1 harboring shRNA expression vector with or without the T7 RNAP locus were compared. As expected, the knockdown efficiency of ST1/pIKRΔT7P-shPLK (bacteria-mediated RNAi only) largely decreased compared to ST1/pIKR-shPLK (inter-kingdom RNAi) at a rather long time (FIG. 32A), which corresponded to a significantly lower level of shPLK expression as measured by quantitative real-time RT-PCR (FIG. 32B, $P=0.006$). These results suggested that systemic administration of ST1 with inter-kingdom RNAi system could induce a potent, specific and continuous gene silencing in mammals after a single treatment.

[0107] The enhanced therapeutic effect of bacteria plus ST1-mediated inter-kingdom RNAi led to a noticeable tumor growth reduction compared to that in controls (FIG. 33). On day 24 following treatments, the tumor volume was 2777.0 ± 371.5 mm³ and 1928.8 ± 520.6 mm³ in the buffer control and ST1/pIKR-shTom group respectively, whereas it was 903.8 ± 303.8 mm³ in the ST1/pIKR-shPLK treated mice. Furthermore, decreased angiogenic marker CD31 expression and increased apoptotic tumor cells were observed in the tumors treated with ST1/pIKR-shPLK, which may contribute to tumor inhibitory effects observed in this study (FIG. 34).

5.8 Systemic Toxicity Testing of ST1-Mediated Therapeutic System

[0108] In order to exclude any unspecific toxic effect responsible for the observed effects, preliminary acute toxicity experiments were conducted. Body weight of each mouse was recorded every other day. Total body weights of ST1 treated mice reduced at the beginning and then recovered to normal conditions (FIG. 35A). Treatment was well tolerated with no gross sign of sepsis and no acute spleen enlargement was detected after ST1 infection (FIG. 35B). To investigate the long-term consequences, serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels, an indicator of liver injury, were measured at the end point. These levels in all treatment groups were in the normal range (FIG. 36), which indicated no detectable hepatic stress. The reason might be that our therapeutic system causes low systemic toxicity and early recovery from the impaired liver function. No detectable pathological damage in the livers, kidney and lungs as shown in the H&E stained tissue sections (FIG. 37). According to these data, we conclude that the observed curative effects of our therapeutic system are unlikely related to systemic cytotoxicity.

5.9 Exosomes Derived from the Mice Infected with ST1 Harboring Inter-Kingdom Therapeutic System

[0109] Provided herein is a novel exosome-based delivery platform that transfers exogenous cargoes to selected tissues. Exosomes are membrane-bound vesicles of nanoparticle size (40-100 nm) of endocytotic origin and act as natural carriers of mRNA, small RNA and proteins. Accumulating evidences indicate the exosomes may play a critical role in cell-to-cell communication. Various bioactive molecules from one cell can be transferred to another cell via exosomes. By taking advantage of its natural carrier capability, the exosome with exogenous genetic cargoes can facilitate a long-distance delivery of therapeutic factors. In addition, one advantage of these natural nanoparticles is an immune evasion allowing for repeat administration.

[0110] Provided herein is a method to load exosomes with exogenous protein, mRNA and shRNA in vivo by ST1 infection and isolated them from murine model. Exosome-like microvesicles were harvested from the tumors treated with ST1/pIKDE (empty vector) and ST1/pIKDE-DTA by ultracentrifugation or differential centrifugation and filtered through a 0.2 μm size filter to remove impurities. The pelleted exosomes were further dissolved in DEPC water for RNA isolation and Electron microscopy or lysis buffer for protein extraction. Electron microscopy and western blot analysis (FIG. 38) of specific marker protein HSP70 confirmed the presence of exosomes. Total RNA isolated from exosomes was subjected to RT-PCR analysis to identify the presence of DT-A mRNA (FIG. 39A). Immunoblot detected a specific band corresponding to DT A chain (FIG. 39B). These data confirmed the presence of microvesicles containing the transgene mRNA as well as protein in the tumor microenvironment. The transfer of bioactive molecules mediated by these exosomes may contribute to the delivery of therapeutic factors to the uninfected cells (FIG. 23B). Additionally, we also detected the presence of exosomes containing shRNA against CTNNB1 in the mice infected by ST1/pIKR-shCAT (FIG. 40A). These endogenous exosomes may transfer shRNA to the uninfected cells and elicit overall reduction of target proteins in the tumor tissues (FIG. 40B). ST1 infection could

generate large quantities of 'self' exosomes loading with therapeutics for intracellular delivery of these factors. The spatial diffusion pattern of these cytotoxic molecules may exert an enhanced oncolytic effect.

5.10 A DNA/RNA Vaccine Encoding H7N9 Virus HA Antigen Delivered by ST1

[0111] The mutant strains of the invention are highly suitable for use in a live attenuated vaccine, as a live vector and a DNA-mediated vaccine. DNA vaccines have been the subject of much promising research against influenza, but the high copy number plasmids required are notoriously unstable in *Salmonella*. To solve this problem, an expression plasmid is provided which encodes (1) a Plasmid Maintenance system and (2) a protein operally linked to a dual promoter (3) a T7 RNAP autogene-based cassette. Therefore the stability and novel inter-kingdom dual expression platform enables the possibility of new vaccination strategies against H7N9.

[0112] Here, we used the hemagglutinin (HA) from the avian influenza H7N9 virus as a model antigen, which is the essential vaccine antigen, to evaluate the ability of our engineered strain to deliver an antigen encoded by the improved DNA vaccine vector to host tissues. A DNA fragment encoding Influenza A virus (A/Shanghai/4664T/2013(H7N9)) hemagglutinin (HA) with Kozak sequence was inserted downstream of the IRES_{EMCV} in the improved DNA vaccine vector to obtain pIKDE-HA. BALB/c mice were immunized intraperitoneally with ST1/pIKDE-HA at the dosage of 10⁷ CFU. In order to evaluate the humoral immune responses mounted against ST1/pIKDE-HA strain, ELISA assays were performed to test the anti-HA IgG responses using blood sera of vaccinated mice the 14th, 35th and 48th day after immunization. Results indicated that anti-HA responses of mice immunized with ST1/pIKDE-HA strain were moderate on the day 14 after immunization. After receiving three boosts on day 14, 21 and 28, the anti-HA IgG response in the mice were greatly increased. 100% mice (all 7 mice) had high anti-HA IgG responses on day 48 (FIG. 41).

5.11 Formulations

[0113] The modified bacteria containing the RNA and/or DNA molecules provided herein can be formulated for a variety of types of administration, including systemic and topical administration. For systemic administration, injection is preferred, including intravenous, intramuscular, intraperitoneal, intrarectal and subcutaneous routes. For injection, the composition can be formulated in liquid solutions, preferably in physiologically compatible buffers such as Hank's solution or Ringer's solution.

[0114] For oral administration, the pharmaceutical compositions may take the form of, for example, tablets or capsules prepared by mixing the effective amount of bacteria and the proper amount of additives according to known methods in pharmaceutical chemistry. Suitable formulations can be prepared by methods commonly employed using conventional, organic or inorganic additives, such as an excipient selected from fillers or diluents (e.g., sucrose, starch, mannitol, glucose, cellulose, calcium phosphate or calcium carbonate and the like), binders (e.g., cellulose, carboxymethylcellulose, gelatin, gum arabic, polyethyleneglycol or starch and the like), disintegrants (e.g., sodium starch glycolate, croscarmellose sodium and the like), lubricants (e.g., magnesium stearate, light anhydrous silicic acid, sodium lauryl sulfate

and the like), flavoring agents (e.g., citric acid, menthol and the like), preservatives (e.g., sodium benzoate, sodium bisulfate, methylparaben and the like), stabilizers (e.g., citric acid, sodium citrate, acetic acid and the like), suspending agents (e.g., methylcellulose, polyvinyl pyrrolidone or aluminum stearate and the like), dispersing agents (e.g., hydroxypropylmethylcellulose and the like), surfactants (e.g., sodium lauryl sulfate, polaxamer, polysorbates and the like), antioxidants (e.g., Ethylene diamine tetraacetic acid (EDTA), butylated hydroxyl toluene (BHT) and the like) or solubilizers (e.g., polyethylene glycols, SOLUTOL™, GELUCIRE™ and the like).

[0115] The modified bacteria provided herein can be administered to a patient in the conventional form of preparations, such as injections and suspensions. Suitable formulations can be prepared by methods commonly employed using conventional, organic or inorganic additives, such as an excipient selected from fillers or diluents, binders, disintegrants, lubricants, flavoring agents, preservatives, stabilizers, suspending agents, dispersing agents, surfactants, antioxidants or solubilizers.

[0116] Excipients that may be selected are known to those skilled in the art and include, but are not limited to fillers or diluents (e.g., sucrose, starch, mannitol, sorbitol, lactose, glucose, cellulose, talc, calcium phosphate or calcium carbonate and the like), a binder (e.g., cellulose, carboxymethylcellulose, methylcellulose, hydroxymethylcellulose, hydroxypropylmethylcellulose, polypropylpyrrolidone, polyvinylpyrrolidone, gelatin, gum arabic, polyethyleneglycol or starch and the like), a disintegrants (e.g., sodium starch glycolate, croscarmellose sodium and the like), a lubricant (e.g., magnesium stearate, light anhydrous silicic acid, talc or sodium lauryl sulfate and the like), a flavoring agent (e.g., citric acid, or menthol and the like), a preservative (e.g., sodium benzoate, sodium bisulfate, methylparaben or propylparaben and the like), a stabilizer (e.g., citric acid, sodium citrate or acetic acid and the like), a suspending agent (e.g., methylcellulose, polyvinyl pyrrolidone or aluminum stearate and the like), a dispersing agent (e.g., hydroxypropylmethylcellulose and the like), surfactants (e.g., sodium lauryl sulfate, polaxamer, polysorbates and the like), antioxidants (e.g., ethylene diamine tetraacetic acid (EDTA), butylated hydroxyl toluene (BHT) and the like) and solubilizers (e.g., polyethylene glycols, SOLUTOL®, GELUCIRE® and the like). The effective amount of the modified bacteria provided herein in the pharmaceutical composition may be at a level that will exercise the desired effect.

[0117] In another embodiment, provided herein are compositions comprising an effective amount of modified bacteria provided herein and a pharmaceutically acceptable carrier or vehicle, wherein a pharmaceutically acceptable carrier or vehicle can comprise an excipient, diluent, or a mixture thereof. In one embodiment, the composition is a pharmaceutical composition.

[0118] Compositions can be formulated to contain a daily dose, or a convenient fraction of a daily dose, in a dosage unit. In general, the composition is prepared according to known methods in pharmaceutical chemistry. Capsules can be prepared by mixing the modified bacteria provided herein with a suitable carrier or diluent and filling the proper amount of the mixture in capsules.

5.12 Method of Use

[0119] Solid tumor cancers that can be treated by the methods provided herein include, but are not limited to, sarcomas, carcinomas, and lymphomas. In specific embodiments, cancers that can be treated in accordance with the methods described herein include, but are not limited to, cancer of the breast, liver, neuroblastoma, head, neck, eye, mouth, throat, esophagus, esophagus, chest, bone, lung, kidney, colon, rectum or other gastrointestinal tract organs, stomach, spleen, skeletal muscle, subcutaneous tissue, prostate, breast, ovaries, testicles or other reproductive organs, skin, thyroid, blood, lymph nodes, kidney, liver, pancreas, and brain or central nervous system. In certain embodiments, the solid tumors that can be treated by the methods provided herein include, but are not limited to, sarcomas, carcinomas, and lymphomas. In specific embodiments, a cancer that can be treated in accordance with the methods described herein include, but are not limited to, cancer of the breast, liver, colon, neuroblastoma, head, neck, eye, mouth, throat, esophagus, chest, bone, lung, kidney, rectum or other gastrointestinal tract organs, stomach, spleen, skeletal muscle, subcutaneous tissue, prostate, ovaries, testicles or other reproductive organs, skin, thyroid, blood, lymph nodes, pancreas and brain.

[0120] In particular embodiments, the methods for treating cancer provided herein inhibit, reduce, diminish, arrest, or stabilize a tumor associated with the cancer. In other embodiments, the methods for treating cancer provided herein inhibit, reduce, diminish, arrest, or stabilize the blood flow, metabolism, or edema in a tumor associated with the cancer or one or more symptoms thereof. In specific embodiments, the methods for treating cancer provided herein cause the regression of a tumor, tumor blood flow, tumor metabolism, or peritumor edema, and/or one or more symptoms associated with the cancer. In other embodiments, the methods for treating cancer provided herein maintain the size of the tumor so that it does not increase, or so that it increases by less than the increase of a tumor after administration of a standard therapy as measured by conventional methods available to one of skill in the art, such as digital rectal exam, ultrasound (e.g., transrectal ultrasound), CT Scan, MRI, dynamic contrast-enhanced MRI, or PET Scan. In specific embodiments, the methods for treating cancer provided herein decrease tumor size. In certain embodiments, the methods for treating cancer provided herein reduce the formation of a tumor. In certain embodiments, the methods for treating cancer provided herein eradicate, remove, or control primary, regional and/or metastatic tumors associated with the cancer. In some embodiments, the methods for treating cancer provided herein decrease the number or size of metastases associated with the cancer.

[0121] In certain embodiments, the methods for treating cancer provided herein reduce the tumor size (e.g., volume or diameter) in a subject by at least about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 80%, 85%, 90%, 95%, 99%, or 100%, relative to tumor size (e.g., volume or diameter) prior to administration of modified bacteria as assessed by methods well known in the art, e.g., CT Scan, MRI, DCE-MRI, or PET Scan. In particular embodiments, the methods for treating cancer provided herein reduce the tumor volume or tumor size (e.g., diameter) in a subject by an amount in the range of about 5% to 20%, 10% to 20%, 10% to 30%, 15% to 40%, 15% to 50%, 20% to 30%, 20% to 40%, 20% to 50%, 30% to 60%, 30% to 70%, 30% to 80%, 30% to 90%, 30% to 95%, 30% to 99%, 30% to 100%, or any range

in between, relative to tumor size (e.g., diameter) in a subject prior to administration of modified bacteria as assessed by methods well known in the art, e.g., CT Scan, MRI, DCE-MRI, or PET Scan.

[0122] In certain embodiments, the methods for treating cancer provided herein reduce the tumor perfusion in a subject by at least about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 80%, 85%, 90%, 95%, 99%, or 100%, relative to tumor perfusion prior to administration of modified bacteria as assessed by methods well known in the art, e.g., MRI, DCE-MRI, or PET Scan. In particular embodiments, the methods for treating cancer provided herein reduce the tumor perfusion in a subject by an amount in the range of about 5% to 20%, 10% to 20%, 10% to 30%, 15% to 40%, 15% to 50%, 20% to 30%, 20% to 40%, 20% to 50%, 30% to 60%, 30% to 70%, 30% to 80%, 30% to 90%, 30% to 95%, 30% to 99%, 30% to 100%, or any range in between, relative to tumor perfusion prior to administration of modified bacteria, as assessed by methods well known in the art, e.g., MRI, DCE-MRI, or PET Scan.

[0123] In particular aspects, the methods for treating cancer provided herein inhibit or decrease tumor metabolism in a subject as assessed by methods well known in the art, e.g., PET scanning. In specific embodiments, the methods for treating cancer provided herein inhibit or decrease tumor metabolism in a subject by at least about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 80%, 85%, 90%, 95%, or 100%, relative to tumor metabolism prior to administration of modified bacteria, as assessed by methods well known in the art, e.g., PET scanning. In particular embodiments, the methods for treating cancer provided herein inhibit or decrease tumor metabolism in a subject in the range of about 5% to 20%, 10% to 20%, 10% to 30%, 15% to 40%, 15% to 50%, 20% to 30%, 20% to 40%, 20% to 50%, 30% to 60%, 30% to 70%, 30% to 80%, 30% to 90%, 30% to 95%, 30% to 99%, 30% to 100%, or any range in between, relative to tumor metabolism prior to administration of modified bacteria, as assessed by methods well known in the art, e.g., PET scan.

5.13 Patient Population

[0124] In some embodiments, a subject treated for cancer in accordance with the methods provided herein is a human who has or is diagnosed with cancer. In other embodiments, a subject treated for cancer in accordance with the methods provided herein is a human predisposed or susceptible to cancer. In some embodiments, a subject treated for cancer in accordance with the methods provided herein is a human at risk of developing cancer.

[0125] In one embodiment, a subject treated for cancer in accordance with the methods provided herein is a human infant. In another embodiment, a subject treated for cancer in accordance with the methods provided herein is a human toddler. In another embodiment, a subject treated for cancer in accordance with the methods provided herein is a human child. In another embodiment, a subject treated for cancer in accordance with the methods provided herein is a human adult. In another embodiment, a subject treated for cancer in accordance with the methods provided herein is a middle-aged human. In another embodiment, a subject treated for cancer in accordance with the methods provided herein is an elderly human.

[0126] In certain embodiments, a subject treated for cancer in accordance with the methods provided herein has a cancer

that metastasized to other areas of the body, such as the bones, lung and liver. In certain embodiments, a subject treated for cancer in accordance with the methods provided herein is in remission from the cancer. In some embodiments, a subject treated for cancer in accordance with the methods provided herein that has a recurrence of the cancer. In certain embodiments, a subject treated in accordance with the methods provided herein is experiencing recurrence of one or more tumors associated with cancer.

[0127] In certain embodiments, a subject treated for cancer in accordance with the methods provided herein is a human that is about 1 to about 5 years old, about 5 to 10 years old, about 10 to about 18 years old, about 18 to about 30 years old, about 25 to about 35 years old, about 35 to about 45 years old, about 40 to about 55 years old, about 50 to about 65 years old, about 60 to about 75 years old, about 70 to about 85 years old, about 80 to about 90 years old, about 90 to about 95 years old or about 95 to about 100 years old, or any age in between. In a specific embodiment, a subject treated for cancer in accordance with the methods provided herein is a human that is 18 years old or older. In a particular embodiment, a subject treated for cancer in accordance with the methods provided herein is a human child that is between the age of 1 year old to 18 years old. In a certain embodiment, a subject treated for cancer in accordance with the methods provided herein is a human that is between the age of 12 years old and 18 years old. In a certain embodiment, the subject is a male human. In another embodiment, the subject is a female human. In one embodiment, the subject is a female human that is not pregnant or is not breastfeeding. In one embodiment, the subject is a female that is pregnant or will/might become pregnant, or is breast feeding.

[0128] In some embodiments, a subject treated for cancer in accordance with the methods provided herein is administered modified bacteria or a pharmaceutical composition thereof, or a combination therapy before any adverse effects or intolerance to therapies other than the modified bacteria develops. In some embodiments, a subject treated for cancer in accordance with the methods provided herein is a refractory patient. In a certain embodiment, a refractory patient is a patient refractory to a standard therapy (e.g., surgery, radiation, anti-androgen therapy and/or drug therapy such as chemotherapy). In certain embodiments, a patient with cancer is refractory to a therapy when the cancer has not significantly been eradicated and/or the one or more symptoms have not been significantly alleviated. The determination of whether a patient is refractory can be made either *in vivo* or *in vitro* by any method known in the art for assaying the effectiveness of a treatment of cancer, using art-accepted meanings of "refractory" in such a context. In various embodiments, a patient with cancer is refractory when one or more tumors associated with cancer, have not decreased or have increased. In various embodiments, a patient with cancer is refractory when one or more tumors metastasize and/or spread to another organ.

[0129] In some embodiments, a subject treated for cancer in accordance with the methods provided herein is a human that has proven refractory to therapies other than treatment with modified bacteria, but is no longer on these therapies. In certain embodiments, a subject treated for cancer in accordance with the methods provided herein is a human already receiving one or more conventional anti-cancer therapies, such as surgery, drug therapy such as chemotherapy, anti-androgen therapy or radiation. Among these patients are refractory patients, patients who are too young for conven-

tional therapies, and patients with recurring tumors despite treatment with existing therapies.

5.14 Dosage

[0130] The effective amount of the modified bacteria provided herein to be administered to a subject will vary depending on the species of the subject, as well as the disease or condition that is being treated. Preferably, the dosage employed will be 10^7 to 10^{10} viable microorganisms per subject.

[0131] In one aspect, a method for treating cancer presented herein involves the administration of a unit dosage of modified bacteria thereof. The dosage may be administered as often as determined effective (e.g., once, twice or three times per day, every other day, once or twice per week, biweekly or monthly). In certain embodiments, a method for treating cancer presented herein involves the administration to a subject in need thereof of a unit dose of modified bacteria that can be determined by one skilled in the art.

[0132] In some embodiments, a unit dose of modified bacteria or a pharmaceutical composition thereof is administered to a subject once per day, twice per day, three times per day; once, twice or three times every other day (i.e., on alternate days); once, twice or three times every two days; once, twice or three times every three days; once, twice or three times every four days; once, twice or three times every five days; once, twice, or three times once a week, biweekly or monthly, and the dosage may be administered orally.

5.15 Combination Therapy

[0133] Presented herein are combination therapies for the treatment of cancer which involve the administration of modified bacteria in combination with one or more additional therapies to a subject in need thereof. In a specific embodiment, presented herein are combination therapies for the treatment of cancer which involve the administration of an effective amount of modified bacteria in combination with an effective amount of another therapy to a subject in need thereof.

[0134] As used herein, the term "in combination," refers, in the context of the administration of modified bacteria, to the administration of modified bacteria prior to, concurrently with, or subsequent to the administration of one or more additional therapies (e.g., agents, surgery, or radiation) for use in treating cancer. The use of the term "in combination" does not restrict the order in which modified bacteria and one or more additional therapies are administered to a subject. In specific embodiments, the interval of time between the administration of modified bacteria and the administration of one or more additional therapies may be about 1-5 minutes, 1-30 minutes, 30 minutes to 60 minutes, 1 hour, 1-2 hours, 2-6 hours, 2-12 hours, 12-24 hours, 1-2 days, 2 days, 3 days, 4 days, 5 days, 6 days, 7 days, 1 week, 2 weeks, 3 weeks, 4 weeks, 5 weeks, 6 weeks, 7 weeks, 8 weeks, 9 weeks, 10 weeks, 15 weeks, 20 weeks, 26 weeks, 52 weeks, 11-15 weeks, 15-20 weeks, 20-30 weeks, 30-40 weeks, 40-50 weeks, 1 month, 2 months, 3 months, 4 months, 5 months, 6 months, 7 months, 8 months, 9 months, 10 months, 11 months, 12 months, 1 year, 2 years, or any period of time in between. In certain embodiments, modified bacteria and one or more additional therapies are administered less than 1 day, 1 week, 2 weeks, 3 weeks, 4 weeks, one month, 2 months, 3 months, 6 months, 1 year, 2 years, or 5 years apart.

[0135] In some embodiments, the combination therapies provided herein involve administering modified bacteria daily, and administering one or more additional therapies once a week, once every 2 weeks, once every 3 weeks, once every 4 weeks, once every month, once every 2 months (e.g., approximately 8 weeks), once every 3 months (e.g., approximately 12 weeks), or once every 4 months (e.g., approximately 16 weeks). In certain embodiments, modified bacteria and one or more additional therapies are cyclically administered to a subject. Cycling therapy involves the administration of modified bacteria for a period of time, followed by the administration of one or more additional therapies for a period of time, and repeating this sequential administration. In certain embodiments, cycling therapy may also include a period of rest where modified bacteria or the additional therapy is not administered for a period of time (e.g., 2 days, 3 days, 4 days, 5 days, 6 days, 7 days, 1 week, 2 weeks, 3 weeks, 4 weeks, 5 weeks, 10 weeks, 20 weeks, 1 month, 2 months, 3 months, 4 months, 5 months, 6 months, 7 months, 8 months, 9 months, 10 months, 11 months, 12 months, 2 years, or 3 years). In an embodiment, the number of cycles administered is from 1 to 12 cycles, from 2 to 10 cycles, or from 2 to 8 cycles.

[0136] In some embodiments, the methods for treating cancer provided herein comprise administering modified bacteria as a single agent for a period of time prior to administering the modified bacteria in combination with an additional therapy. In certain embodiments, the methods for treating cancer provided herein comprise administering an additional therapy alone for a period of time prior to administering modified bacteria in combination with the additional therapy.

[0137] In some embodiments, the administration of modified bacteria and one or more additional therapies in accordance with the methods presented herein have an additive effect relative to the administration of modified bacteria or said one or more additional therapies alone. In some embodiments, the administration of modified bacteria and one or more additional therapies in accordance with the methods presented herein have a synergistic effect relative to the administration of the Compound or said one or more additional therapies alone.

[0138] As used herein, the term "synergistic," refers to the effect of the administration of modified bacteria in combination with one or more additional therapies (e.g., agents), which combination is more effective than the additive effects of any two or more single therapies (e.g., agents). In a specific embodiment, a synergistic effect of a combination therapy permits the use of lower dosages (e.g., sub-optimal doses) of modified bacteria or an additional therapy and/or less frequent administration of modified bacteria or an additional therapy to a subject. In certain embodiments, the ability to utilize lower dosages of modified bacteria or of an additional therapy and/or to administer modified bacteria or said additional therapy less frequently reduces the toxicity associated with the administration of modified bacteria or of said additional therapy, respectively, to a subject without reducing the efficacy of modified bacteria or of said additional therapy, respectively, in the treatment of cancer. In some embodiments, a synergistic effect results in improved efficacy of modified bacteria and each of said additional therapies in treating cancer. In some embodiments, a synergistic effect of a combination of modified bacteria and one or more additional therapies avoids or reduces adverse or unwanted side effects associated with the use of any single therapy.

[0139] The combination of modified bacteria and one or more additional therapies can be administered to a subject in the same pharmaceutical composition. Alternatively, modified bacteria and one or more additional therapies can be administered concurrently to a subject in separate pharmaceutical compositions. Modified bacteria and one or more additional therapies can be administered sequentially to a subject in separate pharmaceutical compositions. Modified bacteria and one or more additional therapies may also be administered to a subject by the same or different routes of administration.

[0140] The combination therapies provided herein involve administering to a subject to in need thereof modified bacteria in combination with conventional, or known, therapies for treating cancer. Other therapies for cancer or a condition associated therewith are aimed at controlling or relieving one or more symptoms. Accordingly, in some embodiments, the combination therapies provided herein involve administering to a subject to in need thereof a pain reliever, or other therapies aimed at alleviating or controlling one or more symptoms associated with or a condition associated therewith.

[0141] Specific examples of anti-cancer agents that may be used in combination with modified bacteria include: a hormonal agent (e.g., aromatase inhibitor, selective estrogen receptor modulator (SERM), and estrogen receptor antagonist), chemotherapeutic agent (e.g., microtubule disassembly blocker, antimetabolite, topoisomerase inhibitor, and DNA crosslinker or damaging agent), anti-angiogenic agent (e.g., VEGF antagonist, receptor antagonist, integrin antagonist, vascular targeting agent (VTA)/vascular disrupting agent (VDA)), radiation therapy, and conventional surgery.

[0142] Non-limiting examples of hormonal agents that may be used in combination with modified bacteria include aromatase inhibitors, SERMs, and estrogen receptor antagonists. Hormonal agents that are aromatase inhibitors may be steroidal or nonsteroidal. Non-limiting examples of nonsteroidal hormonal agents include letrozole, anastrozole, aminoglutethimide, fadrozole, and vorozole. Non-limiting examples of steroidal hormonal agents include aromasin (exemestane), formestane, and testolactone. Non-limiting examples of hormonal agents that are SERMs include tamoxifen (branded/marketed as Nolvadex®), afimoxifene, arzoxifene, bazedoxifene, clomifene, femarelle, lasofoxifene, ormeloxifene, raloxifene, and toremifene. Non-limiting examples of hormonal agents that are estrogen receptor antagonists include fulvestrant. Other hormonal agents include but are not limited to abiraterone and lonaprisan.

[0143] Non-limiting examples of chemotherapeutic agents that may be used in combination with modified bacteria include microtubule disassembly blocker, antimetabolite, topoisomerase inhibitor, and DNA crosslinker or damaging agent. Chemotherapeutic agents that are microtubule disassembly blockers include, but are not limited to, taxenes (e.g., paclitaxel (branded/marketed as TAXOL®), docetaxel, abraxane, larotaxel, ortataxel, and tasetaxel); epothilones (e.g., ixabepilone); and vinca alkaloids (e.g., vinorelbine, vinblastine, vindesine, and vincristine (branded/marketed as ONCOVIN®)).

[0144] Chemotherapeutic agents that are antimetabolites include, but are not limited to, folate antimetabolites (e.g., methotrexate, aminopterin, pemetrexed, raltitrexed); purine antimetabolites (e.g., cladribine, clofarabine, fludarabine, mercaptopurine, pentostatin, thioguanine); pyrimidine antimetabolites (e.g., 5-fluorouracil, capecitabine, gemcitabine

(GEMZAR®), cytarabine, decitabine, floxuridine, tegafur); and deoxyribonucleotide antimetabolites (e.g., hydroxyurea).

[0145] Chemotherapeutic agents that are topoisomerase inhibitors include, but are not limited to, class I (camptotheca) topoisomerase inhibitors (e.g., topotecan (branded/ marketed as HYCAMTIN®) irinotecan, rubitecan, and belotecan); class II (podophyllum) topoisomerase inhibitors (e.g., etoposide or VP-16, and teniposide); anthracyclines (e.g., doxorubicin, epirubicin, Doxil, aclarubicin, amrubicin, daunorubicin, idarubicin, pirarubicin, valrubicin, and zorubicin); and anthracenediones (e.g., mitoxantrone, and pixantrone).

[0146] Chemotherapeutic agents that are DNA crosslinkers (or DNA damaging agents) include, but are not limited to, alkylating agents (e.g., cyclophosphamide, mechlorethamine, ifosfamide (branded/ marketed as IFEX®), trofosfamide, chlorambucil, melphalan, prednimustine, bendamustine, uramustine, estramustine, carmustine (branded/ marketed as BiCNU®), lomustine, semustine, fotemustine, nimustine, ranimustine, streptozocin, busulfan, mannosulfan, treosulfan, carboquone, N,N'-triethylenethiophosphoramide, triaziquone, triethylenemelamine); alkylating-like agents (e.g., carboplatin (branded/ marketed as PARAPLATIN®), cisplatin, oxaliplatin, nedaplatin, triplatin tetranitrate, satraplatin, picoplatin); nonclassical DNA crosslinkers (e.g., procarbazine, dacarbazine, temozolomide (branded/ marketed as TEMODAR®), altretamine, mitobronitol); and intercalating agents (e.g., actinomycin, bleomycin, mitomycin, and plicamycin).

[0147] Non-limiting examples of other therapies that may be administered to a subject in combination with a Compound include:

- (1) a statin such as lovastatin (e.g., branded/ marketed as MEVACOR®);
- (2) an mTOR inhibitor such as sirolimus which is also known as Rapamycin (e.g., branded/ marketed as RAPAMUNE®), temsirolimus (e.g., branded/ marketed as TORISEL®), everolimus (e.g., branded/ marketed as AFINITOR®), and deforolimus;
- (3) a farnesyltransferase inhibitor agent such as tipifarnib;
- (4) an antifibrotic agent such as pirfenidone;
- (5) a pegylated interferon such as PEG-interferon alfa-2b;
- (6) a CNS stimulant such as methylphenidate (branded/ marketed as RITALIN®);
- (7) a HER-2 antagonist such as anti-HER-2 antibody (e.g., trastuzumab) and kinase inhibitor (e.g., lapatinib);
- (8) an IGF-1 antagonist such as an anti-IGF-1 antibody (e.g., AVE1642 and IMC-A11) or an IGF-1 kinase inhibitor;
- (9) EGFR/HER-1 antagonist such as an anti-EGFR antibody (e.g., cetuximab, panitumumab) or EGFR kinase inhibitor (e.g., erlotinib; gefitinib);
- (10) SRC antagonist such as bosutinib;
- (11) cyclin dependent kinase (CDK) inhibitor such as seliciclib;
- (12) Janus kinase 2 inhibitor such as lestaurtinib;
- (13) proteasome inhibitor such as bortezomib;
- (14) phosphodiesterase inhibitor such as anagrelide;
- (15) inosine monophosphate dehydrogenase inhibitor such as tiazofurine;
- (16) lipoxygenase inhibitor such as masoprocol;
- (17) endothelin antagonist;
- (18) retinoid receptor antagonist such as tretinoin or alitretinoin;

(19) immune modulator such as lenalidomide, pomalidomide, or thalidomide;

(20) kinase (e.g., tyrosine kinase) inhibitor such as imatinib, dasatinib, erlotinib, nilotinib, gefitinib, sorafenib, sunitinib, lapatinib, or TG100801;

(21) non-steroidal anti-inflammatory agent such as celecoxib (branded/ marketed as CELEBREX®);

(22) human granulocyte colony-stimulating factor (G-CSF) such as filgrastim (branded/ marketed as NEUPOGEN®);

(23) folic acid or leucovorin calcium;

(24) integrin antagonist such as an integrin $\alpha 5 \beta 1$ -antagonist (e.g., JSM6427);

(25) nuclear factor kappa beta (NF- κ B) antagonist such as OT-551, which is also an anti-oxidant.

(26) hedgehog inhibitor such as CUR61414, cyclopamine, GDC-0449, and anti-hedgehog antibody;

(27) histone deacetylase (HDAC) inhibitor such as SAHA (also known as vorinostat (branded/ marketed as ZOLINZA)), PCI-24781, SB939, CHR-3996, CRA-024781, ITF2357, JNJ-26481585, or PCI-24781;

(28) retinoid such as isotretinoin (e.g., branded/ marketed as ACCUTANE®)

(29) hepatocyte growth factor/scatter factor (HGF/SF) antagonist such as HGF/SF monoclonal antibody (e.g., AMG 102);

(30) synthetic chemical such as antineoplaston;

(31) anti-diabetic such as rosiglitazone (e.g., branded/ marketed as AVANDIA®)

(32) antimalarial and amebicidal drug such as chloroquine (e.g., branded/ marketed as ARALEN®);

(33) synthetic bradykinin such as RMP-7;

(34) platelet-derived growth factor receptor inhibitor such as SU-101;

(35) receptor tyrosine kinase inhibitors of Flk-1/KDR/VEGFR2, FGFR1 and PDGFR beta such as SU5416 and SU6668;

(36) anti-inflammatory agent such as sulfasalazine (e.g., branded/ marketed as AZULFIDINE®); and

(37) TGF-beta antisense therapy.

The following non-limiting examples are merely illustrative of the preferred embodiments of the present invention, and are not to be construed as limiting the invention.

6. EXAMPLES

6.1: Construction of Tumor-Targeting *Salmonella* ST1 for Delivery and Expression

[0148] With careful genetic engineering, *S. typhimurium* was modified to target solid tumor and express multiple therapeutic molecules. The starting parental strain is the auxotrophic *Salmonella enterica* serovar *typhimurium* 7207 strain (*S. typhimurium* 2337-65 derivative hisG46, DEL407 [aroA::Tn 10{Tc-s}], made by λ -Red mediated recombinering, selecting for the appropriate antibiotic resistance markers. Strain SL001 was constructed by first replacing the *gmd* open reading frame by a RCR-amplified cassette containing a chloramphenicol resistance gene and a T7 RNAP gene (Δ *gmd*::T7 RNAP-cat). Then the PCR product target *gmd* gene was electroporated into recombination-competent cells and selected on LB plates containing 25 μ g/ml of chloramphenicol. Then the excision of the antibiotic gene has been achieved here using plasmid p705cre to produce a recombinase that eliminates DNA fragment flanked by two loxP sites, generating the strain SL001.

[0149] For the integration of hlyA gene into the genome, first, pYB-asd (a pBSK derivative with 1 kb flanking regions of asd sites) has been generated to target the essential gene. Subsequently, an in vivo inducible promoter PsseA sequence was cloned from the *Salmonella* Pathogenicity Island 2 (SPI2) and ligated into plasmid pYB-asd through the NotI and HindIII cutting sites. PCR product loxp-cat-loxp was amplified from plasmid ploxp-cat-loxp and ligated into pYB-asd-PsseA at the XhoI site to create plasmid pYB-asd-PsseA-cat. A hlyA gene encoding LLO was PCR-amplified from *Listeria* genomic DNA and digested with HindIII and XhoI, then ligated into pYB-asd-PsseA-cat, to construct plasmid pYB-asd-hlyA (FIG. 42).

[0150] Then the DNA-targeting cassette has been digested with KpnI and SacI from pYB-asd-hlyA. The fragment was purified and transformed into electro-competent SL001 cells induced for the phage λ Red-mediated recombineering system. After recombineering, the correct colonies were identi-

fied by colony PCR conformation, using a pair of primers: asd-test-f and PsseA-r. Chloramphenicol resistance gene was removed by site-specific Cre/loxP mediated recombination by transformation of plasmid p705cre-Km, generating the strain SL002. Similarly, an anti-stress related gene htrA was replaced by cat-PpepT-asd-sodA cassette. The essential gene with tightly anaerobic control was cloned back to develop strain SL003.

[0151] Furthermore, to maintain the therapeutic plasmids without antibiotic selections, a "precise" deletion of the entire structural gene of initiation factor 1 (encoded by infA) from SL003 chromosome has been performed, which is presented in Example 6.5. After a series of genetic manipulations (FIG. 1), SL7207 has been engineered to be a tumor-targeting delivery and expression vector which was termed ST1. ST1 has the following genotype: *S. typhimurium* 2337-65 derivative hisG46, Δ aroA::Tn10 (Tcs), Δ gmd::T7 RNAP, Δ asd::PsseA-hlyA, Δ htrA::cat-PpepT-asd-sodA, Δ infA::tetR.

TABLE 3

Oligonucleotides		
Name	Sequence (5' - 3')	Purpose
loxp-F-XhoI	CCGCTCGAGCCGATCATATTCAATAACCCCT	pBSK-cat
loxp-R-XhoI	CCGCTCGAGGACTAGTGAACCTCTTCGAGGG	pBSK-cat
Hind3-T7-ploy-F	CCCAAGCTTCCGGATTTACTAACTGGAAGAGGCACT AAATG	Ts-Plac-T7P
LacIZ-T7-ploy-R	CCGCTCGAGAAGGGGATCCGGAGTCGTATTGATTTG	Ts-Plac-T7P
gmd50-Plac-F	AAGTCGCTCTCATTACTGGCGTAACCGGACAGGATG GGTCTTACCTGGCAGTGTGCAAGGCGATTAAAGTTGG	T7 RNAP-cat
gmd50-T7-R	TCTCAAGGAACCACTGGTAAGTACCGCAAGCCCT GCCTCCAGTGAATCTGTGGATAACCGTATTACCGC CT	T7 RNAP-cat
PsseA-F-NotI	ATTTGCGCCGAGAGAACAACGGCAAGTTAC	pYB-asd-hlyA
PsseA-R-HindIII	CCAAGCTTACGATAGATAGATAATTAACGTCG	pYB-asd-hlyA
hlyA-F-HindIII	CCCAAGCTTATGAAAAAATAATGCTAGTT	pYB-asd-hlyA
hlyA-R-XhoI	CCGCTCGAGCGCCGCTACTAGTAAGCTTTTAAATC AGCAGGG	pYB-asd-hlyA
asd-LA-F-SacI	TCCGAGCTCGTAGACATGATGGAAACTATCCTCGGC ACG	pYB-asd-hlyA
asd-LA-R-SacII	TCCCCGCGCGACATCAACATCAGGCTAACGGT	pYB-asd-hlyA
asd-RA-F-XhoI	CCGCTCGAGCGGAAACCAACAAGATCAAGATCCTA CAATA	pYB-asd-hlyA
asd-RA-R-KpnI	CGGGTACCCTCGACGACACTTCTTTGACCTGAACG GCG	pYB-asd-hlyA
htrA-LA-F-SacI	TCCGAGCTCGTCGACGCTACGTGGAAGTCGTCAGTA	pYB-htrA-asd
htrA-LA-R-SacII	TCCCCGCGCGTCGGTCTGAATAAAGTTCTCGTAA	pYB-htrA-asd
htrA-RA-F-XhoI	CCGCTCGAGGGATGTCATTACCTCGCTGAACGGG	pYB-htrA-asd
htrA-RA-R-KpnI	CGGGTACCCTCGACTCCCTAAACGCTGTCGCCATTC	pYB-htrA-asd
cat-F-NotI	ATTTGCGGCCGCGGATCATATTCAATAACCCCT	pYB-htrA-asd
cat-R-NotI	ATTTGCGGCCGCGACTAGTGAACCTCTTCGAGGG	pYB-htrA-asd
P _{pepT} -F-NotI	ATTTGCGGCCGCGTAAACGCAACGGATGGCTGACCCG	pYB-htrA-asd
P _{pepT} -R-HindIII	CCCAAGCTTCTTTTCGTGACAACATTATTAATAAG	pYB-htrA-asd
asd-F-HindIII	CCCAAGCTTTGGAGCGAACCAGATGAAAATGTTG GTTTTATCGGCTGGC	pYB-htrA-asd
asd-R-XhoI	CCGCTCGAGCTACGCCAACTGGCGCAGCATTCTGA	pYB-htrA-asd
PsodA-F	GACGAAAGTACGGCATTGATAATCATTTTCAATATCA TTTAATTAACATAATGAACCAAC	pYB-htrA-asd
PsodA-R	TCGAGTTGGTTTATTAGTTAATAAATGATATTGAA AATGATTATCAATGCGGCTACTTTTCGTCGACA	pYB-htrA-asd
infA-LA-F-XbaI	GCCTCTAGATAAAAGGTCGGTTTAACCGGCC	pYB-infA-tetR
infA-LA-R-SacII	ACACCGCGCACTGTAAGCGATGCTGGT	pYB-infA-tetR
infA-RA-F-XhoI	TCTACTCGAGATCCTCTGGGGTATCACTACC	pYB-infA-tetR
infA-RA-R-KpnI	TTCTGGGTACCACGATGCTTGT	pYB-infA-tetR

TABLE 3-continued

Oligonucleotides		
Name	Sequence (5'-3')	Purpose
gmd-test-F	GTTCAGAAAGTTACTCCC	Verification
htrA-test-F	GTCGACGCCCTACGTGGAAGTCGTCGTCAGTA	Verification
asd-test-F	GTCGACATGATGGAACTATCCTCGGCACG	Verification
infA-test-F	CTTGCGTACTGGAGTTTCG	Verification
EGFP-pA-R-PstI	GCGCTGCAGTTTTTTTTTTTTTTTTTTTACTTGTACA GCTCGTC	pT7-EGFP
P_{CMV} -F-NdeI	TATCATATGCCAAGTACG	pSE3
P_{T7} -R-NheI	AACGCTAGCCAGCTTGG	pSE3
T7 RNAP-F-XbaI	TCTAGAATGAACACGATTAACATCGCTAAG	pIKDE, pIKRP
T7 RNAP-R-NotI	CTGCAGCGGCCGCTACTAGTTACGCGAACGCGAAGT CCGACT	pIKDE, pIKRP
IRES-F-XbaI	ATATCTAGAGCCCCCTCCTCCCCCCCC	pIKDE, pIKRP
IRES-R-NheI- EcoRI	CGCGAATTCGCTAGCATATTATCATCGTGT	pIKDE, pIKRP
IRES-R-SpeI	GGCACTAGTTGTGGCCATATTATCATCGT	pIKDE, pIKRP
BGHpA- P_{T7} -R- SacII	ATACCGCGGTCTCCCTATAGTGAGTCGTATTACCATA GAGCCACCGCATCC	pIKDE, pIKRP
Kozak-EGFP-F-NheI	GCTAGCACAAACCATGGTGAGCAAG	pIKDE-EGFP
Lpp_ompA-F- EcoRI	GGGAATTCATATGAAAGCTACTAAACTGGTACTGG GCGCGGTAAACCCGATGTTGGCTTTGAAATGGG	pLpp_ompA_sTRAIL
Lpp_ompA-R- NotI	CCGCTCGAGTTATGCGGCCGCGTTGTCCGGACGAGT GCCGATGGTGT	pLpp_ompA_sTRAIL
sTRAIL-F-NotI	GCGGCCGAGTGAGAGAAAGAGGTCCTCA	pLpp_ompA_sTRAIL
sTRAIL-R-XhoI	CTCGAGGCCAACTAAAAGGCCCGA	pLpp_ompA_sTRAIL
sTRAIL-F-NheI- NdeI	CGTGCTAGCATATGGTGAGAGAAAGAGGTCCTCA	pSspH2-sTRAIL
sTRAIL-R-PstI- HindIII	CTGAAGCTTCTGCAGTTAGCCAACTAAAAGGCC	pSspH2-sTRAIL
SspH2-F-NcoI	ATACCATGGCACCCCTTTCATATTGGAAGC	pSspH2-sTRAIL
SspH2-R-NcoI	GTACCATGGACCCCGATGCCCTTCCGCG	pSspH2-sTRAIL
mEnd-R-PstI- HindIII	AAGCTTCTGCAGTTATTTGGAGAAAGAGGTCATG	pSspH2-Endo statin
3xFlag-F-NcoI	GACTATACCATGGACTACAAAGACCATGACGGTG	pIKDE-DTA
3xFlag-partial seq-F	ACAAAGACCATGACGGTGATTATAAAGATCATGACA TCGATTACAAGGATGACGAC	pIKDE-DTA
3XFlag-R-NcoI	AGAAGGAGATATACCATGGATTACAAGGATGACGAC GATAAGCATATG	pIKDE-DTA
Kozak-3xFlag-F- XbaI	TCTAGACCACCATGGACTACAAAGACCATGACGGTG	pIKDE-DTA
DTA-R-PstI	AAGCTTCTGCAGTTATCGCCTGACACGATTTCC	pIKDE-DTA
HA-F-SpeI	CATT CTAGAGCCACCATGGGAAACTCAAATCC	pIKDE-HA
HA-R-XbaI	AGATCTAGACTCGACTGCAGTTAGTGCTTCAACTTAT ATACAAAT AGTGCACCGC	pIKDE-HA
DTA-For	AAAGGTTTCGATGATGGTGCTTCGC	qRT-PCR
DTA-Rev	TCTACGCTTAACGCTTTCGCCTGT	qRT-PCR
URP-DTA-R	TGGTGTTCGTGGAGTCGTCGCTGACACGATTTCC	RT-PCR
P_{T7} -F-BglII	CGAAGATCTAATACGACTCACTATAG	pIKR-shRNA
t7 term-R-BglII	CGAAGATCTCAAAAAACCCCTCAAGACC	pIKR-shRNA
P_{T7} -shTom-F- BglII	AGATCTAATACGACTCACTATAGGGCCAAGAAGCCC GTGCAATTCAAGAGATTGC	pIKR-shTom
shTom-t7 term	TGCAATCAAGAGATTGCACGGGCTTCTTGGCCTTT TTAGCATAACCCCTTGGG	pIKR-shTom
P_{T7} -shPLK-F	TAATACGACTCACTATAGGGAGATCACCCCTCTTAAA TATTTTCAAGAGAAATAT	pIKR-shPLK
HDV-shPLK-R	GGAGATGCCATGCGACCCAAAAGATCACCCCTCCT TAAATATTTCTCTTGAAAATAT	pIKR-shPLK

TABLE 3-continued

Oligonucleotides		
Name	Sequence (5'-3')	Purpose
P _{T7} -let-7-F	AGATCTTAATACGACTCACTATAGGAGACAGGAAGC TTTGGGATGAGGTAGT	pIKR-let-7
HDV-let-7-R	TGGAGATGCCATGCCGACCCAACTCGAGAAAAAAT AGGAAAG	pIKR-let-7
P _{T7} -her-2-F	AGATCTTAATACGACTCACTATAGGAGACAGGGTCA CAGGGGCCCTCCCAGG	pIKR-her-2
HDV-her-2-R	TGGAGATGCCATGCCGACCCAAATCACAGGGGCCCTC CCCAGGT	pIKR-her-2
HDV ribo seq-R	CTTCTCCCTTAGCCTACCGAAGTAGCCAGGTCGGA CCGCGAGGAGGTGGAGATGCCATGCCGACCC	pIKR-shPLK
t7 term-HDV-R	CAAAAAACCCCTCAAGACCCGTTTAGAGGCCCCAA GGGGTTATGCTAACTTCTCCCTTAGCCTACCGA	pIKR-shPLK
bla-For	CTACGATACGGGAGGGCTTA	qRT-PCR
bla-Rev	ATAAATCTGGAGCCGGTGAG	qRT-PCR
CTNNB1-For	GACAATGGCTACTCAAGCTG	qRT-PCR
CTNNB1-Rev	CAGGTCAATCAAACCCAGG	qRT-PCR
dxs-For	CGAGAACTGGCGATCCTTA	qRT-PCR
dxs-Rev	CTTCATCAAGCGTTTCACA	qRT-PCR
GAPDH-For	AGCCACATCGCTCAGACAC	qRT-PCR
GAPDH-Rev	GCCCAATACGACCAATCC	qRT-PCR
PLK1-For	CACAGTGTCAATGCCTCCA	qRT-PCR
PLK1-Rev	TTGCTGACCCAGAAGATGG	qRT-PCR
s1-siCAT-RT	CTCAACTGGTGTCTGGAGTCGGCAATTCAGTTGAG AGCTGATA	RT-PCR
s1-siPLK-RT	CTCAACTGGTGTCTGGAGTCGGCAATTCAGTTGAG AGATCACC	RT-PCR
URP	TGGTGTCTGGAGTCG	qRT-PCR
siCAT-For	ACACTCCAGCTGGGCTGTCCATCAA	qRT-PCR
siPLK-For	ACACTCCAGCTGGGAATATTTAAGGAGGGT	qRT-PCR
UAP poly G	CGCGTCGACTAGTACGGGGGGGGG	5' RACE
PLK GSP1	GGGCAGCTATTAGGAGGCCCTTGAGACG	5' RACE
UAP	CGCGTCGACTAGTACG	5' RACE
PLK1 GSP2	AGTCCGGAGGGGGAGGGCAGC	5' RACE
CTNNB1 GSP2	CGCATGATAGCGTGTCTGGAAGCTT	5' RACE

6.2: Generation of SL006, SL007 and SL008 Mutants

[0152] These mutants are attracted to tumors, can penetrate into tumor tissue, but do not exclusively colonize tumor hypoxic cores. A series of ST1 mutants have been developed by a similar strategy.

[0153] To decrease the fitness of tumor-targeting *Salmonella*, a replication-incompetent strain SL006 (diaminopimelic acid auxotrophy) has been engineered. SL006 has the following genotype: *S. typhimurium* 2337-65 derivative hisG46, ^ΔaroA::Tn10 (Tcs), ^Δgmd::T7 RNAP, ^Δasd::PsseA-hlyA, ^ΔinfA::cat, which was derived from SL002 strain by removing an essential gene. The infA::cat cassette generated using PCR with pYB-infA-cat was purified and transformed into electro-competent SL002 cells harboring plasmid pET28a-infA and psim6 for λ Red-recombination. Samples of the electroporation mixture were spread on LB plates supplemented with 25 mg/ml chloramphenicol. PCR amplification of the new junctions between the drug marker and infA homology arm-flanking DNA was performed to confirm that the wild-type copy of infA was removed.

[0154] Because early metastases and viable tumor cells outside necrotic regions are well or partially oxygenated, they

are inaccessible to obligate anaerobic bacteria. To increase the fitness in the non-hypoxic, outer rim of the solid tumor and metastases, replication-competent strains SL007 and SL008 have been developed. SL007 has the following genotype: *S. typhimurium* 2337-65 derivative hisG46, ^ΔaroA::Tn10 (Tcs), ^Δgmd::T7 RNAP, ^ΔhtrA::PsseA-hlyA, ^ΔinfA::cat, which was derived from SLOW strain through two procedures. In the first step, pYB-htrA (a pBSK derivative with 1 kb flanking regions of htrA locus) has been generated. The DNA sequence encoding PsseA-hlyA and loxp-cat-loxp were ligated into plasmid pYB-asd through multiple cutting sites to construct plasmid pYB-htrA-hlyA. Then the DNA-targeting cassette has been digested with KpnI and SacII from pYB-htrA-hlyA. The fragment was purified and transformed into electro-competent SL001 cells induced for the phage λ Red-mediated recombineering system. After overnight incubation, the correct colonies were identified by colony PCR conformation. Chloramphenicol resistance gene was removed by induction of Cre recombinase. In the second step, in the new mutant strain, the essential gene infA was replaced by cat cassette, generating SL007. ^ΔhtrA mutation causes low growth defects in growth at high or low temperatures and stationary phase further attenuating SL007 strain.

[0155] SL008 has the following genotype: *S. typhimurium* 2337-65 derivative hisG46, ^ΔaroA::Tn10(Tcs), ^Δgmd::T7

RNAP, Δ asd::P_{seA}-hlyA, Δ htrA::P_{pepT}-asd-sodA, Δ infA::tetR, which was derived from SL003 strain. Chloramphenicol resistance gene in SL003 was eliminated by induction of Cre recombinase. Once the loxP sites have been removed, the transcription of asd gene is controlled by the upstream htrA promoter, which resulted in a leaky expression under normal oxygen levels. Then the infA gene was replaced by tetR expression cassette via recombineering, generating SL008 strain. This mutant is attracted to tumors, can penetrate into tumor tissue and effectively colonize viable regions of tumors otherwise unaffected by standard cancer therapy (FIG. 43).

6.3: The Growth of ST1 and its Mutant Strains Under Anaerobic Conditions

[0156] Tight control of the expression of the essential gene asd under hypoxic conditions requires a precise genetic regulation. Based on the design of the “obligate” anaerobic *S. typhimurium* strain YB1, FNR regulated anaerobic capable promoter P_{pepT} and aerobic promoter P_{sodA} (antisense) were used to control asd transcription in ST1. If asd does not express, the bacteria will die in the absence of additional DAP. Survival of ST1 under normal and low oxygen conditions was tested. For anaerobic growth on LB agar plates, an anaerobic jar was applied to maintain low oxygen concentration (0.5% O₂) by absorption of AnaeroPacks and monitored by an oxygen meter. ST1 showed the combination of growth under 0.5% oxygen concentration and repression in the aerobic environment without exogenous DAP supplement (FIG. 3A). In comparison, replication-competent SL007 and SL008 can grow in all conditions. Replication-competent SL008 showed growth only on the plates supplied with DAP (Data not shown).

6.4: Accumulation of ST1, SL007 and SL008 in Tumor and Normal Tissues In Vivo

[0157] Three groups of six-week-old BALB/c mice were inoculated with CT26 colon cancer cells and, when tumor volumes reached 300–500 mm³, a single dose (5×10⁷) of ST1, SL007 or SL008 was injected via the tail vein. On day 14 post injections, mice were euthanized and most organs and tumor were collected, homogenized and cultured on LB agar plates with antibiotics. CFU/gram was used as a relative measure of the degree of colonization of the tissues with bacteria (FIG. 44).

[0158] For SL007 inoculated mice, 10³ to 10⁵ CFU/gram of bacteria were found in normal organs. On day 14, SL007 levels in tumor reached 2.1×10⁸ CFU/gram. In comparison, by 2 weeks following injections, ST1 and SL008 were totally eliminated from spleen, liver and kidney, which could prevent a risk for sepsis in the clinical setting. Tuning survival gene asd expression level in SL008 strain leads to migration throughout the tumor (in both viable and necrotic region) (FIG. 43), with increased accumulation within tumors. The amounts of SL008 in the tumors retained at 10⁷–10⁸ levels during several weeks after systemic administration and were approximately two orders of magnitude higher than ST1 detected at the same time point. These high titers may enhance the therapeutic effects, as high amounts of therapeutic factors generated and delivered by bacteria.

6.5: Establishment of an In Vivo Plasmid Maintenance System Based on infA

[0159] As live carriers, therapeutic efficacy of these bacteria is always related to the amount of protein presented or the

dose of DNA delivered. Therefore, plasmid stability is the most critical parameter for the successful delivery of cargos. In this study, we describe the development of a balanced-lethal vector/host system based on an enzyme essential for protein synthesis in *E. coli* and *S. typhimurium*. In strain ST1, the exogenous plasmid is maintained, since it harbors a small essential gene infA, which has been removed from the chromosome. As a consequence, only plasmid-carrying infA⁻ mutant cells can survive, making this strain totally dependent on the maintenance of the infA⁺ plasmids.

[0160] First, the plasmid pET28a-infA containing infA cassette clone from *E. coli* MG1655 strain has been constructed and co-transformed with psim6 into SL003 (FIG. 45). Then plasmid pYB-infA-tetR containing tetracycline resistance gene flanked with 1 kb long homology arms of infA sites has been constructed and digested with KpnI and SacII. The selection cassette was purified and transformed into electro-competent SL003 cells carrying pET-infA and psim6 for recombineering. Samples of the electroporation mixture were spread on LB plates supplemented with 12.5 µg/ml tetracycline. PCR amplification of the new junctions between the drug marker and infA homology arm-flanking DNA was performed to confirm that the wild-type copy of infA was removed (FIG. 1B).

[0161] After 5-day growth in antibiotic free medium, all the infA⁻ mutant cells carried the exogenous infA⁺ plasmids. However, more than 80% of the parental SL003 cells had lost the plasmid (FIG. 46). The same results also obtained when the bacteria were cultured in minimal medium in absence of antibiotics selection pressure.

[0162] Since it has been shown that both ST1 and SL008 are capable of targeting and proliferating in tumor tissue, we assessed the plasmid stability of ST1 and SL008 harboring high-copy-number plasmid pcDNA3.1-infA (pUC origin, Amp^R) or low-copy-number plasmid pET32-infA (pER322 origin, Amp^R) in tumor tissues after systemic administration. A mouse tumor model was created by implanting CT26 mouse colon cancer cells in the right thigh of BALB/c mice. No statistic difference was detected in the total number of bacteria and total account of ST1 containing high or low-copy-number plasmids harboring infA gene. Validating by CFU test on ampicillin-rich plates indicated that the plasmids containing ampicillin resistance gene still remained in ST1 strains after 3 weeks following injections (FIG. 7). In comparison, no high-copy-number plasmid harboring SL003 cells were recovered from tumors on 2 day post injection. It suggested that the infA⁺ plasmids in the ST1 and SL008 were stable while those in its paternal strain were disappeared quickly. This observation was consistent with the Gahan's report which indicated that these high-copy plasmids were unstable in *Salmonella* strains. In addition, the copy number of high-copy-plasmids (pUC origin) inside the ST1 three weeks after initial injection was still high in mice.

6.6: The Ability of ST1 to Invade and Deliver Exogenous Proteins in Mammalian Cells

[0163] To confirm of the bacterial invasion of ST1, mouse colon cancer CT26 cell monolayer (80–90% confluence) was incubated with ST1 (at an MOI of 200) for 3 h under 0.5% oxygen concentration. Subsequently, the culture medium was removed and replaced with fresh medium supplemented with gentamicin (50 µg/mL) to kill external bacteria. After 2 and 4 hours, cells were washed and treated with lysis buffer (1% Triton X-100 in PBS) for 30 min on ice. The amount of

intracellular bacteria was measured by plating serial dilutions of cell lysates on LB plates with streptomycin and DAP. Invasion rate (%) = number of internalized *Salmonella*/number of mammalian cells per well × 100.

[0164] At 2 h post infection, more than 80% of cells contained one or more bacteria. At a later time point (4 h) the number of infected cells kept unchanged, however, the amount of bacteria inside the infected cells increased approximately 2-fold, suggesting that ST1 can replicate within the tumor cells (FIG. 5).

Furthermore, to ascertain the ability of ST1-mediated delivery of protein, GFP was used as a marker. Tumor cells were incubated with ST1 carrying a prokaryotic plasmid psgfp. After a 3 h-incubation, ST1/psgfp invaded nearly 80% of epithelial cells which was quantified by gentamicin protection assay, and elicit >50% fluorescent cells detected by flow cytometry using a FACScalibur cytometer (FIG. 47).

6.7: Reporter Protein Expression and Translocation Through Bacterial Surface Display or Type III Secretion System

[0165] Plasmid pLpp_ompA_GFP encodes a hybrid protein consisting of (a) a signal sequence and first nine N-terminal amino acids of the major *E. coli* lipoprotein Lpp, (b) amino acids 46-159 of the outer membrane protein A (ompA) and the GFP domain. Fluorescence visualization of ST1/pLpp_ompA_GFP indicated the insertion of GFP on the outer membrane (FIG. 48A).

[0166] Another plasmid pSspH2-GFP was constructed to fuse the marker protein with the secretion and translocation effectors SspH2 (1-142 aa domain) from the type III secretion system. Here, pGFP without the signal domain was constructed as control. Compared to the vector control, noticeably high fluorescence intensity and diffused location of reporter protein in the cytosol were detected in ST1/pSspH2-GFP infected cells, indicating that a more effective and efficient delivery of exogenous proteins can be achieved through the type III secretion system (FIG. 48B).

6.8: Anti-Angiogenic Effect by Combination of Tumor-Targeting *Salmonella* SL008 and Endostatin in a Murine Model

[0167] Endostatin, a 20-kDa carboxy-terminal fragment of collagen XVIII, is a potent anti-angiogenic agent currently being evaluated in clinical trials. However, a discrepancy remained unresolved: sustained tumor regression has only been observed with a non-soluble, precipitated form of recombinant endostatin produced in bacteria. To shed light on this question and establish a model of systemic anti-angiogenic gene therapy of cancer that may surmount obstacles in protein production and delivery, we transformed SL008 with a plasmid pSspH2-Endostatin encoding a secreted form of murine endostatin (FIG. 49). Endostatin expression was tested by western blotting (FIG. 50A), and the biological activity of the secreted endostatin by tumor-targeting *Salmonella* was confirmed by anti-proliferative effect on blood vessels (FIG. 50B). As show in growth cures, by comparing mock control and ST1/control vector treated groups attenuated *Salmonella* itself has some inhibition effects on tumor growth. The mechanism is still not certain and there are some possible reasons. A large amount of *Salmonella* accumulated in the tumor sites could induce cell death by competing with the tumor for nutrients and releasing virulent factors. The

bacteria may also stimulate the inflammatory response, recruit immune cells and provoke the activation of macrophages.

[0168] Furthermore, the ability of *Salmonella* SL008 to secrete biologically active antiangiogenic factors at therapeutically sufficient levels largely enhanced the antitumor effects. Taking advantage of the tumor-targeting characteristics of SL008, secretion expression of Endostatin by SL008 throughout the tumors avoided systemic toxicity and markedly stopped tumor growth in mice (FIG. 51) These findings suggested that the combination of tumor-targeting bacteria with angiogenesis inhibitor might be effective in a variety of solid tumors.

6.9: Functional mRNA Delivery by T7 RNAP-Expressing ST1 Strain

[0169] Beside exogenous proteins and eukaryotic plasmids, ST1 is capable of delivering of RNA. By integration of the T7 RNAP gene into the genome, ST1 is able to mediate a transcription of functional mRNA encoding proteins or shRNA. After adhesion and entry, ST1 could escape from the host-cell vacuole by equipping them with LLO and replicate in the cytoplasm of host cells. Some bacteria disrupt and release nucleic acids and proteins inside the cytosol. To investigate whether ST1 could deliver functional mRNA in host cells, EGFP was used as an indicator for monitoring target gene expression in our system. The released unmodified mRNAs without a 5'7-methylguanosine cap structure and a 3' poly translation in mammals and block prokaryotic translation. Kozak consensus sequence as well as an IRES sequence of the encephalomyocarditis virus were cloned upstream of gene of interest.

[0170] For mRNA delivery, the inserted cassettes P_{T7}-kozak-IRES-EGFP-pA₂₀ fragment was amplified from pIRES-EGFP and digested by BglII and NotI. The stabilized vector backbone was generated from the plasmid pET32-infA. pET32a-infA was digested with BglII and NotI and the backbone was purified using agarose gel extraction. The backbone was ligated with P_{T7}-kozak-IRES-EGFP-pA₂₀ fragment and after transformation; the plasmid pT7-EGFP was confirmed by sequencing. Then the prokaryotic plasmid was transformed into engineered ST1 strain to yield ST1/pT7-EGFP (FIG. 52A).

[0171] RNA delivery of ST1/pT7-EGFP was examined. Infection of cultured mouse CT26 cells was performed using ST1/pT7-EGFP at an MOI of 200. At 48 h post infection, cells were detached by trypsinisation and EGFP expression was detected by western blot (FIG. 52B). In addition, no EGFP expression was observed in ST1/pT7-EGFP either by using fluorescence microscopy or by then the more sensitive western blot. Accordingly, our data indicated that EGFP expression after mRNA delivery completely based on the transcription/translation in mammalian cells, but not in prokaryotic cells. These results strongly showed the potential of ST1 in the functional delivery of nucleic acids to mammalian cells. We showed, for the first time, that the phagosome-disrupting *S. typhmuri* strain ST1 can directly deliver both plasmid DNA and translation-competent mRNA into the cytosol, leading to model gene expression. The established DNA/RNA delivery system in engineered bacteria has the potential to develop into a novel kind of inter-kingdom dual expression system based on the interaction of bacteria and host cells.

6.10: ST1/pIKDE-EGFP Mediating EGFP Expression in CT26 Mouse Colon Cancer Cells

[0172] To monitor the ST1-mediated inter-kingdom expression in CT26 mouse colon cancer cells, EGFP was used as a marker. ST1/pIKDE-EGFP was co-cultured with a monolayer of CT26 mouse cancer cells at an MOI of 200 for 3 h. After removal of extracellular bacteria, cells were cultured in a hypoxic incubator containing 0.5% O₂ and 5% CO₂ at 37° C. for 48 h and then fixed. Fluorescence microscopy revealed fluorescence in the cytosol cells infected with ST1/pIKDE-EGFP and a lack of fluorescent signal from ST1/pIKDE treated cells (FIG. 53).

6.11: Systemic Administration of ST1/pIKDE-DTA and SL008/pIKDE-DTA and Significantly Shrinks CT26 Colon Tumors

[0173] CT26 colon tumors are highly malignant and often lead to death. To investigate the anti-tumor effects of ST1/pIKDE-DTA and SL008/pIKDE-DTA, a colon tumor model was established in the immunocompetent mice. CT26 tumor models were prepared by subcutaneous injection of 1×10⁵ cells into the BALB/c inbred mice (6–8 weeks of age). When the tumor reached 500 mm³ in size, the mice were received treatments. ST1 or SL008 harboring pIKDE-DTA (FIG. 54) or control vector were injected through the tail vein (5×10⁷ cfu/100 μl PBS). In control animals, PBS was injected in the same volume. Mice were examined and the tumor diameters were measured every other day in two dimensions with an external microcaliper. Subcutaneous tumor size was calculated by using the formula: Tumor volume=length×width²×0.52. In the saline-treated groups, the CT26 tumors grew rapidly and exceeded a mean of 4000 mm³ within 10 days (FIG. 55A). Then the mice were killed due to the excessive tumor burden. Mice receiving ST1/pIKDE-EGFP exhibited a depressed effect, when were compared with PBS controls. In comparison, a single intravenous injection of ST1/pIKDE-DTA into CT26 bearing mice resulted in sustained regression of established tumors by cytotoxic toxins (FIG. 55B). Upon ST1/pIKDE-DTA infection, a large necrotic area was left behind in which bacteria resided and thrived. Some tumors even formed to severe scabs in ST1/pIKDE-DTA treated mice (FIG. 55C). Similar result was also observed in SL008 treated groups (Data not shown).

6.12: Analysis of HA-Antigen Expression and In Vivo Immune Response for Replication Defective *Salmonella* ST1/pIKDE-HA Strain

[0174] The hemagglutinin protein (HA) gene of avian influenza virus was amplified by PCR from the cDNA of avian influenza H7N9 virus, and sub-cloned into eukaryotic expression vector pIKDE. The HA gene was identified by sequencing. The recombinant plasmid was transformed into asd mutant ST1 (replication-incompetent), and the recombinants were designed as ST1/pIKDE-HA (FIGS. 56A-F). In a study in mice, BALB/c mice were immunized intraperitoneally at the dosage of 10⁷ CFU/mouse. Blood samples were collected from the tail artery of the mice. Anti-HA IgG titers were determined by means of enzyme-linked immunosorbent assay (ELISA) using HA for coating. Comparison between sera of mice immunized with ST1/pIKDE-HA showed that in both cases anti-HA serum IgG responses were elicited. After receiving three boosts on day 14, 21 and 28, the anti-HA IgG

response in the mice were greatly increased. 100% mice had anti-HA IgG responses with an average titer 1:4000 on day 48 (FIG. 41).

6.13: ST1-Mediated CTNNB1-Specific shRNA Expression Inhibits the Target Gene Expression and Suppresses Cell Proliferation

[0175] To determine whether specific gene silencing can be achieved by ST1 harboring an inter-kingdom RNAi system after its intracellular entry, MDA-MB-231 cancer cells were infected with ST1 carrying plasmid pIKR-shCAT encoding shRNA against the gene of β-Catenin (FIGS. 57A-E), which is the key mediator of Wnt/β-Catenin pathway. The targeting sequence of human CTNNB1 (GenBank accession no. NM_001904) is AGCTGATATTGATGGACAG, corresponding to the coding regions of positions 505 to 523. Subsequently, the targeted gene expression at mRNA and protein levels were examined at 48 h post infection. Semi-quantitative RT-PCR demonstrated that CTNNB1 mRNA decreased by ~90% in ST1/pIKR-shCAT infected cells. Compared with the vector control, ST1/pIKR-shCAT reduced the level of target proteins by ~50% at an MOI of 200 (FIG. 58A). Since, an increase of β-Catenin leads to an increased proliferation in many solid tumors, a time course of cell proliferation in vitro was assessed. ST1/pIKR-shCAT led to a 23.9% reduction in cell growth (FIG. 58B) and a 14.8% increase in cell death (FIG. 58C), which corresponded with a decline in the expression of β-Catenin and its downstream gene myc as well as the activation of caspase-3 expression (FIG. 58A). These results suggested that the ST1-mediated knockdown of the key signal transducer β-Catenin inhibits tumor cell proliferation, at least partly by the induction of caspase-dependent apoptosis.

6.14: Intravenous Administration of ST1/pIKR-shCAT Reduces Human MDA-MB-231 Xenograft Tumor Growth

[0176] The MDA-MB-231 xenograft model was established in which 10⁶ cells were injected into BALB/c female nude mice. Then the mice with established tumors (~250 mm³) were intravenously injected with PBS, ST1/pIKR-shTom or ST1/pIKR-shCAT. The monitoring of tumor growth for 20 days showed a substantial reduction in ST1/pIKR-shCAT treated mice (FIG. 59A). At the end point, the average tumor volume in this group was markedly decreased by 60.9% compared to the PBS group. In comparison, the difference between PBS treated and ST1/pIKR-shTom were not statistically significant, indicating that ST1 amplification is insufficient for tumor inhibition. As is shown in FIG. 59B, mice treated with ST1/pIKR-shCAT showed the smallest tumor sizes resulted by synergic effects of bacteria and inter-kingdom RNAi (FIG. 59C).

[0177] Subsequently, the bacterial distribution was analyzed for safety issues. On day 20 post infection, all the mice were sacrificed and tumor, liver, spleen, kidney, lymph node, lung and heart were removed and weighted. Organs were homogenized in 9 volumes of H₂O and CFU tests of viable *Salmonella* in each organ were determined by plating serial dilutions on LB agar plates supplemented with streptomycin or the antibiotic corresponding to the construction plasmid as well as DAP. The mean number of ST1 per gram tumor exceeded 10⁷–10⁸ (FIG. 59D). In contrast, ST1 was totally eliminated from other normal organs. Furthermore, the shRNA expression vector backbone was modified from a

high-copy plasmid pcDNA3.1 (+) (pUC ori, Amp^R), which is unstable in *Salmonella* (Galen, Pasetti et al. 2009). Validating by CFU tests on ampicillin-rich plates indicated that the therapeutic plasmids containing resistance gene still remained in ST1 in vivo after 3 weeks following injections, despite the absence of any antibiotic selection (FIG. 59D).

6.15: Determination of the Copy Number of pIKR-shCAT in ST1

[0178] Relative analysis was tested with two ST1/pIKR-shCAT colonies harboring pIKR-shCAT which were separated from tumors on day 20 post injections. The separate detection of pIKR-shCAT and host chromosomal DNA were achieved using two separate primer sets, specific for the plasmid β -lactamase gene (bla) and for the chromosomal D-1-deoxyxylulose 5-phosphate synthase gene (dxs). Since both bla and dxs gene are single-copy in the plasmid pIKR-shCAT and *Salmonella* chromosomal DNA, respectively. Thus the plasmid copy number can be determined as the copy ratio of bla to dxs. The result was consistent with the previously reported value of pUC copy number within bacterial host cells, 500~700 (Table. 4).

TABLE 4

Estimate pIKR-shCAT copy number by relative qualifications						
Colony	C_T		ΔC_T	Calibrator	$\Delta\Delta C_T$	Copies/cell $2^{-\Delta\Delta C_T}$
	Bla	Dxs				
1	21.52 \pm 0.19	30.41 \pm 0.11	-8.90 \pm 0.22	0.01 \pm 0.06	-8.91 \pm 0.22	480.7 (15.0%)
2	21.22 \pm 0.08	30.36 \pm 0.13	30.36 \pm 0.13	0.01 \pm 0.06	-9.37 \pm 0.15	571.7 (10.1%)

[0179] The invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described will become apparent to those skilled in the art from the foregoing description and accompanying figures. Such modifications are intended to fall within the scope of the appended claims.

[0180] All references cited herein are incorporated herein by reference in their entirety and for all purposes to the same extent as if each individual publication or patent or patent application was specifically and individually indicated to be incorporated by reference in its entirety for all purposes.

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<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<400> SEQUENCE: 27

gacgaaagta cggcattgat aatcattttc aatatcattt aattaactat aatgaaccaa 60
c 61

<210> SEQ ID NO 28
<211> LENGTH: 71
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<400> SEQUENCE: 28

tcgagttggt tcattatagt taattaaatg atattgaaaa tgattatcaa tgcctgactt 60
ttcgtctgac a 71

<210> SEQ ID NO 29
<211> LENGTH: 31
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

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<400> SEQUENCE: 29

gcctctagat aaaaggtcgg ttaaccggc c 31

<210> SEQ ID NO 30

<211> LENGTH: 29

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<400> SEQUENCE: 30

acaccgaggc actgtaaagc gatgctggt 29

<210> SEQ ID NO 31

<211> LENGTH: 31

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<400> SEQUENCE: 31

tctactcgag atcctctggg gtatcactac c 31

<210> SEQ ID NO 32

<211> LENGTH: 22

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<400> SEQUENCE: 32

ttctgggtac cacgatgctt gt 22

<210> SEQ ID NO 33

<211> LENGTH: 18

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<400> SEQUENCE: 33

gttcagaaag ttactccc 18

<210> SEQ ID NO 34

<211> LENGTH: 31

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<400> SEQUENCE: 34

gtcgacgcct acgtggaagt cgctgcagt a 31

<210> SEQ ID NO 35

<211> LENGTH: 30

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic

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oligonucleotide

<400> SEQUENCE: 35

gtcgacatga tggaaactat cctcggcacg 30

<210> SEQ ID NO 36
<211> LENGTH: 19
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<400> SEQUENCE: 36

cttgcgtact ggagtttcg 19

<210> SEQ ID NO 37
<211> LENGTH: 45
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<400> SEQUENCE: 37

gcgctgcagt tttttttttt ttttttttta cttgtacagc tcgtc 45

<210> SEQ ID NO 38
<211> LENGTH: 18
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<400> SEQUENCE: 38

tatcatatgc caagtacg 18

<210> SEQ ID NO 39
<211> LENGTH: 17
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<400> SEQUENCE: 39

aacgctagcc agcttgg 17

<210> SEQ ID NO 40
<211> LENGTH: 30
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<400> SEQUENCE: 40

tctagaatga acacgattaa catcgctaag 30

<210> SEQ ID NO 41
<211> LENGTH: 42
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence

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<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<400> SEQUENCE: 41

ctgcagcggc cgctactagt tacggaacg cgaagtccga ct 42

<210> SEQ ID NO 42
<211> LENGTH: 28
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<400> SEQUENCE: 42

atatctagag cccctctccc tccccccc 28

<210> SEQ ID NO 43
<211> LENGTH: 33
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<400> SEQUENCE: 43

cgcgaattcg ctagcatatt atcatcgtgt ttt 33

<210> SEQ ID NO 44
<211> LENGTH: 29
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<400> SEQUENCE: 44

ggcactagtt gtggccatat tatcatcgt 29

<210> SEQ ID NO 45
<211> LENGTH: 52
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<400> SEQUENCE: 45

ataccgpgt ctcctatag tgagtcgtat taccatagag cccaccgcat cc 52

<210> SEQ ID NO 46
<211> LENGTH: 24
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<400> SEQUENCE: 46

gctagcacia ccatggtgag caag 24

<210> SEQ ID NO 47
<211> LENGTH: 70

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<212> TYPE: DNA
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
 oligonucleotide

 <400> SEQUENCE: 47

 gggaattcca tatgaaagct actaaactgg tactgggccc ggtaaaccgc tatgttggct 60
 ttgaaatggg 70

<210> SEQ ID NO 48
 <211> LENGTH: 47
 <212> TYPE: DNA
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
 oligonucleotide

 <400> SEQUENCE: 48

 ccgctcgagt tatgcccgcg cgttgctccg acgagtgcgc atggtgt 47

<210> SEQ ID NO 49
 <211> LENGTH: 29
 <212> TYPE: DNA
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
 oligonucleotide

 <400> SEQUENCE: 49

 gcggccgcag tgagagaaag aggtcctca 29

<210> SEQ ID NO 50
 <211> LENGTH: 26
 <212> TYPE: DNA
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
 oligonucleotide

 <400> SEQUENCE: 50

 ctcgaggcca actaaaaagg ccccgca 26

<210> SEQ ID NO 51
 <211> LENGTH: 34
 <212> TYPE: DNA
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
 oligonucleotide

 <400> SEQUENCE: 51

 cgtgctagca tatggtgaga gaaagaggtc ctca 34

<210> SEQ ID NO 52
 <211> LENGTH: 35
 <212> TYPE: DNA
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
 oligonucleotide

 <400> SEQUENCE: 52

 ctgaagcttc tgcagttagc caactaaaaa ggccc 35

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<210> SEQ ID NO 53
<211> LENGTH: 29
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
oligonucleotide

<400> SEQUENCE: 53

ataccatggc accctttcat attggaagc 29

<210> SEQ ID NO 54
<211> LENGTH: 29
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
oligonucleotide

<400> SEQUENCE: 54

gtaccatgga cccggatgcc ccttccgcg 29

<210> SEQ ID NO 55
<211> LENGTH: 34
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
oligonucleotide

<400> SEQUENCE: 55

aagcttctgc agttatttgg agaaagaggt catg 34

<210> SEQ ID NO 56
<211> LENGTH: 34
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
oligonucleotide

<400> SEQUENCE: 56

gactatacca tggactacaa agaccatgac ggtg 34

<210> SEQ ID NO 57
<211> LENGTH: 56
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
oligonucleotide

<400> SEQUENCE: 57

acaaagacca tgacggtgat tataaagatc atgacatcga ttacaaggat gacgac 56

<210> SEQ ID NO 58
<211> LENGTH: 48
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
oligonucleotide

<400> SEQUENCE: 58

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agaaggagat ataccatgga ttacaaggat gacgacgata agcatatg 48

<210> SEQ ID NO 59
<211> LENGTH: 36
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
oligonucleotide

<400> SEQUENCE: 59

tctagaccac catggactac aaagaccatg acggtg 36

<210> SEQ ID NO 60
<211> LENGTH: 33
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
oligonucleotide

<400> SEQUENCE: 60

aagcttctgc agttatcgcc tgacacgatt tcc 33

<210> SEQ ID NO 61
<211> LENGTH: 34
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
oligonucleotide

<400> SEQUENCE: 61

cattctagag ccaccatggg aaacctcaa atcc 34

<210> SEQ ID NO 62
<211> LENGTH: 55
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
oligonucleotide

<400> SEQUENCE: 62

agatctagac tcgactgcag ttagtgcttc aacttatata caaatagtc accgc 55

<210> SEQ ID NO 63
<211> LENGTH: 24
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
oligonucleotide

<400> SEQUENCE: 63

aaaggttcga tgatggtgct tcgc 24

<210> SEQ ID NO 64
<211> LENGTH: 24
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
oligonucleotide

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<400> SEQUENCE: 64

tctacgctta acgcttctgc ctgt 24

<210> SEQ ID NO 65

<211> LENGTH: 34

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<400> SEQUENCE: 65

tggtgctgag gagtcgctgc ctgacacgat ttcc 34

<210> SEQ ID NO 66

<211> LENGTH: 26

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<400> SEQUENCE: 66

cgaagatcta atacgactca ctatag 26

<210> SEQ ID NO 67

<211> LENGTH: 28

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<400> SEQUENCE: 67

cgaagatctc aaaaaacccc tcaagacc 28

<210> SEQ ID NO 68

<211> LENGTH: 55

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<400> SEQUENCE: 68

agatctaata cgactcacta tagggccaag aagccctgag aattcaagag attgc 55

<210> SEQ ID NO 69

<211> LENGTH: 54

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<400> SEQUENCE: 69

tgcaattcaa gagattgcac gggcttcttg gccttttttag cataaccctc tggg 54

<210> SEQ ID NO 70

<211> LENGTH: 55

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

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<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<400> SEQUENCE: 70

taatacgcact cactataggg agatcacccct ccttaaataat tttcaagaga aatat 55

<210> SEQ ID NO 71

<211> LENGTH: 58

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<400> SEQUENCE: 71

ggagatgccca tgccgaccca aaaagatcac cctccttaaa tatttctctt gaaaatat 58

<210> SEQ ID NO 72

<211> LENGTH: 52

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<400> SEQUENCE: 72

agatcttaat acgactcact ataggagaca ggaagctttg ggatgaggta gt 52

<210> SEQ ID NO 73

<211> LENGTH: 43

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<400> SEQUENCE: 73

tggagatgcc atgccgaccc aaactcgaga aaaaatagga aag 43

<210> SEQ ID NO 74

<211> LENGTH: 52

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<400> SEQUENCE: 74

agatcttaat acgactcact ataggagaca gggtcacagg ggcctcccca gg 52

<210> SEQ ID NO 75

<211> LENGTH: 43

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<400> SEQUENCE: 75

tggagatgcc atgccgaccc aaatcacagg ggcctcccca ggt 43

<210> SEQ ID NO 76

<211> LENGTH: 67

<212> TYPE: DNA

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<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<400> SEQUENCE: 76

cttctccctt agcctaccga agtagcccag gtcggaccgc gaggaggtgg agatgccatg 60
ccgaccc 67

<210> SEQ ID NO 77
<211> LENGTH: 68
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<400> SEQUENCE: 77

caaaaaaccc ctcaagacc gtttagaggc cccaaggggt tatgctaact tctcccttag 60
cctaccga 68

<210> SEQ ID NO 78
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<400> SEQUENCE: 78

ctacgatacy ggagggtta 20

<210> SEQ ID NO 79
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<400> SEQUENCE: 79

ataaatctgg agccgggtgag 20

<210> SEQ ID NO 80
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<400> SEQUENCE: 80

gacaatggct actcaagctg 20

<210> SEQ ID NO 81
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<400> SEQUENCE: 81

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caggtcagta tcaaaccagg 20

<210> SEQ ID NO 82
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
oligonucleotide

<400> SEQUENCE: 82

cgagaaactg gcgatcctta 20

<210> SEQ ID NO 83
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
oligonucleotide

<400> SEQUENCE: 83

cttcatcaag cggtttcaca 20

<210> SEQ ID NO 84
<211> LENGTH: 19
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
oligonucleotide

<400> SEQUENCE: 84

agccacatcg ctcagacac 19

<210> SEQ ID NO 85
<211> LENGTH: 19
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
oligonucleotide

<400> SEQUENCE: 85

gcccaatag acccaatcc 19

<210> SEQ ID NO 86
<211> LENGTH: 19
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
oligonucleotide

<400> SEQUENCE: 86

cacagtgta atgcctcca 19

<210> SEQ ID NO 87
<211> LENGTH: 19
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
oligonucleotide

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<400> SEQUENCE: 87

ttgctgaccc agaagatgg 19

<210> SEQ ID NO 88

<211> LENGTH: 44

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<400> SEQUENCE: 88

ctcaactggt gtcgtggagt cggcaattca gttgagagct gata 44

<210> SEQ ID NO 89

<211> LENGTH: 44

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<400> SEQUENCE: 89

ctcaactggt gtcgtggagt cggcaattca gttgagagat cacc 44

<210> SEQ ID NO 90

<211> LENGTH: 16

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<400> SEQUENCE: 90

tggtgtcgtg gagtcg 16

<210> SEQ ID NO 91

<211> LENGTH: 25

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<400> SEQUENCE: 91

acactccagc tgggctgtcc atcaa 25

<210> SEQ ID NO 92

<211> LENGTH: 30

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<400> SEQUENCE: 92

acactccagc tgggaatatt taaggagggt 30

<210> SEQ ID NO 93

<211> LENGTH: 25

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic

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oligonucleotide

<400> SEQUENCE: 93

cgcgctcgact agtacggggg ggggg 25

<210> SEQ ID NO 94
<211> LENGTH: 27
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<400> SEQUENCE: 94

gggcagctat taggaggcct tgagacg 27

<210> SEQ ID NO 95
<211> LENGTH: 16
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<400> SEQUENCE: 95

cgcgctcgact agtacg 16

<210> SEQ ID NO 96
<211> LENGTH: 21
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<400> SEQUENCE: 96

agtcgaggagg gggagggcag c 21

<210> SEQ ID NO 97
<211> LENGTH: 25
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic oligonucleotide

<400> SEQUENCE: 97

cgcatgatag cgtgtctgga agctt 25

<210> SEQ ID NO 98
<211> LENGTH: 19
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 98

agctgatatt gatggacag 19

<210> SEQ ID NO 99
<211> LENGTH: 6620
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic polynucleotide

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<400> SEQUENCE: 99

tggcgaatgg gacgcgccct gtagcggcgc attaagcgcg gcggtgtgg tggttacgcg	60
cagcgtgacc gctacaactg ccagcgcctt agcgcgccct cctttcgtt tcttcccttc	120
ctttctcgc accgttcgccc gctttcccc tcaagctcta aatcgggggc tccctttagg	180
gttccgattt agtgctttac ggcacctcga ccccaaaaaa cttgattagg gtgatggttc	240
acgtagtggg ccacgcgcc gatagacggt ttttcgccct ttgacgttgg agtccacggt	300
ctttaatagt ggactcttgt tccaaactgg aacaacactc aacctatct cgtctattc	360
ttttgattta taagggattt tgccgatttc ggctatttgg ttaaaaaatg agctgattta	420
acaaaaattt aacgcgaatt ttaacaaaat attaacgttt acaatttcag gtggcacttt	480
tcggggaat gtgcgcggaa ccctatttg tttatttttc taaatacatt caaatatgta	540
tccgctcatg aattaattct tagaaaaact catcgagcat caaatgaaac tgcaatttat	600
tcatatcagg attatcaata ccatattttt gaaaaagccg tttctgtaat gaaggagaaa	660
actcaccgag gcagttccat aggatggcaa gatcctggta tcggtctgcg attccgactc	720
gtccaacatc aatacaacct attaatcttc cctcgtcaaa aataaggtta tcaagtgaga	780
aatcaccatg agtgacgact gaatccggtg agaatggcaa aagtttatgc atttctttcc	840
agacttgctc aacagggcag ccattacgct cgtcatcaaa atcactcgc tcaaccaaac	900
cgttattcat tcgtgattgc gcctgagcga gacgaaatac gcgatcgcg ttaaaaggac	960
aattacaaac aggaatcgaa tgcaaccggc gcaggaacac tgccagcgc tcaacaatat	1020
tttccactga atcaggatat tcttctaata cctggaatgc tgttttcccg gggatcgcag	1080
tggtgagtaa ccacgcatca tcaggagtac ggataaaatg cttgatggtc ggaagaggca	1140
taaattccgt cagccagttt agtctgacca tctcatctgt aacatcattg gcaacgctac	1200
ctttgccatg tttcagaaac aactctggcg catcgggctt cccatacaat cgatagattg	1260
tcgcacatga ttgcccgaca ttatcgcgag cccatttata cccatataaa tcagcatcca	1320
tgttgaatt taatcgcggc ctgagcaag acgtttcccg ttgaatatgg ctcataaac	1380
ccctgtatt actgtttatg taagcagaca gttttattgt tcatgaccaa aatcccttaa	1440
cgtagtattt cgttccactg agcgtcagac cccgtagaaa agatcaaagg atcttcttga	1500
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1-19. (canceled)

20. A method to transfer a therapeutic vector from a bacteria to a eukaryotic cell, said method comprising the steps of:

(A) providing the bacteria, said bacteria comprising in its chromosome: (a) a deletion of an initiation factor 1 gene and a GDP-D-mannose dehydratase gene, and replacement with a hypoxia-controlled asd expression cassette, and (b) integration of T7 RNAP, a cytolysin expression cassette, and the therapeutic vector; wherein said therapeutic vector has the following characteristics: (i) a genomic size of less than 10 kb; (ii) an origin of replication; (iii) an *infA* locus; (iv) encodes one or more therapeutic molecules; and (v) P_{CMV} and P_{T7} dual promoters driving the expression of the one or more therapeutic molecules; and

(B) exposing the eukaryotic cell to the bacteria.

21-39. (canceled)

40. The method of claim 20, wherein the bacteria is a *Salmonella* spp.

41. The method of claim 40, wherein the bacteria is a *S. typhimurium* ST1 strain.

42. The method of claim 20, wherein the bacteria further comprises in its chromosome, a deletion of an amino acid biosynthesis-related gene.

43. The method of claim 42, wherein the amino acid biosynthesis-related gene is *aroA*.

44. The method of claim 20, wherein said bacteria further comprises in its chromosome, a deletion of a stress response gene.

45. The method of claim 44, wherein the stress response gene is *htrA*.

46. The method of claim 20, wherein the therapeutic vector further comprises a DT-A gene.

47. The method of claim 20, wherein the therapeutic vector further comprises a 72-bp element of an SV40 enhancer.

48. The method of claim 20, wherein the initiation factor 1 gene is *infA*.

49. The method of claim 20, wherein the GDP-D-mannose dehydratase gene is *gmd*.

50. The method of claim 20, wherein the cytolysin expression cassette comprises a listeriolysin O gene *hlyA*.

51. The method of claim 20, wherein the hypoxia-controlled asd expression cassette comprises:

a forward anaerobic inducible promoter *PpepT*;

an essential gene *asd*; and

an antisense promoter *PsodA*.

52. The method of claim 20, wherein the one or more therapeutic molecules are DNAs.

53. The method of claim 20, wherein the one or more therapeutic molecules are RNAs.

54. The method of claim 53, wherein the RNAs are mRNAs.

55. The method of claim 54, wherein said mRNAs are driven by the T7 RNAP in the bacteria.

56. The method of claim 54, wherein said mRNAs are adapted for eukaryotic translation by linking an IRES element before the mRNA encoding sequence.

57. The method of claim 56, wherein the IRES element is $IRES_{EMCV}$.

58. A method to transfer a therapeutic vector comprising DT-A from a bacteria to a eukaryotic cell, said method comprises the steps of:

(A) providing the bacteria, said bacteria comprising in its chromosome: (a) a deletion of an initiation factor 1 gene *infA* and GDP-D-mannose dehydratase gene *gmd*, and replacement with a hypoxia-controlled asd expression cassette, and (b) integration of T7 RNAP, a cytolysin expression cassette comprising *hlyA*, and the therapeutic vector; wherein said therapeutic vector has the following characteristics: (i) a genomic size of less than 10 kb; (ii) an origin of replication; (iii) an *infA* locus; (iv) encodes one or more therapeutic molecules; (v) a T7 RNAP autogene cassette, (vi) a 72-bp element of an

SV40 enhancer; and (vii) P_{CMV} and P_{T7} dual promoters driving the expression of the one or more therapeutic molecules; and

(B) exposing the eukaryotic cell to the bacteria.

59. The method of claim 58, wherein said bacteria further comprises in its chromosome: a deletion of a stress response gene *htrA*.

60. The method of claim 58, wherein said bacteria further comprises in its chromosome, a deletion of an amino acid biosynthesis-related gene *aroA*.

61. The method of claim 58, wherein the hypoxia-controlled *asd* expression cassette comprises:

- a forward anaerobic inducible promoter *PpepT*;
- an essential gene *asd*; and
- an antisense promoter *PsodA*.

62. The method of claim 58, wherein the bacteria is a *Salmonella* spp.

63. The method of claim 58, wherein the bacteria is a *S. typhimurium* ST1 strain.

64. The method of claim 58, wherein the one or more therapeutic molecules are mRNAs.

65. The method of claim 64, wherein said mRNAs are adapted for eukaryotic translation by linking an IRES_{EMCV} element before the mRNA encoding sequence.

66. A method to transfer a therapeutic vector from a bacteria to a eukaryotic cell, said method comprises the steps of:

(A) providing the bacteria, said bacteria comprising in its chromosome: (a) a deletion of an initiation factor 1 gene, a mutation of a biofilm-producing gene, and an insertion of a hypoxia-controlled *asd* expression cassette; and (b) integration of T7 RNAP, cytolysin expression cassette; and the therapeutic vector, wherein said therapeutic vector has the following characteristics: (i) an *infA* locus;

(ii) encodes therapeutic molecules; (iii) P_{CMV} and P_{T7} dual promoters driving the expression of the one or more therapeutic molecules; and (iv) a 72-bp element of an SV40 enhancer; and

(B) exposing the eukaryotic cell to the bacteria.

67. The method of claim 66, wherein said bacteria further comprises in its chromosome, a deletion of a stress response gene.

68. The method of claim 67, wherein the stress response gene is *htrA*.

69. The method of claim 66, wherein said bacteria further comprises in its chromosome, a deletion of an amino acid biosynthesis-related gene.

70. The method of claim 69, wherein the amino acid biosynthesis-related gene is *aroA*.

71. The method of claim 66, wherein said therapeutic molecules are DNAs and RNAs.

72. The method of claim 71, wherein the RNAs are mRNAs.

73. The method of claim 66, wherein the initiation factor 1 gene is *infA*.

74. The method of claim 66, wherein the biofilm-producing gene is *gmd*.

75. The method of claim 66, wherein the therapeutic vector has a genomic size of less than 10 kb.

76. The method of claim 66, wherein the cytolysin expression cassette comprises a *hlyA* gene.

77. The method of claim 66, wherein the hypoxia-controlled *asd* expression cassette comprises:

- a forward anaerobic inducible promoter *PpepT*;
- an essential gene *asd*; and
- an antisense promoter *PsodA*.

* * * * *